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Aqueous ammonia soaking (AAS) as a biomass pretreatment method: pilot-scale study with switchgrass, bench-scale use with poplar, and methane potential from anaerobic digestion of pretreated switchgrass

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Aqueous ammonia soaking (AAS) as a biomass pretreatment method: pilot-scale study with switchgrass, bench-scale use with poplar, and methane potential from anaerobic digestion of pretreated switchgrass

by

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ABSTRACT

The main objective of this thesis was to evaluate aqueous ammonia soaking (AAS) as a pretreatment method for lignocellulosic biomass preparation for biofuel production, in a variety of settings. This thesis, partially fulfills the Master of Science degree requirement, is prepared in the journal paper format, and includes three papers that have been published in or are prepared for submission to a journal.

The objective of the first chapter was to design and fabricate a pilot-scale soaking and washing system to safely and effectively generate AAS-pretreated switchgrass. Based on economic, safety and convenience factors, a 75-L soaking vessel was constructed and demonstrated to be effective in pretreating 4 kg of dry switchgrass per run with 20-L of aqueous ammonia. This pilot-scale system increased cellulose content and decreased hemicellulose and Klason lignin content of the remaining solids in a similar manner as observed in bench-scale experiments. To our knowledge, this is the first description and report of design, operation, and handling of a pilot-scale AAS biomass pretreatment system.

The objective of the second research paper was to quantify acid soluble lignin and acid insoluble lignin content following four pretreatment methods of eight transgenic and one wild type poplar varieties. The transgenic varieties of poplar (*Populus* spp) had modifications in the lignin biosynthesis pathway to reduce lignin content or make varieties more susceptible to delignification. All pretreatment techniques were successful in removing a fraction of both acid soluble lignin (ASL) and acid insoluble lignin (AIL) from the transgenic varieties removing 12-70% ASL and 5-52% AIL.

The objective of the last paper was to evaluate the energy yields from the anaerobic digestion (AD) of AAS-pretreated switchgrass and AAS-pretreated switchgrass plus

hydrolytic enzymes. The results show that anaerobic digestion of AAS-pretreated switchgrass significantly increases biogas energy production over the AD of untreated switchgrass, and that the addition of sufficient commercially available hydrolytic enzymes greatly increased biogas yields, methane concentration, and total methane yields. At the highest enzyme loading, gross energy production from AD was over twice the gross energy production from ethanol fermentation of the same material.

CHAPTER 1. GENERAL INTRODUCTION

Nearly 100 years ago, the first oil refineries started processing crude oil into gasoline and other useful products. Today, the petroleum industry in the United States refines nearly 21 million barrels of oil per day, 60% of which we import, into numerous fuels and hundreds of petroleum based products (Energy Information Administration, 2009). Our economy relies heavily on this integrated system. With this reliance on a limited and depleting supply of fossil fuels comes environmental impacts and dependence on foreign imports threatening our national security (Brown, 2007). The combustion of fossil fuels accounts for the largest source of carbon dioxide in the earth's atmosphere (EPA, 2009). Carbon dioxide and other greenhouse gases trap heat inside the earth's atmosphere which contributes to climate change (EPA, 2009). At the current fossil fuel consumption and emission rates, we are on target to double atmospheric carbon dioxide concentration in the next 50 years, however, many believe that we can meet the energy needs of the world and stabilized CO_2 emissions by using a portfolio of technologies (Pacala and Socolow, 2004). One such technology involves utilizing and converting biorenewable resources to meet our petroleum fuel and product demands (Wyman, 1999) which will substantially reduce net greenhouse gas emissions (DOE, 2009). The development of this biobased economy will reduce our dependence on petroleum, create new domestic job opportunities, and improve environmental quality.

The first-generation approach to a biobased economy focuses on utilizing plant material for biofuels production. Corn-ethanol production has been promoted as an alternative to gasoline derived from crude oil. As of January 2009, 179 biorefineries were in operation in the United States, producing 9.2 billion gallons of ethanol from 3.3 billion bu of corn per year (RFA, 2009). A recent study suggests that corn-ethanol petroleum replacement

could increase substantially with progressive farming techniques and closed-loop biorefineries (Liska et al., 2008). However, negative public perception of corn ethanol and conflicting demands as a food, fuel, and feed sources limit the use of corn ethanol as a fossil fuel replacement. Lessons learned from the corn (starch) ethanol industry serve as a stepping block for the advancement of second-generation biofuels derived from renewable non-food plant material such as lignocellulose.

Utilizing inexpensive and abundant lignocellulosic biomass appears to be a promising alterative to edible feedstocks and is expected to provide environmental and economic benefits (Perlack et al., 2005). Recently, collaborations between government, universities, and industries have been formed to accelerate advancement of cellulosic biofuels (Schwietze et al., 2008). Several firms are engaged in the demanding task of introducing new technologies into the marketplace in hopes that demonstration plants will be on-line in 2010 (DOE, 2009).

The complexity of the lignocellulosic material poses several problems that hinder commercialization (Wyman et al., 2005). Pretreatment is needed to disrupt the lignin structure and expose the cellulose to hydrolysis (Mosier et al., 2005). The pretreatment step is expected to account for a third of the total processing costs in second-generation lignocellulosic biorefineries (Wyman et al., 2005; Isci 2008), despite over two decades of active research examining multiple pretreatment methods. Pretreatment research focuses on developing processes that enhance conversion rates, reduce the need for hydrolytic enzymes, and increase ethanol yields (Mosier et al., 2005). Defining a single most efficient method of pretreatment is not feasible due to the diverse nature of lignocellulosic biomass (Mosier et al., 2005) thus crop-specific research is needed in order to promote the commercialization of

second generation biofuels. This thesis evaluates one pretreatment approach of aqueous ammonia soaking (AAS) and aids in gathering information that can guide in the commercialization process.

Objectives

The research objectives for this work are:

- To design, fabricate, test, and operate a pilot-scale AAS biomass pretreatment system.
- To compare lignin removal following AAS, and three other pretreatment methods, of transgenic varieties of poplar.
- To assess the biochemical methane potential (BMP) and energy yield of AAS-pretreated switchgrass and AAS-pretreated switchgrass plus enzymes.

Thesis Organization

This thesis contains a general introduction, three research articles, a general conclusion, as well as cited references and acknowledgments. The general introduction includes the objectives of this thesis, thesis organization, a description of the authors' role in each article and a brief literature review.

The first article, entitled "Design and Testing of a Pilot-Scale Aqueous Ammonia Soaking Biomass Pretreatment System," was submitted to the *Journal of Applied Engineering in Agriculture*. This article demonstrated that AAS could be safely conducted at pilot-scale with a low-cost system. The second article entitled "Aqueous Ammonia Soaking and Other Pretreatment of Transgenic Varieties of Poplar," was prepared as a summary report as part of the Biorenewable Resources and Technology International Exchange Program at the University of Gent. In this article, eight varieties of transgenic poplar, with

modifications to enzymes in the lignin biosynthetic pathway, and a wild type were pretreated using four different techniques and compared based on acid soluble lignin (ASL) and acid insoluble lignin (AIL) removal. Aqueous ammonia soaking (AAS), aqueous ammonia soaking with hydrogen peroxide supplement (AAS-HP), and organosolv pretreatment methods were successful in removing both ASL and AIL. Transgenic sample with modification in the caffeic acid/5-hydroxyferulic acid O-methyltrasferace (COMT) enzyme biosynthesis pathway resulted in the greatest delignification. The last research article "Energy Yield of Anaerobically Digested, Aqueous Ammonia Soaked Switchgrass: A Bench-Scale Biochemical Methane Potential Study" demonstrates that AAS and AAS plus enzymes can significantly improve the energy yield from the anaerobic digestion of switchgrass. It is expected that this article will be submitted to *Biomass and Bioenergy* in April 2009. Reference for the general introduction and each paper are included at the end of each chapter.

Authors' Role

The primary author, with the guidance, support, and assistance of co-authors composed all of the research articles presented in this thesis. Unless otherwise indicated, all methods were performed by the primary author.

Asli Isci (PhD graduate, Iowa State University) contributed to the first article (Chapter 2) by aiding the primary author with the fabrication of the pilot scale soaking system as well as with the execution of the experiments. Dr. D. Raj Raman (Associate Professor, Department of Agricultural and Biosystems Engineering, Iowa State University) provided guidance and assistance during the design of the system and assisted with the execution of experiments. Dr. Robert P. Anex (Associate Professor, Department of

Agricultural and Biosystems Engineering, Iowa State University) conceived the original study and assisted with experiments.

The second article (Chapter 3) was conceived under the direction of Prof. Wim Soetaert (Associate Professor, Faculty of Bioscience Engineering, University of Gent) as part of the Biorenewable Resource and Technology International Exchange Program for which research was performed at the University of Gent in Gent, Belgium. Dr. D. Raj Raman provided guidance throughout the study.

Dr. D. Raj Raman and Dr. Robert Burns conceived the fourth article (Chapter 4) and provided extensive guidance in the results analysis. Dr. Robert Burns provided laboratory equipment and assistance during the study. Dr. Robert Anex provided guidance in the energy analysis.

Literature Review

Petroleum is the largest single energy source in the United States, supplying approximately 40% of our energy (Wyman et al., 2005). For this energy, we rely on imports from unstable countries that hold the reserves (Wyman, 2007). A sustainable alternative is needed to shift away from industrial society's dependence on petroleum. Biofuels derived from biomass offer significant environmental and economic advantages as a sustainable source for the production of transportation fuel (Ragauskas et al., 2006). Currently, US companies commercially process corn grain into ethanol (Gray et al., 2006) but the limited supply of corn and its multiple roles as a feed, fuel, and food source limit the expansion of grain based ethanol production. As an alternative to corn derived ethanol, renewable lignocellulosic biomass offers a particularly well-suited feedstock for biofuels production as it is widely available, low cost, and does not interfere with the food chain (Lynd et al., 2005).

Moreover, a renewable feedstock platform offers energy security along with economic and environmental benefits (Ragauskas et al., 2006). For example, many lignocellulosic-based energy production and utilization cycles have promising net greenhouse gas emissions (Lynd et al., 2005) as compared to petroleum fuels and even corn ethanol. Furthermore, this shift away from fossil fuels to renewable energy sources has been a major focus of policy and agricultural production during the past decade. Hundreds of research efforts are in progress striving for the development of a sustainable agricultural society and efficient method of producing energy and other products from renewable source (Wyman, 2005). Following is a brief review of some of the most relevant work to this thesis.

Lignocellulosic Biomass

Biomass represents an abundant carbon-neutral, inexpensive renewable resource for the production of bioenergy. Cellulosic materials including agricultural and forestry residues, perennial crops, herbaceous and woody crops are sufficiently abundant to provide a major resource for producing biofuels, assuming the appropriate technology is in place (US DOE, 2009). Ideal lignocellulosic crops are indentified by high yields, low costs, and the ability to grow on low quality land (Hamelinck et al., 2003). The demand for lignocellulosic biomass as an industrial feedstock, instead of traditional commodity crops, creates opportunities for redesigning agricultural systems allowing the introduction of new crops and farming practices (Anex et al., 2007).

Feedstocks

Switchgrass (*Panicum virgatum L.*) is a perennial, warm-season (C_4) species that is resistance to harsh environmental conditions, pests, and diseases. Successful development of bioenergy industry will depend on identifying switchgrass cultivars with high-yield potential

and acceptable fuel quality (Schmer et al., 2008). Lemus et al. (2002) evaluated 20 different cultivars based on their bioenergy potential. Parrish and Fike (2005) provide an extensive review of the agronomy of switchgrass based on management practice for producing biofuels. They found that successful establishment and production depends on location, seed dormancy, weed control, planting depth, and date of planting and harvesting (Parrish and Fike, 2005). Carbon and energy balances of biofuel systems are favorable for switchgrass, because of resistance to harsh conditions, disease and pest and its ability to produce high yields at low fertilizer application rates. Schmer et al. (2008) found some varieties of established switchgrass to produce 540% more renewable energy than non-renewable energy consumed.

Poplar is another potentially viable feedstock for bioenergy production. Poplar (*Populus* spp.) are fast growing trees produced for pulp, lumber, strand board, plywood, fuel, wildlife habitat, and for ornamental reasons (Baucher et al, 2003). The carbon neutral, perennial hybrid poplar trees require minimal chemical inputs and are relatively low cost crops, making them potential candidates for biofuels production (Wyman et al., 2005). Genetic modifications to poplar, targeting enzymes in the lignin biosynthesis pathway, can alter lignin composition or reduce lignin content creating plants more susceptible to delignification (Baucher et al., 2003). The pulp and paper industry prompted these first attempts to reduce lignin content, because lignin must be extracted by expensive and environmentally hazardous processing in order to produce high quality paper (Boerjan et al., 2003). Therefore, a reduction in lignin would result in a reduction in input chemicals. Similarly, increasing interests in biofuels from lignocellulosic biomass has encouraged

further research in overcoming the hindrance caused by lignin by genetically modifying plants for biofuels (Ragauskas et al., 2006).

Composition of Lignocellulosic Material

Lignocellulosic biomass is a complex matrix of approximately 30-50% cellulose, 15-35% hemicellulose, 15-35% lignin and trace amounts of minerals, oils, soluble sugars and other components (Wyman et al., 2005). The cell wall of lignocellulosic material consists of complex matrix of these components impeding the hydrolysis of carbohydrates into fermentable sugars.

Cellulose $[C_6H_{10}O_5]_n$ is a straight-chain polysaccharide containing covalently linked ß-1,4 glycosidic bonds (Gray et al., 2006). It is the most abundant organic polymer found in nature and is located, nearly entirely, within the plant cell wall where it is embedded in a complex lignin structure. Cellulose is synthesized in nature as individual molecules, comprised of linear chains of glucosyl residues (Lynd et al., 2002). Chains of cellulose molecules form within the plant cell wall, which connect with other polymers to form strong linear chains called microfibrils (Baucher et al., 2003). These cellulose chains are generally made up of approximately 30 individual cellulose molecules, creating a crystalline core that is surrounded by hemicellulose. Hemicellulose cross-links with individual microfibrils (Lynd et al., 2002).

Hydrogen bonding within cellulose creates a crystalline structure, which significantly hinders enzymatic hydrolysis (Lynd et al., 2002) and creates a lattice-like matrix in which penetration with enzymes or water is difficult (Petrus and Noordermeer, 2006). Although these bonds form a distinct crystalline structure, cellulose fibers found in nature are not

purely crystalline. This enables hemicellulose fibers to be at least partially hydrated by water and even allow some enzymes to access substrate during hydrolysis (Baucher et al., 2003).

Hemicellulose (20-30%) consists of a heterogeneous mixture of short, highly branched chains of pentoses and hexoses including xylose, arabinose, galactose, glucose, and mannose. The degree of branching and composition of sugars in hemicellulose vary with different feedstocks (Gray et al., 2006).

Lignin, along with cellulose, is a major constituent of lignocellulosic material and is the second most abundant biopolymer on Earth (Wyman, 1999). Lignin is composed of a heterogeneous mixture of polymers, derived from cinnamyl alcohols that are covalently linked, making a glue-like matrix within the secondary cell wall of a plant. The composition of lignin varies with biomass and composition can be influenced by environmental conditions such as soil type, growing conditions, and climate (Baucher et al., 2003).

The pulp and paper industry gained extensive knowledge about lignin over the last 40 years. During the manufacture of paper, lignin is chemically separated from the polysaccharide components of wood by pulping and bleaching reactions (Baucher et al., 2003). This extraction of lignin requires large quantities of expensive chemicals and energy but is necessary in order to avoid discoloration of the paper. It is also desirable to remove the lignin fraction in the bioprocessing of lignocellulosic biomass (Mosier et al., 2005). Although hindersome to biochemical processing for cellulosic ethanol, lignin has some desirable characteristics as a relatively energy dense solid fuel (Petrus and Noordermeer, 2006) and in other applications such as additives in cement, dyes, water treatment and as a dust suppressant for gravel roads (Baucher et al., 2003).

Lignocellulosic Ethanol Production

Processing lignocellulosic material (Figure 1) to ethanol via a biochemical route requires five important steps: 1) growing, harvesting, processing, and transporting lignocellulosic material, 2) pretreatment degrade the cell wall structure, removes lignin, make cellulose accessible, and solubilize hemicellulose, 3) hydrolysis of sugars by enzymes, 4) fermentation of sugars, and 5) separation of ethanol produced (US DOE 2009). Although each of these step are collectively important, pretreatment is the primary focus of this work.

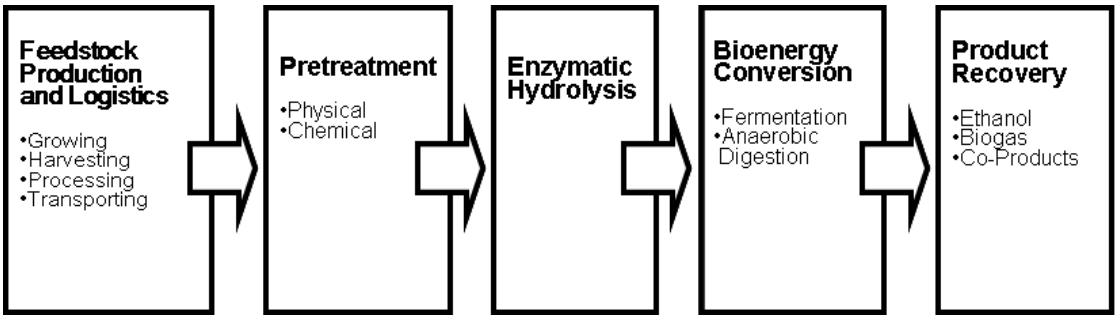

Figure 1. Process flow diagram of lignocellulosic biofuels (adapted from DOE, 2009)

Pretreatment

Methods of lignocellulosic biomass pretreatment are well developed but remain one of the most costly steps in the ethanol conversion process (Wyman 1999). Many different methods have been developed to pretreat lignocellulosic material with the goal of increasing enzymatic digestibility by freeing fermentable sugars and removing fermentative inhibitors (Mosier et al., 2005). Such pretreatment methods include mechanical treatments of grinding or milling and chemical treatments often using alkali, acid, or steam. Mosier et al. (2005) provides an excellent review of lignocellulosic pretreatment techniques including steam explosion, liquid hot water, dilute acid, and alkali pretreatments.

Steam explosion involves rapidly heating with high-pressure steam to a temperature at which water acts as an acid. At this point, hemicellulose is hydrolyzed increasing the enzymatic digestibility of the resulting material (Bari et al., 2002). Liquid hot water pretreatment utilizes pressure to keep water in a liquid state at temperatures around 200^{o}C. This cleaves the hemiacetal and ether linkages in the biomass dissolving 40-60% of the total biomass. Acid pretreatment methods are used to remove hemicellulose significantly increasing the digestibility of cellulose (Zhu et al., 2008). The corrosive nature of dilute sulfuric acid requires expensive pipes and vessels. The intense conditions also require neutralization prior to fermentation. Alkali pretreatment processes use lower temperatures and pressures compared to other pretreatment technologies (Mosier et al., 2005). The process of lime pretreatment involves slurrying the lime with water, spraying it onto the biomass material, and storing the material for a specific duration, usually from days to weeks (Mosier et al., 2005). Pretreatment by ammonia fiber explosion (AFEX) uses a combination of high temperature and pressure to simultaneously reduce lignin content, partially remove hemicellulose, and break the crystalline structure of cellulose (Mosier et al., 2005). Another type of alkali pretreatment involves the use of aqueous ammonia at ambient conditions, which is described in detail below.

Many of these pretreatment technologies require high temperatures and/or high pressures. The extreme conditions increase digestibility of the biomass and decrease the reaction time required for pretreatment (Wyman et al., 1999). However, theses conditions significantly increase the capital and operating costs of proposed integrated biorefineries. Therefore, pretreatment technologies at ambient temperatures and pressures are of interest.

Aqueous Ammonia Soaking (AAS)

Ammonia has desirable characteristics as a pretreatment reagent in that it is an effective swelling reagent for lignocellulosic materials and is highly selectivity for reactions with lignin over those with carbohydrates (Wyman et al., 2005). Ammonia pretreatment works by breaking apart crystalline cellulose and acetyl linkages (Gollapalli et al., 2002). Pretreatment using ammonia has the potential to be efficient because ammonia is highly volatile, which suggests that it could be easily recycled (Wyman et al., 2005). Dale et al. (1986) suggests that residual amounts of ammonia following pretreatment may enhance the fermentation due to increased nitrogen content.

AAS is an ambient pressure and temperature process that is the pretreatment method selected for these studies. Kim and Lee (2005) pioneered soaking in aqueous ammonia as a means of pretreating corn stover. More recently, Kim et al. (2008) found a pretreatment of barley hull with 15 wt% aqueous ammonia at 75^{o}C for 48h at a 1:12 solid to liquid ratio removed 66% of lignin and retained the xylan and glucan fractions. Isci et al. (2007) explored different liquid to solid ratios and soaking durations for switchgrass by operating a soaking and rinsing system designed and fabricated by our group. It was concluded that a liquid to solids ratio of 5 L/kg for 5 d with 20x volume rinsing followed by simultaneous saccharification and fermentation (SSF) was effective for ethanol production (Isci et al., 2007). Using aqueous ammonia as a pretreatment reagent at ambient conditions retains the cellulose and hemicellulose approach to increase the fermentation yield and simplify the bioconversion scheme (Isci et al., 2007).

Scale

AAS and other pretreatment techniques have been explored at the bench-scale (Isci et al., 2007; Mosier et al., 2005; Kim and Lee, 2005). However, pilot-scale experiments are a necessary intermediate step between bench- and full-scale experiments (Isci et al., 2008). These experiments help estimate operational parameters and identify potential material handling and operational problems associated with scale-up prior to investing in expensive full-scale equipment. Only a handful of pilot-scale lignocellulosic biomass pretreatment systems have been previously described (e.g., Schell et al., 2003; Marchal et al., 1992). One objective of this work was to design, fabricate, test and operate a pilot-scale AAS biomass pretreatment system to safely and effectively generate pretreated biomass.

Biogas from Anaerobic Digestion

Anaerobic digestion (AD) takes place through the sequential action of four types of microorganisms (Figure 2): hydrolytic, fermentative, acidogenic, and methanogenic bacteria (Adney et al., 1991). Hydrolytic bacteria use cellulase enzymes to depolymerize cellulose in carbohydrates to simple sugars (Speece, 1996). Other compounds in the feedstock such as hemicellulose, proteins and lipids are also subject to enzymatic degradation. Fermentative bacteria convert simple organic compounds to organic acids through acidogenesis (Adney et al., 1991). Organic acids are then converted to hydrogen, carbon dioxide, and acetate by acetogenesis, which are then utilized in methanogenesis to produce methane and carbon dioxide, the end products of the reaction and constituents of the energy-rich biogas (Speece, 1996). Lignin within feedstocks can significantly inhibit biogas production rates because cellulose is unavailable to the hydrolytic bacteria (Adney et al., 1991). Introducing

pretreatment as a preprocessing step to AD frees cellulose and hemicellulose from the lignin structure (Taherzadeh and Karimi 2008).

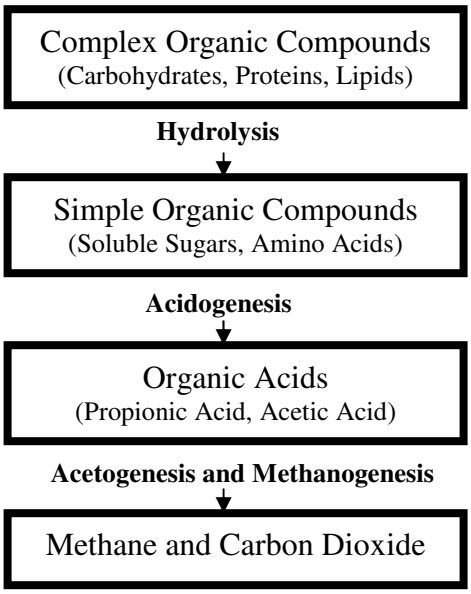

Figure 2. Biological pathway for anaerobic digestion (adapted from Adney et al., 1991)

As previously discussed, current ethanol technologies requires feedstocks with high fermentable carbohydrate levels (e.g. corn and sugarcane), or require pretreatment and enzymatic hydrolysis in order to release and convert fermentable sugars (Wyman et al., 1999). This technology requires extensive pre-processing of feedstocks and only yields fuel from a portion of the native biomass material. In contrast, biogas can be made from most biomass and waste materials, regardless of the composition, and over a large range of moisture contents, with limited feedstock preparation (Speece, 1996). Feedstocks for biogas production may be solid, slurries, and both concentrated and dilute liquids (Adney et al., 1991).

Most of the existing AD systems in the United States are processing residual sludge from wastewater treatment plants and while other facilities process wastes from chicken processing, juice processing, brewing, and dairy production (Schwietzke et al., 2006). The range of potential waste feedstocks is quite broad including: municipal wastewater, residual sludge, food waste, food processing wastewater, dairy manure, poultry manure, aquaculture wastewater, seafood processing wastewater, yard wastes, and municipal solid wastes (Labatut and Scott, 2008). Lignocellulosic material is the most abundant organic resource on earth thus a promising raw material for bioenergy production. Gunaseelan (1997), Chynoweth (1993), and Smith et al. (1992) provide extensive reviews of AD of various feedstocks, including lignocellulosic material for methane production. Lignin has been indentified to severely hinder cellulose decomposition under anaerobic condition in lignocellulosic biomass (Stinson and Ham, 1995) resulting in methane yields inversely related to lignin content (Smith et al., 1992). Pretreatment of lignocellulosic material modifies the lignin bonds freeing cellulose and hemicellulose enhancing the biodegradability and possibly increasing biogas production (Yadvika et al., 2004). Furthermore, the addition of commercial hydrolytic enzymes could potentially increase biogas composition and methane yields.

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CHAPTER 2. DESIGN AND TESTING OF A PILOT-SCALE AQUEOUS AMMONIA SOAKING BIOMASS PRETREATMENT SYSTEM

A paper submitted to *Applied Engineering in Agriculture* Journal

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Abstract

Scale-up of the aqueous ammonia soaking (AAS) biomass pretreatment method to 75-L soaking vessel size was accomplished in this work. The rationale for this effort grew out of need for approximately 6 to 10 kg of dry fermentation residues to feed a small gasifier on the Iowa State University campus. A novel, pilot-scale AAS system capable of pretreating 4 kg of switchgrass per cycle was designed, fabricated, and tested. Following pretreatment in the pilot-scale pretreatment reactors, the feedstock was subjected to simultaneous saccharification and fermentation (SSF) and subsequently gasified. The pretreatment process involved soaking biomass in reagent-grade 29.5% aqueous ammonium hydroxide at a liquid:solid ratio of 5 L/kg. Major reactor design criteria included the following: (1) limiting safety hazards by minimizing potential leakage of ammonia fumes from the system; (2) allowing thorough washing of the soaked biomass in the pretreatment reactor; and (3) simple, low-cost fabrication. Based on these constraints, commercially available 75-L HDPE tanks were selected as the primary vessels for pretreatment, with 2 mm fiberglass mesh screening on the vessel outlets to prevent biomass washout during rinsing. The vessels were operated outside, without agitation during the summer months in Iowa, with ambient temperatures ranging from 15 to 33°C during the experiments. During the first experimental cycle,

clogging of the outlet resulted in leakage from the vessel during rinsing, and led to redesign of the washout prevention system. The redesigned system used a “teabag” approach in which dry biomass was preloaded into a cylindrical mesh bag, and the filled bag placed into the soaking vessel. This modification eliminated outlet clogging, simplified biomass loading and unloading, but slightly reduced washing efficiency. Through five soaking cycles, an average of 22 to 25% delignification was achieved (Klason lignin basis) compared to the 35% removal seen at the bench-scale as reported by our group. Approximately 50 to 60% of the pretreated switchgrass was recovered, compared to 75% previously achieved at the bench-scale (Isci et al., 2007). Results were slightly lower than previously reported data by our group for a 1-L bench-scale AAS process, but were adequate for the subsequent SSF process as reported in Isci et al., 2008. Overall, the system provided effective and safe AAS pretreatment and washing of switchgrass prior to pilot-scale fermentation. This represents the first description of such a system in the literature. It is not suggested that this approach would be practical at full-scale, but rather that it is a realistic method of generating moderate quantities (ca. 10 kg/wk) of pretreated biomass for pilot-scale fermentation experiments and identifying potential obstacles that must be addressed as pretreatment methods are scaled-up to commercial scale in the move to second generation biofuels.

Introduction

Integrated biorefineries are expected to extract value from a complex feedstock through a variety of processing steps. For example, in one possible biochemical/thermochemical biorefinery, lignocellulosic biomass would be pretreated, hydrolyzed, and fermented to produce ethanol, subsequently the fermentation residue would be thermochemically converted to yield additional fuels, process heat, and a nutrient-rich, ash

residue suitable as a soil amendment. Returning this ash to crop fields closes nutrient cycles, reducing the energetic and economic costs of fertilization, and creating a more sustainable system (Anex et al., 2007). A proof-of-concept demonstration of this integrated biorefinery concept with nutrient recovery was undertaken using switchgrass feedstock, aqueous ammonia soaking (AAS) pretreatment, simultaneous saccharification and fermentation (SSF) to ethanol and conversion of the fermentation residue in a 5 kg/hr air blown fluidized bed gasifier located on the campus of Iowa State University . This gasification system required a minimum of approximately 10 kg of dry fermentation residue to achieve steady-state operation (Do et al., 2007). Upstream of the gasifier, 50- and 350-L fermentors were available for SSF. What was lacking was a means to pretreat sufficient quantities of biomass for fermentation that would meet the gasifier federate requirements.

Pretreatment of cellulosic materials is required to breakdown its complex structure making the cellulose and hemicellulose more accessible to enzymatic hydrolysis (Heitz et al., 1991). Many pretreatment methods have been developed and tested at the lab scale. Ammonia fiber explosion (AFEX), water with pH control, dilute acid, and lime treatment are all pretreatment methods capable of increasing biomass digestibility, but most require high temperatures and/or pressure, increasing capital and operating costs. Some alkali pretreatments use lower temperature and pressure while adequately removing lignin from biomass and maintaining the polysaccharides required for conversion downstream in biological processing (Mosier et al., 2005).

AAS – pioneered by Kim and Lee (2005) as a method of pretreating corn stover – was selected over other pretreatment methods for our work, because of its relative simplicity and effectiveness at ambient temperatures and pressures. Kim et al. (2008) have recently

explored the use of AAS to pretreat barley hull, while Isci et al. (2007) explored the use of AAS on switchgrass. In previously reported work (Isci et al. 2007), we designed and fabricated a system to soak and rinse switchgrass at the bench-scale (1-L vessel volume) and analyzed the effect of soaking time and liquid:solid ratios on lignin removal from switchgrass. We found a liquid to solids ratio of 5 L/kg and 5 d soaking time to be effective. To produce the 10 kg of dry residue required for the subject biorefinery concept demonstration using the bench-scale system described in Isci et al. (2007), would require approximately one thousand runs of the 1-L AAS system, which would have been both time and cost prohibitive. To save time and reduce costs, a pilot-scale pretreatment system was developed, and is described below.

AAS and other pretreatment techniques have been explored at the bench-scale (Isci et al., 2007; Mosier et al., 2005; Kim and Lee, 2005). However, pilot-scale experiments are a necessary intermediate step between bench- and full-scale experiments because they help estimate operational parameters and identify potential problems associated with scale-up prior to investing in expensive full-scale equipment. Although a handful of pilot-scale lignocellulosic biomass pretreatment systems have been previously described (e.g., Schell et al., 2003; Marchal et al., 1992), none of these systems could be easily adapted to handle a volatile corrosive pretreatment chemical like aqueous ammonia. Therefore, the objective of this study was to design and fabricate a pilot-scale soaking and washing system to safely and effectively generate aqueous ammonia pretreated switchgrass, and in doing so to indentify and report design, operation, and handling issues in order to aid others in future work.

Materials and Methods

Sizing of Soaking Vessels

Results from our previous study suggested a 75% recovery of biomass following AAS pretreatment (Isci et al., 2007) and a 50% residue recovery following SSF of the pretreated biomass (unreported results). Meeting the 10-kg feedstock requirement of the gasification system, and accounting for an anticipated reduction in recovery efficiency at pilot-scale (compared to bench-scale), yielded a target dry matter pretreatment capacity of 40 kg. Processing this amount of material could be done in a small number of large vessels or a large number of small vessels. Selecting the optimum number and size of vessels was done via an economic analysis with the goal of minimizing the total overall cost while taking into consideration less-quantifiable considerations such as safety and ease of material handling.

To begin the economic analysis, nine tank sizes were selected based on commercial availability and compatibility with ammonium hydroxide. The estimated price per container was determined for commercially available products, all of which were high density polyethylene (HDPE) (Options 1-3: Nalgene, Fisher Scientific, Hanover Park, Ill.; Options 4-6: Plastic Drums, Dawg, Inc., Terryville, Conn.; Options 7-9: Schutz IBC Indusrun Totes, Theisen's, Dubuque, Iowa). Vessel fabrication time was estimated based on the number of shop operations required. Fabrication time was then converted to a cost based on an estimated labor rate of $9/hr. Operational cost was again based upon an assumed labor rate ($9/hr) multiplied by the total time needed to process the biomass. Factors such as the number of times a vessel would be reused, the vessel cleaning time, setup time, and monitoring time were included in this computation. The total cost to process the requisite 40

kg of switchgrass was found by summing the capital cost, fabrication cost, and operation cost estimates.

Not surprisingly, the economic analysis indicated that the 1-L vessel was the most expensive option due to the labor costs associated with fabricating 20 vessels and with operating and cleaning them all 100 times. At the other end of the spectrum, the high capital cost of the 2000-L vessel, and it's relatively low use rate, led to a high total cost. Furthermore, the safety risks associated with high volumes of ammonium hydroxide in the 2000-L vessel were deemed unacceptable. For these reasons, both the 1-L and 2000-L vessels were eliminated from further consideration.

The remaining vessels were compared based on cost as shown in Figure 1. Four of the options were estimated to cost less than $500 per use, and we believed the difference in these were negligible compared to the uncertainty inherent in these estimates. We selected the 75-L vessel, primarily based on the expected ease of transportation, fabrication, and operation as compared with the larger but slightly cheaper alternatives.

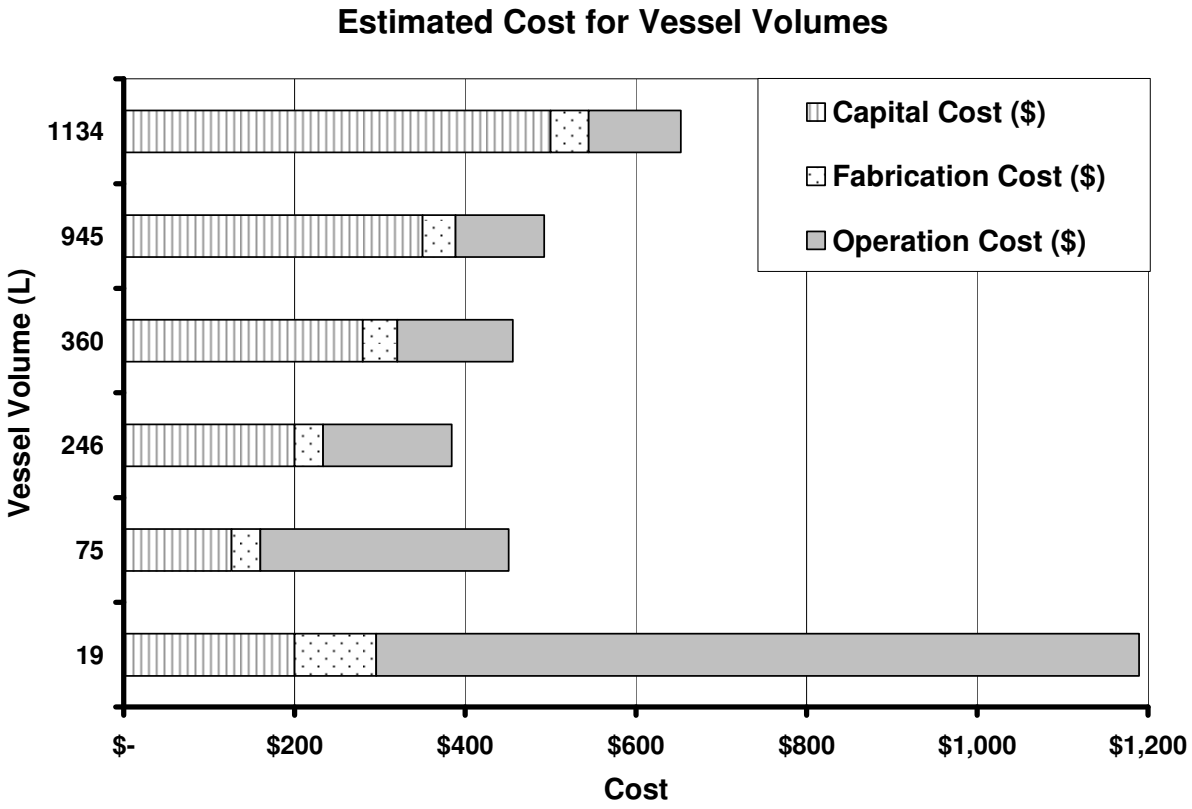


Figure 1: Total cost to process 40 kg dry biomass at 6 vessel sizes based on capital, fabrication and operation costs.

Biomass Washing System

Having selected a 75-L soaking vessel, the remainder of the system was designed and fabricated, with a goal of operating similarly to the bench-scale model described by Isci et al. (2007). A challenge in this regard was to ensure sufficient stirring of the biomass during the washing phase.

For proper stirring and washing of the switchgrass, agitation is needed, this in of itself proposed a potential challenge. At the bench-scale, a magnetic stir bar augmented the mixing created by the wash-water flushing through the vessel, but implementing mechanical mixing at the pilot-scale would be expensive and hazardous due to the necessity of positioning the

mechanical mixer directly in the aqueous ammonia. Therefore, agitation was provided solely by the flow of rinse-water through the reactor. For intense agitation, mixing power densities of 0.8 to 2.0 W/L are recommended (Geankoplis 1993). Accounting for the pump losses, and aiming for the high end of this range, because of the slurry-like nature of the soaked biomass, a 250 W pump (Model 43577, Wayne Reliant One, Harrison, Ohio) was selected to provide fresh water into the system and to agitate the solution. Wash water was introduced into the bottom of the soaking vessel via a PVC manifold with 3.2 mm (1/8”) holes on 2 cm centers (approx 60 total holes). Supplying rinse water on the bottom the vessel at high flow rates with a drain port near the top of the reactor provided thorough washing and agitation of the switchgrass. In preliminary testing, the effectiveness of this washing system was visually verified by adding red dye to the bottom of the system as water was pumped through the reactor (data not shown).

Additional Design Considerations

A 75-L container (Model PAK120, Dawg Inc., Terryville, Conn.) with a screw-top lid was used as the primary vessel for the soaking system. Since rinse-water was pumped into the soaking vessel, it was necessary to evaluate the pressure limits of the vessel. Based on material properties for HDPE, the estimated burst pressure for the vessel was 82 psi. Because the supply pump was rated at 11 psi, the system was considered safe from a burst standpoint. However, the screw top lid would likely leak at significantly lower pressures, estimated to be around 0.1 psi. Considering this during the design of the system suggested placement of the water inlet and outlet below the 75-L containers’ screw top lid rather than in the lid itself.

Safety Emphasis

Handling, storing, and disposing of ammonium hydroxide in a safe and environmentally acceptable manner was a major consideration at all stages of this experiment. Ammonia gas volatilized from the ammonium hydroxide solution poses a significant health hazard due to irritation or burning of skin or eyes. Inhalation of concentrated ammonia fumes causes similar damage to the upper respiratory tract and can be fatal at moderate exposure levels. A multi-step approach was employed to mitigate this risk, including the use of engineering controls, administrative controls, and personal protective equipment. Specifically, because the primary risk was due to the volatile nature of the ammonia, the soaking vessel and handling systems were designed to minimize the possibility of gaseous emissions. The experiment was carried out at a cordoned-off location away from buildings and populated areas. Major equipment was labeled with content and contact information, and the soaking vessels were placed in secondary containment vessels to avoid ground contamination, if leaks occurred. Whenever ammonium hydroxide was handled, there were always more than two people on site with one serving as an observer and safety monitor. Full-face respirators (6000 series with ammonia cartridges, 3M, St. Paul, Minn.), ammonia compatible gloves (0.016 in non-flocked nitrile gloves, Fisher Scientific), non-permeable aprons (cat. S47382, Fisher Reusable Vinyl Aprons, Fisher Scientific), and lab coats were worn by the personnel at all times working with the vessels, while they contained ammonium hydroxide or when handling the fresh or spent ammonium hydroxide.

Experimental Procedure

The original intent was to operate the soaking system six times to treat the desired 40 kg of dry switchgrass. After the soaking system had been designed and constructed, the estimated biomass requirement was reevaluated at 24 kg dry switchgrass. However, because of problems encountered during the first run, a design change was made. Because the operational problems did not reduce pretreated biomass quality, the biomass from the first run was used as the pretreated feedstock for a preliminary 50-L pilot-scale fermentation (Isci et al., 2008).

The biomass used in these experiments was *Cave-in-Rock* cultivar switchgrass harvested from dormant mature stands in Chariton, IA. Its composition was determined by the Iowa State University Department of Agronomy using the ANKOM method (ANKOM Technol. Corp., Fairport, N.Y.) as described by Vogel et al., (1999). Klason lignin was determined as described by Crawford and Pometto (1988), slightly modified by Isci et al. (2007). Composition of the untreated switchgrass was 32% cellulose, 31% hemicellulose, 4.4% acid detergent lignin, 27% Klason lignin and 0.7% ash.

In the first soaking run (Run 1), switchgrass was loaded directly into the soaking system. After loading 4 kg of switchgrass into each soaking system, a screening system constructed of 2 mm fiberglass mesh (Fiberglass Screen, New York Wire, Mount Wolf, Pa.) was installed above the switchgrass to keep the switchgrass from clogging the outlet during rinsing. This screening system was attached above the inlet and below the outlet in the inside of the container with screen retainer strips (US Patent 6250040, Screen Tight, Georgetown, S.C.), with the hook retainer surface attached to the vessel interior using adhesive (Quick Gel Super Glue, Duro, Avon, Ohio). In addition to the bulk switchgrass loaded into the container,

six mesh bags containing 20 g switchgrass each were installed in various locations (radially and at two heights) in the vessel to determine the spatial uniformity of the soaking and washing processes.

Because of problems encountered with the direct-loading method, in soaking runs 2 – 5, switchgrass was loaded into a large cylindrical mesh bag (Fiberglass Screen, New York Wire, Mount Wolf, Pa.) that was then placed in the soaking vessel (fig. 2). To test the uniformity of this method, sample bags containing 20 g of switchgrass each were placed in even increments along the length of the large cylindrical mesh bag; when the large bag was coiled into the vessel, this meant that the sample bags were distributed as shown in Figure 3. Because the biomass was constrained within the bag, no screening system was installed over the vessel outlet for these runs.

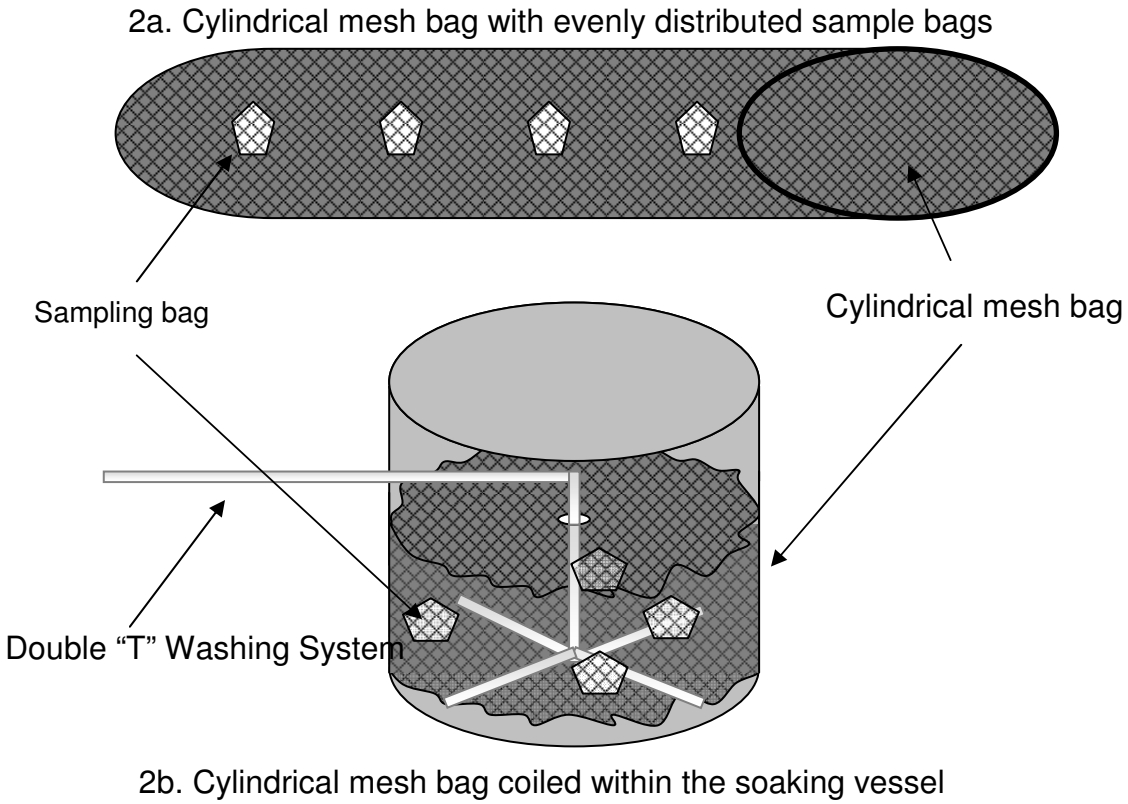


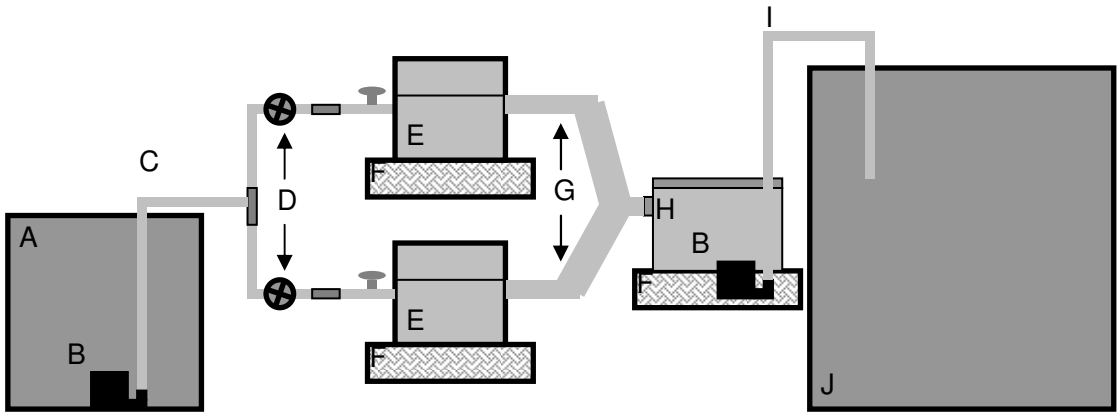
Figure 2a: Cylindrical mesh bag was loaded with 4 kg switchgrass and sample bags at even increments and the end of the mesh bag was cinched closed by elastic drawstring. 2b. The cylindrical mesh bag was loaded into the soaking vessel around the vertical inlet pipe of the double "T" washing system.

In both methods of operation, the process began by removing the vessel top, loading 4.0 kg of switchgrass, and adding reagent grade 29.5% aqueous ammonium hydroxide to achieve a ratio of 5 L/kg. The lid was then replaced and secured, and the switchgrass soaked for 5 d. The reagent grade aqueous ammonia was purchased in a 196 L drum (cat A669-385LB, Fisher Scientific) and was pumped into the soaking vessels using a hand pump (PMP 101, Dawg Inc., Terryville, Conn.) with a buttress fitting (70mm buttress adapter BRE, BA-Industrial, Muldrow, Okla.). During soaking, the PVC outlet of the system was covered using

a plastic bag to reduce ammonia volatilization from the vessel. We intentionally avoided a truly airtight seal to avoid accidental pressurization of the vessel.

The experimental site was set up as shown in Figure 3. Following the 5-d soaking process, the rinse pump was submerged in the 250-L full-scale reservoir; the pump was connected to the vessel inlet via a 3-cm diameter corrugated hose, energized, and used to flush the treated switchgrass. Ball valves on the inlet allowed for rinse-water flow rate control and simultaneous rinsing of both soaking vessels. During flushing, rinsate flowed into the 75-L outlet container via a 4-cm PVC pipe (PVC-1120, Silver-Line, Asheville, N.C.). A second 250 W pump was used to transfer the ammonia-laden rinsate to the 2000-L holding tank.

Approximately 250 L of fresh water flushed through each soaking vessel to remove the ammonia from the switchgrass, yielding a rinse volume of approximately 12x the initial aqueous ammonium hydroxide dose. This level of rinsing was demonstrated to be adequate in previous bench-scale experiments (Isci et al., 2007). At this rinsing level, a significant amount of nitrogen-rich rinsate was generated which was land applied at an agronomic rate at the research site, with approval from the Iowa State University Environmental Safety and Health unit.



A: 250 L Fresh water containment
B: 250 W Submersible pump
C: Inlet hose
D: Flow Valve
E: 75-L Soaking vessel with screw top lid
F: Secondary vessel containment
G: Soaking outlet hose
H: 75-L Outlet container with cover
I: Rinsate outlet hose
J: 2000 L Rinsate containment

Figure 3: Pilot-Scale Soaking System

Following washing, the cylindrical mesh bags were removed from soaking vessels and drained. Pretreated switchgrass at approximately 80% moisture content was then transferred to 4-L poly bags (poly bag, cat. 288807, Associated Bag Company, Milwaukee, W.I.) and frozen at -20^{o}C until needed for pilot-scale fermentation. The switchgrass sampling bags were oven dried overnight at 105^{o}C and ground to 1 mm for fiber and Klason lignin analysis (per Isci et al., 2007).

Results and Discussion

In Run 1, the screen, which had excess fabric, was forced into the outlet by the upwelling switchgrass and rinsate. This in turn partially clogged the outlet and caused

pressurization of the vessel and leakage of rinsate from the cap seal. Placing a weight atop the screen temporarily solved this problem during Run 1, but additional challenges in loading and unloading the switchgrass motivated a redesign. The six sample bags, containing 20 g of switchgrass from Run 1, were analyzed to determine cellulose and hemicellulose content (fig. 4). Consistent cellulose and hemicellulose content in various sample locations within the soaking vessel (fig. 4) demonstrated uniformity of both soaking and washing operations. In Run 1, the average post-soaking cellulose and hemicellulose concentrations were 48% and 23% respectively, with a variance among the samples of 2 and 4%, respectively. These results are similar to those we reported at the bench-scale: 56.6% cellulose and 23% hemicellulose (Isci et al., 2007). We attribute the slightly lower cellulose concentrations at the pilot-scale to the loss of fine particles from the system during washing.

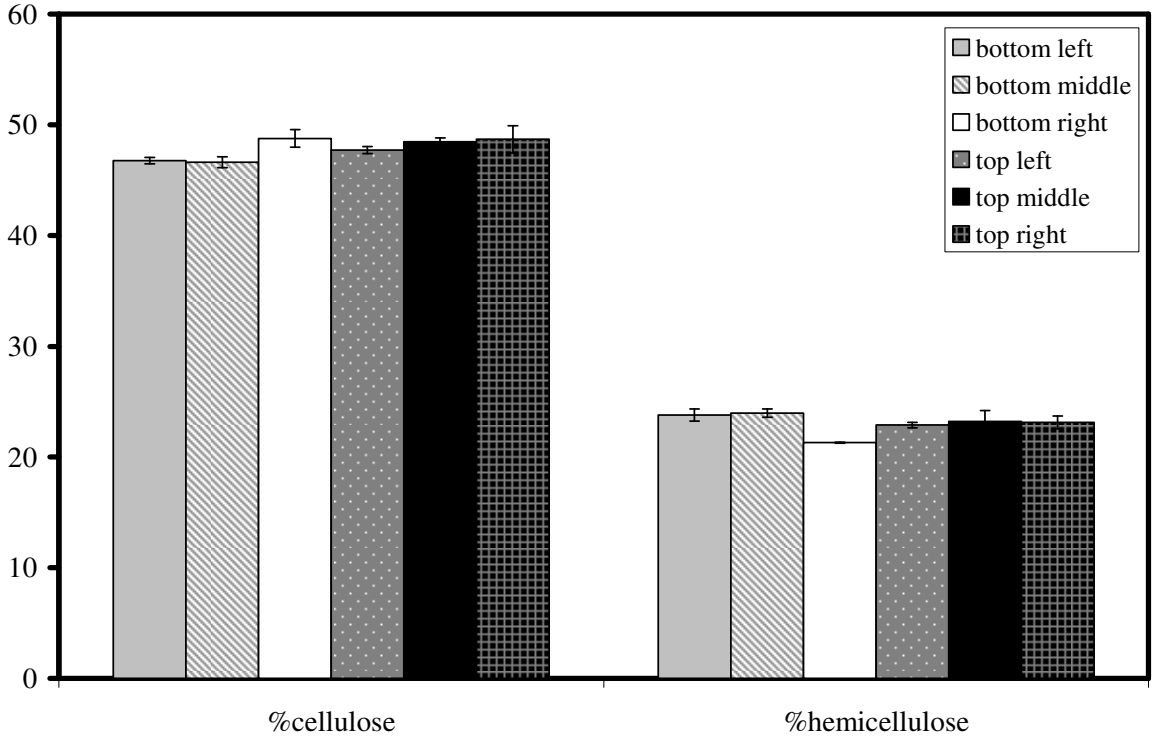


Figure 4. Percentage (w/w) cellulose and hemicellulose at various locations in one soaking vessel during the first run (n=3).

The redesigned vessel was operated with a cylindrical mesh bag into which the switchgrass was loaded (the "teabag" approach). The uniformity of pretreatment using the teabag method was evaluated by Ankom fiber analysis of small sample bags containing 20 g of switchgrass distributed throughout the biomass during pretreatment. Cellulose (45%) and hemicellulose (23%) content were slightly less consistent in the cylindrical mesh bag runs (fig. 5) with a variance among the samples of 6 % for cellulose and 2 % for hemicellulose. One disadvantage of the mesh bag approach used in Runs 2–5 was that agitation during rinsing did not appear to be as thorough as in the initial design. This was suggested by visual

observations at the end of the rinsing (when ammonia concentrations were low), and by a faint smell of ammonia from the rinsed switchgrass which was not noted in Run 1. Future designs could overcome this by reducing the amount of switchgrass in each vessel or by providing better sealing on the vessel top and allowing for higher rinse-water flow rates for greater agitation.

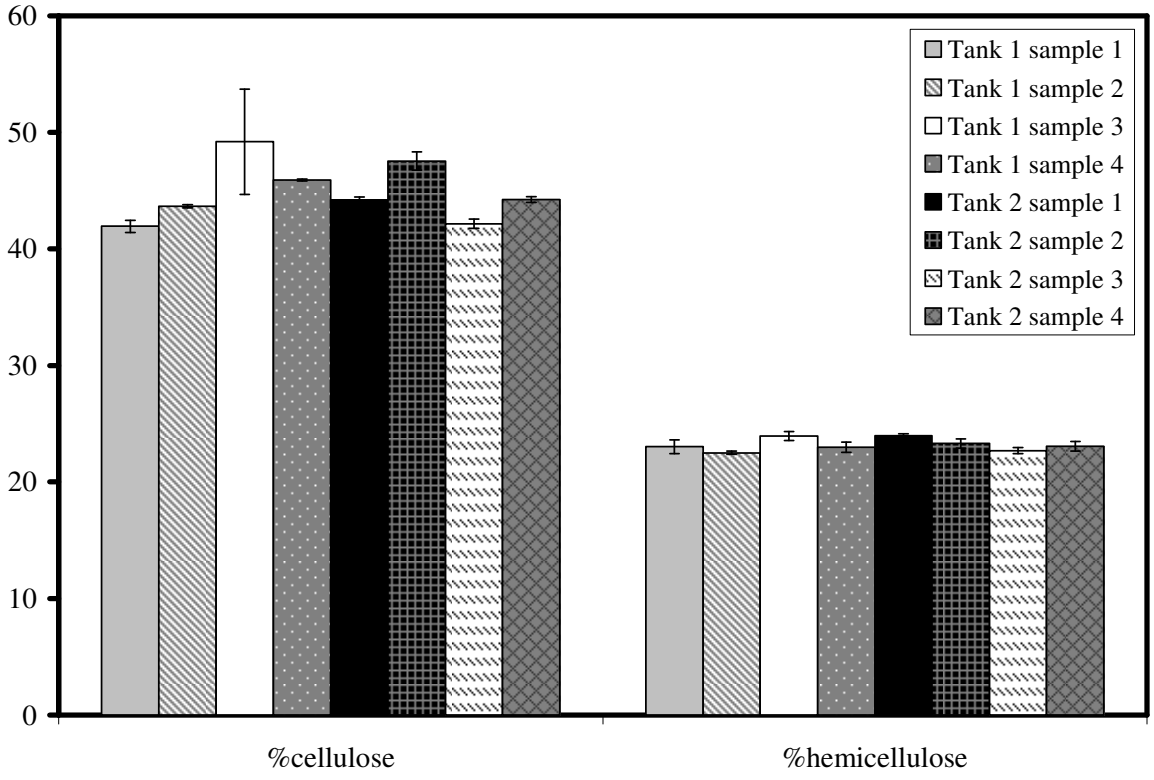


Figure 5. Percentage (w/w) cellulose and hemicellulose from various locations in two soaking vessels during the second trial using cylindrical mesh bag (n=3).

As at the bench-scale, pilot-scale AAS proved to be an effective method for preserving the cellulose fraction in the switchgrass. Percent cellulose increased in both runs, from 32 to 48% in Run 1 and to 45% in Runs 2–5, these changes were similar to those

reported by Isci et al. (2007) at the bench-scale. Percent hemicellulose decreased in all runs, from 31 to 23% based on the untreated biomass weight, this decrease is an expected characteristic of AAS of switchgrass (Isci et al., 2007). Klason lignin decreased by nearly 25% in the pilot-scale experiments, a smaller drop than the 37% decrease seen at bench-scale (Isci et al., 2007). We attribute this reduced delignification to the less thorough rinsing, particularly with the teabag method implemented in Runs 2 – 5. Breaking the structure and partially removing lignin is a desired characteristic of biomass pretreatment, because it allows the cellulose and hemicellulose to be more accessible to enzymatic hydrolysis. Isci et al. (2008) demonstrated that the pilot-scale AAS system adequately pretreated switchgrass for subsequent SSF yielding 52-74% of maximum theoretical ethanol yields.

The redesigned vessel significantly improved the ease of fabrication and operation of the system over the initial design and only slightly reduced pretreatment efficacy. The cylindrical mesh bag vessel reduced safety hazards because the system was less likely to leak due to clogging. Clearly, the methods developed and described herein are not suitable to full-scale AAS systems, which will likely rely on metal vessels and automated solids handling systems. However, the methods described here work well for small-pilot-scale projects needing AAS pretreated biomass.

Conclusion

A method for generating kilogram-quantities of aqueous ammonia soaked pretreated biomass was developed and demonstrated. The experiment showed that aqueous ammonia soaking can be operated at pilot-scale with relatively inexpensive equipment. Based on economic, safety and convenience factors, a 75-L soaking vessel was selected and shown to be effective in pretreating 4 kg of switchgrass with 20-L of aqueous ammonia. Multiple such

soaking vessels can be run at one time; in this work, we ran two simultaneously. Ammonia soaking for 5 d at 5 L/kg at the pilot-scale increased cellulose content and decreased hemicellulose and Klason lignin content of the remaining solids in a similar manner as observed in bench-scale experiments. The pretreated switchgrass was successfully used in subsequent pilot-scale fermentations (results reported elsewhere). To our knowledge, this is the first description of pilot-scale aqueous ammonia soaking biomass pretreatment system. Key challenges overcome in our effort included the handling of multi-liter quantities of aqueous ammonia, the separation of biomass from rinsate, and the disposal of over 1000-L of ammonia-enriched rinsate. Large-scale application of the AAS method will need to address safety, separation, and ammonia recycling issues that were encountered here.

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CHAPTER 3. AQUEOUS AMMONIA SOAKING AND OTHER PRETREATMENTS OF TRANSGENIC VARITIES OF POPLAR

Prepared as a summary report as part of the Biorenewable Resources and Technology International Exchange Program at the University of Gent

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Abstract

Multiple genetic modifications have been made to poplar (*Populus spp*) to reduce their lignin content in hopes of facilitating their processing into fiber and fuel. In this study, one wild type and eight transgenic strains of poplar were sampled and pretreated by five techniques: untreated, aqueous ammonia soaking (AAS), dilute acid, aqueous ammonia soaking with hydrogen peroxide supplement (AAS-HP), and the organosolv method. Acid soluble lignin and acid insoluble lignin were compared from each tree and each pretreatment. Pretreatment techniques were successful in removing both acid soluble lignin (ASL) and acid insoluble lignin (AIL) from the wild type and transgenic varieties. AAS pretreatment was successful in removing approximately 15% of ASL and AIL. Dilute acid pretreatment removed ASL but less than 5% of AIL. AAS-HP was successful in removing ASL and AIL, particularly in the case of plants with modifications to the CCoAOMT enzyme which is a methylating enzyme for lignin precursors. Organosolv pretreatment was the most successfully in deligninification, removing an average of 65% ASL and 43% AIL throughout the wild type and transgenic plants varieties. Among the eight transgenic lines evaluated,

ASOMT2B(ASCOMT) was the most successful variety with regards to delignification across the board of pretreatments. In general, modifications to the lignin biosynthesis pathways in transgenic poplar plants improve the delignification of subsequent pretreatment methods by removing 12-70% ASL and 5-52% AIL.

Introduction

Even with the potentially promising future of lignocellulosic biofuels and renewable products, major technical obstacles hinder the large-scale adoption and economic feasibility of these developing technologies (Wyman, 2007). A major hindrance arises from recalcitrant structure of lignocellulosic plant cell walls that contain embedded cellulose (Mosier et al., 2005). To ensure successful biological conversion of lignocellulosic biomass, the chemical linkages between lignin, cellulose, and hemicellulose components of the cell wall must be broken through a pretreatment step (Mosier et al., 2005). Pretreatment is expected to be one of the most costly steps in the conversion of lignocellulosic material to biofuels and bioproducts (Wyman et al., 2005). Costly pretreatment has motivated the genetic engineering of potential bioenergy crops, to make their cell walls susceptible to pretreatment and thus more amenable to hydrolysis (Ragauskas et al., 2006). It is hoped that such approaches will improve the economic viability of lignocellulosic ethanol.

Cellulose and lignin are the two most abundant biopolymers on earth (Boerjan et al., 2003) and as a major component of plant cell walls, lignin has a far-reaching impact on agriculture, industry, and the future of lignocellulosic biofuels. For example, in the pulp and paper industry, lignin must be extracted by expensive and environmentally hazardous processing to produce a high-quality paper (Brown, 2003). Driven by its significance in the economics of these and other industries, lignin has been studied intently over the last century,

with breakthroughs in the last decade allowing manipulation of lignin structure, composition, and content in a variety of plant species including poplar (Boerjan et al., 2003).

Poplar (*Populus* spp.) is a fast growing trees produced for pulp, lumber, strand board, plywood, fuel, wildlife habitat, and ornamental reasons. This perennial tree requires minimal chemical inputs, making it a promising candidate for biofuels production (Baucher et al., 2003).

Transgenic poplars with modified lignin biosynthesis pathways were evaluated in this study. The lignin polymer is primarly produced via the dehydrogenative polymerization of three different cinnamyl alcohols (*p*-coumayl, coniferyl, and sinapyl alcohol) (Boerjan et al., 2003). In lignin, these alcohols are polymerized to form *p*-hydroxyphenyl (H), guaicyl (G), and syringyl (S) units (Baucher et al., 2003), the building blocks of lignin (Chen and Dixon, 2007). The composition of polymer units depends on the parent material and the enzymatic pathways (Baucher et al., 2003). Li et al., (2008) specified the 10 primary enzymes used in lignin biosynthesis, three of which that are focused on in this paper: caffeoyl CoAO-methyltransferase (CCoAOMT), hydroxy-cinnamoyl CoA reductase (CCR), and caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT).

Baucher et al. (2003) reviewed the up- and down-regulation of these enzymes, which are thought to reduce lignin content or modify lignin composition. Enzyme down-regulation has been shown to affect lignin content and composition thereby impacting the efficiency of pulping. In one trial, laboratory-scale Kraft pulping was performed on two lines of field-grown transgenic poplar with down-regulated COMT enzyme and two lines with down-regulated CAD enzyme. This study demonstrated that the COMT transgenic plants were

more resistant to Kraft delignification than the control, however, the CAD transgenic plants were more easily delignified than the control (Baucher et al., 2003).

Altering the enzyme expression in the lignin biosynthesis pathway in poplar resulted in variable effects on the lignin modification and pulping (e.g. Baucher et al., 2003; Chen et al, 2001; Li et al., 2008; Chen and Dixon 2007). Some enzyme modifications produced improved delignification following Kraft pulping (Chen et al., 2001) and some enzyme modification resulted in no change or worse Kraft delignification (Baucher et al., 2003). Similar to paper pulping, the lignocellulosic ethanol industry could benefit from selecting transgenic plants more susceptible to delignification (Chen and Dixon, 2007). However, an array of enzyme modifications and various pretreatment methods must be examined.

Mosier et al. (2005) provides an extensive overview of leading technologies for lignocellulosic biomass pretreatment for bioethanol production. However, defining a single "most efficient" method of pretreatment is not feasible due to the diverse nature of lignocellulosic biomass (Mosier et al., 2005), thus crop-specific research is needed in order to promote the commercialization of second generation biofuels. For this reason, four pretreatment methods were selected to provide a basic understanding of the delignification of transgenic poplar plants.

Removing lignin with alkaline chemicals to improve cellulose digestibility has been evaluated on several types of biomass (Mosier et al., 2005). Aqueous ammonia soaking (AAS) is an ambient pressure and temperature process that has been successful in the delignification of various feedstocks (Isci et al., 2007). Kim and Lee (2005) pioneered soaking in aqueous ammonia as a means of pretreating corn stover. More recently, Kim et al. (2008) found a pretreatment of barley hull with 15 wt% aqueous ammonia at 75^{o}C for 48h at

a 1:12 solid to liquid ratio removed 66% of lignin and retained the xylan and glucan fractions. Isci et al., (2007) used switchgrass as a feedstock and explored different liquid to solid ratios and soaking durations by operating a biomass pretreatment system designed and fabricated by our group. It was concluded that a liquid to solids ratio of 5 L/kg for 5 d with 20x volume rinsing followed by simultaneous saccharification and fermentation (SSF) to be effective for ethanol production (Isci et al., 2007).

Acid pretreatment methods are extensively researched on a variety of feedstocks over a range of different acids, concentrations, temperatures, and durations. Jacobsen and Wyman (1999) and Lee et al. (1999) provide reviews of acid pretreatment methods. Most commonly, sulfuric acid is added to remove hemicellulose, increasing the digestibility of cellulose in the remaining biomass.

Hydrogen peroxide has been used as a supplement to reagents used in pretreatment in both acidic and alkaline methods (Mosier et al., 2005). Aqueous ammonia with the addition of hydrogen peroxide has previously been tested on combination of corn stover and cobs using a percolation reactor with an array of concentrations and temperatures (Kim and Lee, 1996). The addition of hydrogen peroxide increased the degradation of lignin by breaking carbon-carbon linkages in lignin and is commonly used in the pulp and paper industry as bleaching agent (Kim and Lee, 1996).

In the organosolv process, an organic or aqueous organic solvent mixture with inorganic acid catalysts is used to break the internal lignin and hemicellulose bonds. Organosolv is considered attractive because it allows for the fractionation of lignocellulosic biomass into a series of valuable chemical products, which have a combined commercial value exceeding that of the biofuel alone (Pan et al., 2006). The biomass fraction composed

of lignin and hemicellulose is partially hydrolyzed and dissolved into a liquor resulting in a cellulose-rich fiber in the remaining biomass which is ready for subsequent SSF. The lignin and hemicellulose liquor is processed to recover separate streams of high-purity lignin (for use in resins and a large number of other applications), as well as furfural, acetic acid from the hemicellulose. The ethanol used in the process is recovered by distillation and recycled back to the process. This treatment has traditionally been utilized in the pulp and paper industry. Pan et al. (2006) investigated this organosolv fractionation process on poplar at various temperatures, time, catalyst dose and ethanol concentration using an elaborate four-vessel, rotating digester, resulting in a 74% lignin removal at the highest catalyst dose for the longest duration and highest temperature.

The objective of this study was to determine the difference in lignin, acid soluble lignin (ASL) and acid insoluble lignin (AIL), for eight transgenic poplar varieties and the wild type following AAS, dilute acid, AAS with hydrogen peroxide, and organosolv pretreatment.

Materials and Methods

Feedstock Samples

In Ardon, France, poplars were micropropagated, and then acclimated in a greenhouse study. Following greenhouse studies and authorization from the Minister of Agriculture, transgenic poplar trees were planted in the field at 1.5 x 3 m density, as described by Baucher et al. (2003) and Pilate et al. (2002). During this study, tree phenology was recorded each spring. No difference in bud burst timing was evident for the transgenic lines in any year. None of the transgenic lines showed any significant difference from wild-type trees in height or trunk diameter (Pilate et al., 2002). Trees from this study as described

by Baucher et al. (2003) and Pilate et al. (2002) were obtained and were selected to focus on transgenic poplar plants with downregulation modifications of the CCoAOMT, CCR, or COMT enzymes in the lignin biosynthesis pathway.

Trees were de-limbed and manually debarked. Initial size reduction was done in a wood grinder. Further size reduction was done by a Retsch cutting mill SM 2000 with a 2 mm screen. Five trees of each modification were pooled to make up one sample resulting in one wild type sample and eight transgenic samples with different genetic modifications (Table 1).

Table 1: Description of sample ID and gene modification

Sample #	Lignin Mutant in Transgenic Plant
1	Wild type
2	101 (ASCCoAOMT)
3	416 (SCCoAOMT)
4	429 (SCCoAOMT)
5	WT52-3 (SCCR)
6	WT62-13 (ASCCR)
7	ASOMT2B (ASCOMT)
8	ASOMT10B (ASCOMT)
9	823 (ASOMT10B/SCCoAOMT)

Transgenic samples 2-4 and 9 had modifications to the CCoAOMT enzyme, samples 5-6 had modifications to the CCR enzyme, and samples 7-8 had modifications to the COMT enzyme.

Pretreatment

Four pretreatment techniques were selected to be performed on the 9 samples. Compositional analysis following each pretreatment was performed to determine the effect of the specific pretreatment.

AAS was performed by soaking samples at 5 L/kg in 30% ammonium hydroxide for 5 days at ambient conditions. Following soaking, the pretreated biomass was thoroughly washed, using distilled water, with 10x the initial volume of ammonium hydroxide.

Dilute acid pretreatment was performed using 1 N hydrochloric acid at a solid to liquid loading ratio of 1:100 at 80^{o}C for 2 h in double-neck round-bottom flasks equipped with reflux columns and magnetic stir bars. Reflux columns were used to prevent the evaporation of acid during the heating period. Following the incubation period, the pretreated poplar was washed extensively with 10x the initial acid volume with distilled water.

Samples were pretreated by soaking in 5 L of 30% ammonium hydroxide/kg switchgrass with the addition of 1 L of 10% hydrogen peroxide/kg switchgrass. Following the soaking, the pretreated biomass was thoroughly washed, using distilled water, with 10x the initial solution volume.

Samples were cooked in 50% aqueous ethanol at a solid to liquid loading ratio of 1:50 and 2% concentrated sulfuric acid for 1 hour at 121^{o}C in an autoclave to achieve organosolv pretreatment. Ten grams of sample were cooked in each 100 mL serum bottles that were capped and sealed. Samples were cooled to room temperature and washed three times using 10 mL of 50% aqueous ethanol at 60^{o}C and then with 100 mL of DI water.

Compositional analysis

Total solids were determined by a Precisa XM60 automatic infrared moisture analyzer according to the National Renewable Energy Laboratory procedure (NREL LAP001). The automatic infrared moisture analyzer was programmed for a standby temperature of 70^{o}C and an analysis temperature of 105^{o}C. The endpoint of analysis was

selected as a weight change of less than 0.05% in one minute. All samples were analyzed in triplicate.

The composition of untreated samples of poplar was analyzed using two-step acid hydrolysis according to the procedure published by the NREL. The dried samples were treated with 3 ml of 72% H_2SO_4 and placed in a water bath at 30^{o}C for 1 h and were stirred every 5 minutes. The samples were diluted to 4% H_2SO_4 by adding 84 ml of Milli-Q water and were autoclaved for 1 h at 121^{o}C. After cooling, the liquid and solid fractions were separated using pre-weighted glass fiber filters under negative pressure. The liquid fraction was collected from which a 20 mL aliquot was neutralized with $CaCO_3$ to pH 5-6 and decanted. The decanted was centrifuged and filtered this fraction is referred to the carbohydrate liquor.

Lignin content was determined in two steps. The remaining liquid fraction from the acid hydrolysis was diluted and analyzed on a UV-Visible spectrophotometer at 240 nm to determine acid soluble lignin (ASL). Acid insoluble lignin (AIL was determined by weighing the dried glass fiber filters containing the solid fraction. This same procedure was repeated on all samples following designated pretreatment.

Experimental data were statistically analyzed using the GLM procedure (SAS Institute, Cary, NC). The effects of pretreatment and transgenic plant on the ASL and AIL were analyzed using least square means procedure ($P < 0.05$).

Results and Discussion

Lignin was fractionated and quantified into two components: ASL and AIL. This section begins by discussing the ASL results across genotypes and pretreatments, then

follows with a similar section for AIL. Finally, this section ends, with a discussion comparing the ASL and AIL results.

The ASL composition of untreated samples varied from 2.7 to 3.0 % (Figure 1). No difference in ASL content was found between the wild type samples and the 101(ASCCoAOMT) and WT52-3(SCCR) transgenic plant samples. The transgenic plant with modification ASOMT10B(ASCOMT) had the least ASL but varied from the wild type by 10% (w/w).

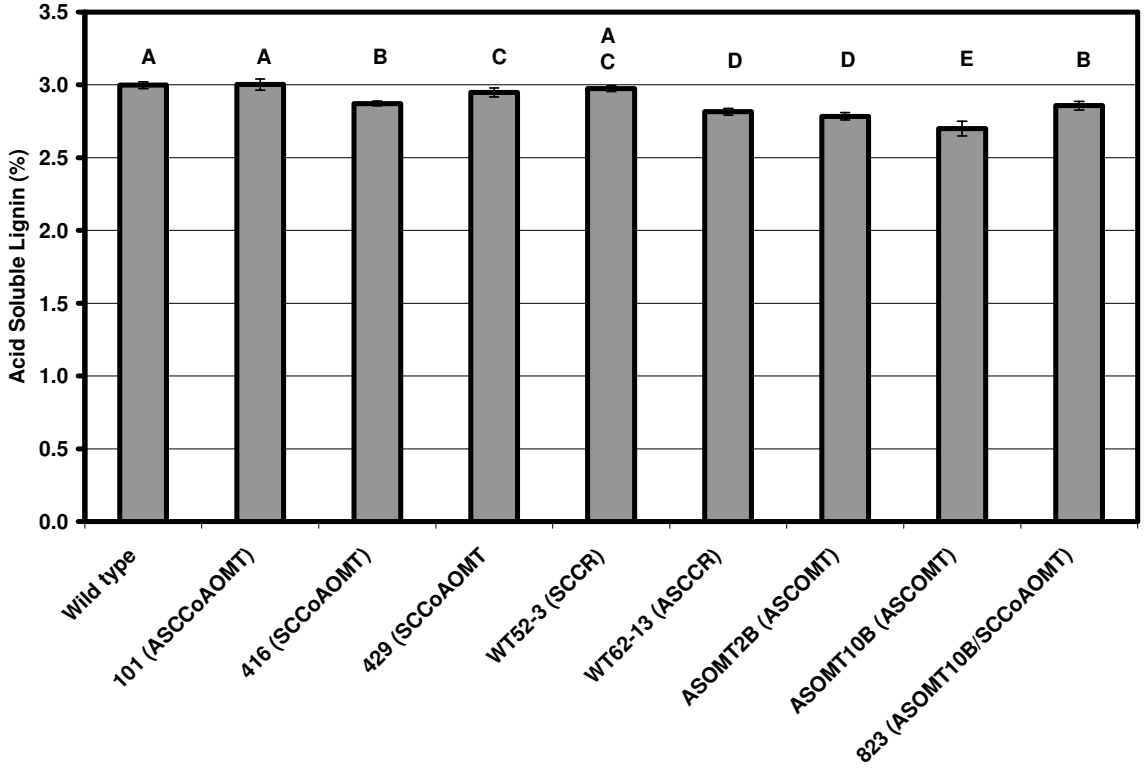

Figure 1: ASL of Untreated Samples (n=3) where each letter signifies a significant difference (P <0.5)

The ASL variations in AAS-pretreated poplar were larger than in the untreated samples, ranging from 1.8 to 2.6% (Figure 2). AAS pretreatment produced no significance

difference between the wild type and the WTS2-3(SCCR) transgenic plant compared to untreated samples, both the wild type and the WTS2-3(SCCR) transgenic plant had 15% reductions of ASL after AAS pretreatment. However, the AAS pretreated transgenic plant 101(ASCCoAOMT) displayed a 40% reduction in ASL over untreated samples of the same variety which was the greatest removal of ASL following AAS pretreatment.

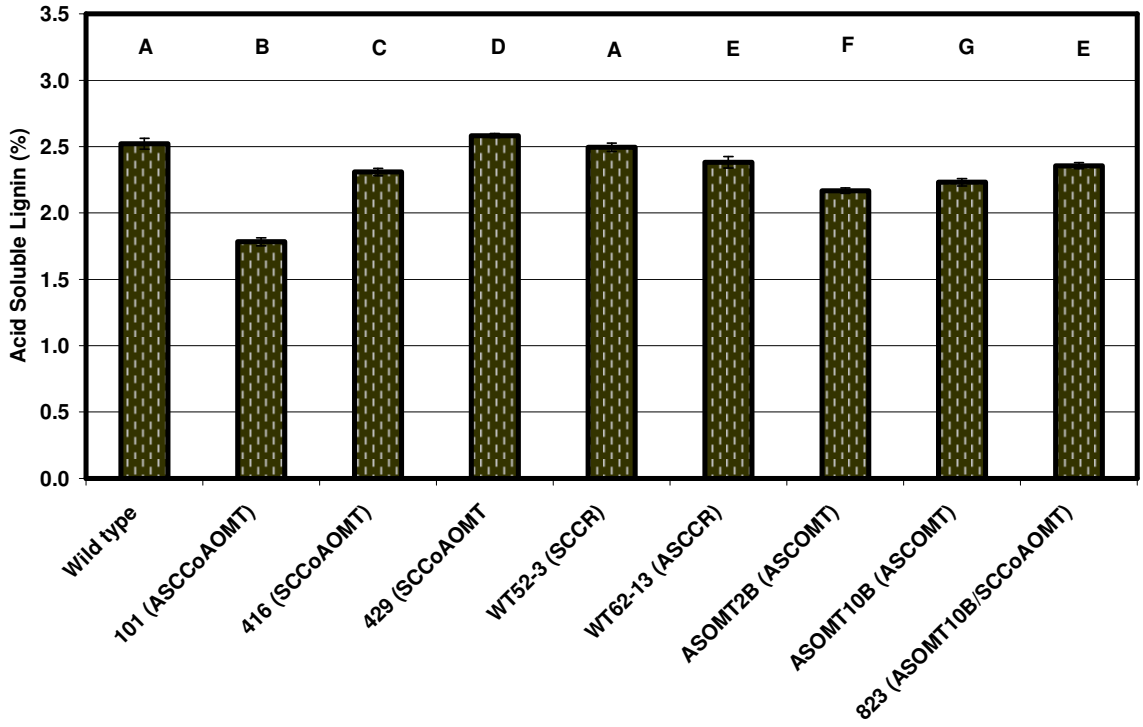

Figure 2: Acid soluble lignin after AAS pretreatment (n=3) where each letter signifies a significant difference (P <0.5)

Unsurprisingly, dilute acid pretreatment produced the greatest reduction in ASL and the greatest variability in remaining ASL (1.1-2.0%) (Figure 3). Interestingly the lowest ASL were in the wild type and the 416(SCCoAOMT), averaging approximately 61% reduction

over the untreated samples. The 101(ASCCoAOMT) which was effectively treated by AAS achieved a 50% reduction with dilute acid.

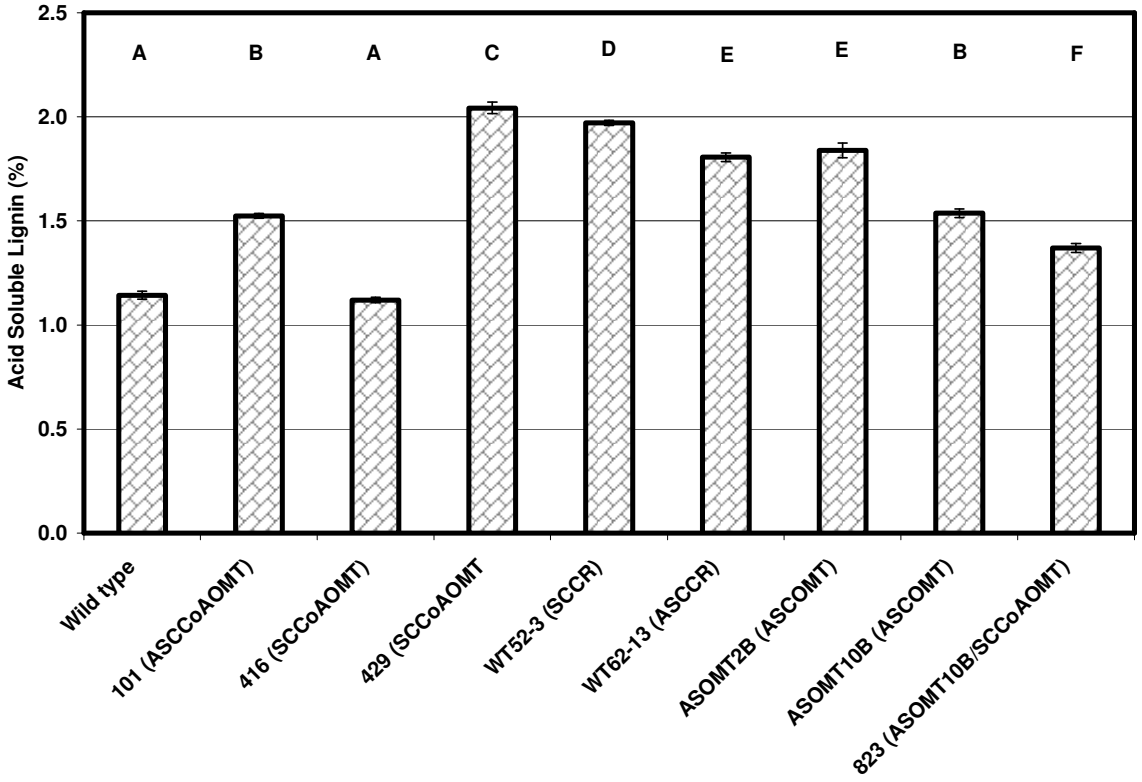

Figure 3: Acid soluble lignin after dilute acid pretreatment (n=3) where each letter signifies a significant difference (P <0.5)

The ASL variations in AAS with hydrogen peroxide supplement ranged from 1.6 to 2.5% (Figure 4). AAS-HP pretreatment produced consistent result amongst the modification to the CCoAOMT enzyme in the lignin biosynthesis pathway. The 101(ASCCoAOMT), 416(SCCoAOMT), 429(SCCoAMT), and 823(ASOMT10B/SCCoAMT) all had a reduced ASL content of approximately 50% over the corresponding untreated varieties suggesting that AAS-HP was successful in consistently removing ASL in plants with the CCoAOMT enzyme modification. Transgenic samples had a lower ASL content than the wild type variety following AAS-HP pretreatment except for the ASOMT10B(ASCOMT) sample.

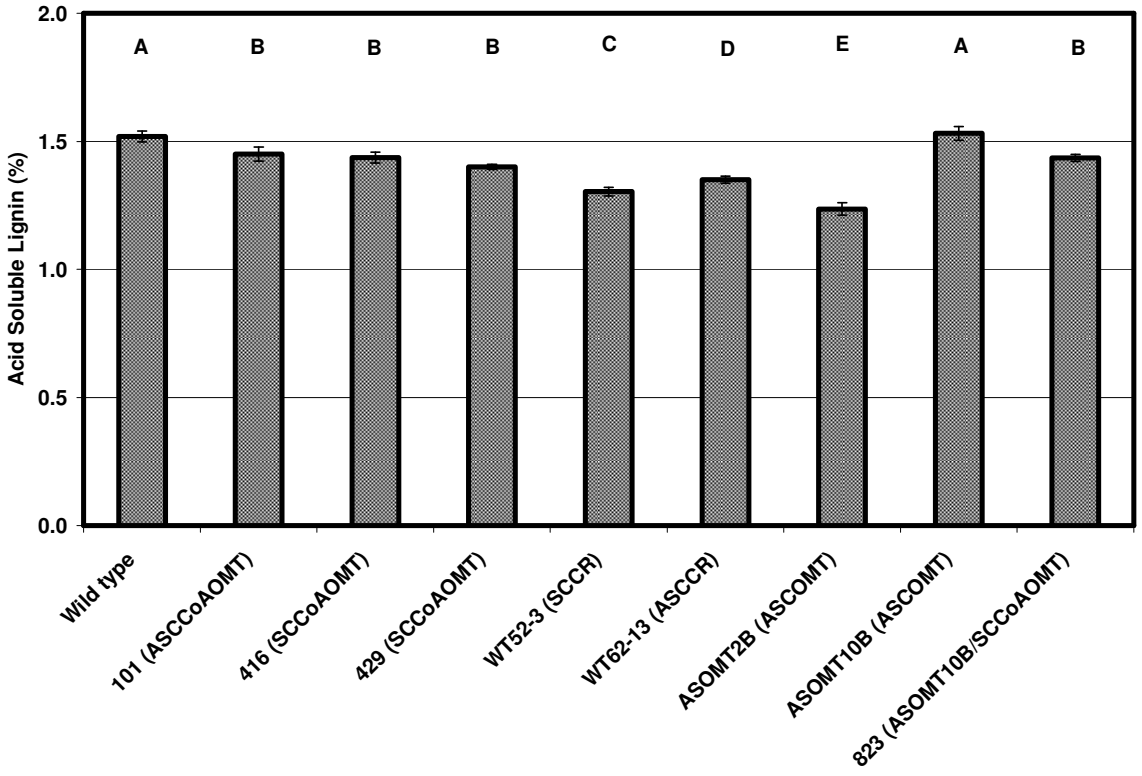

Figure 4: Acid soluble lignin content after AAS with hydrogen peroxide supplement (n=3) where each letter signifies a significant difference (P <0.5)

Organosolv pretreatment reduced lignin content through the wild type and transgenic samples (Figure 5), with a range of 63-69% reduction in ASL as compared to the corresponding untreated sample. All transgenic samples had lower ASL content than the wild type variety following organosolv pretreatment.

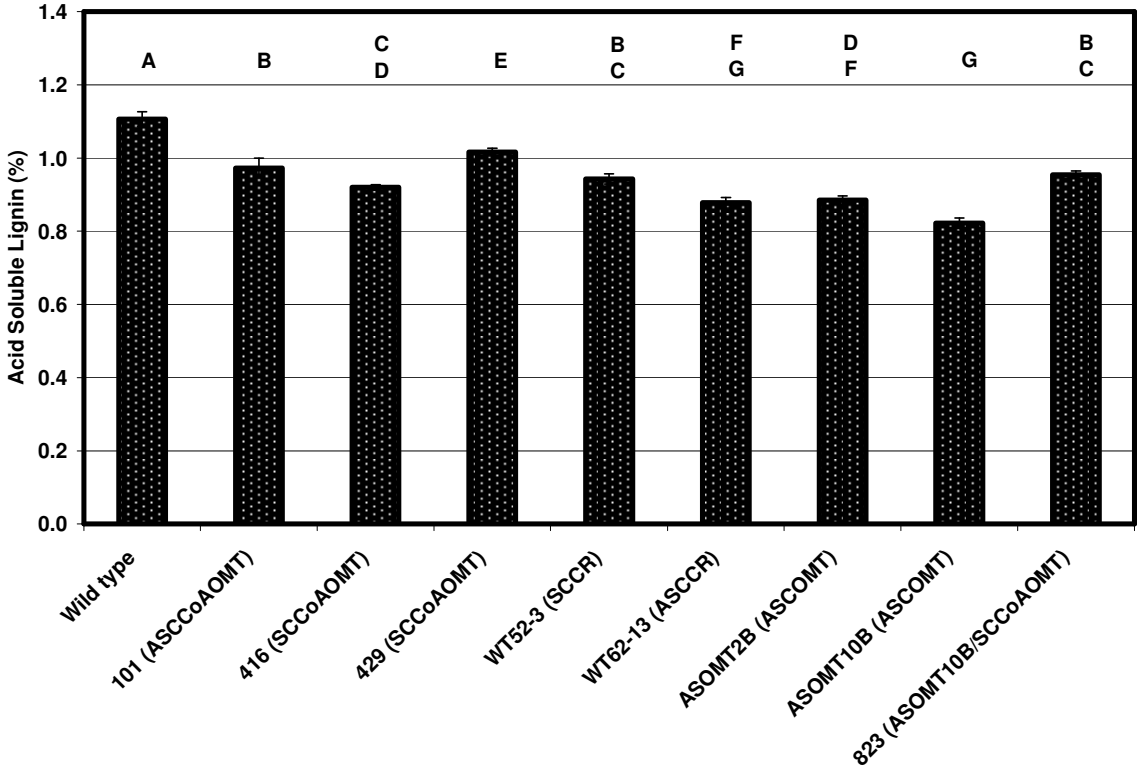

Figure 5: ASL after Organosolv pretreatment (n=3) where each letter signifies a significant difference (P <0.5)

In contrast to the ASL data, no significant difference in AIL content was found throughout the wild type and transgenic samples ($p>0.05$) which averaged 30% AIL. These results are consistent with Pilate et al. (2002), who reports that lignin values for the same ASOMT2B(ASCOMT) and ASOMT10B(ASCOMT) transgenic plants were also similar to the wild type following field trials. The lignin structure of the ASCOMT modification was found to have a greatly reduced proportion of S to G units as determined by thioacidolysis (Pilate et al., 2002). Contrary to this study, Chen et al. (2001) reported a 12% reduction in AIL lignin content in the CCoAOMT transgenic plants over the wild type in a greenhouse study. The average AIL content among samples was 30.1% ± 0.72%. Variability in the results suggest that it cannot be claimed that the AIL content of the transgenic samples were

different from the wild type. However, as shown below, the ability to remove the AIL by pretreatment was significant throughout the transgenic samples.

Aqueous ammonia soaking of the CCoAOMT modification resulted in consistent lignin removal for the 416(SCCoAOMT), 429(SCCoAMT), and 823(ASOMT10B/SCCoAMT) varieties resulting in approximately 26% AIL. AAS pretreatment of ASOMT2B(ASCOMT) removed 28% of the AIL over the untreated sample of the same variety. Modification to the CCR enzyme in the WT52-3(SCCR) and WT62-13(ASCCR) were consistent with each other following AAS pretreatment. Furthermore, AAS pretreatment was successful for reducing ASL and AIL in the ASOMT2B(ASCOMT).

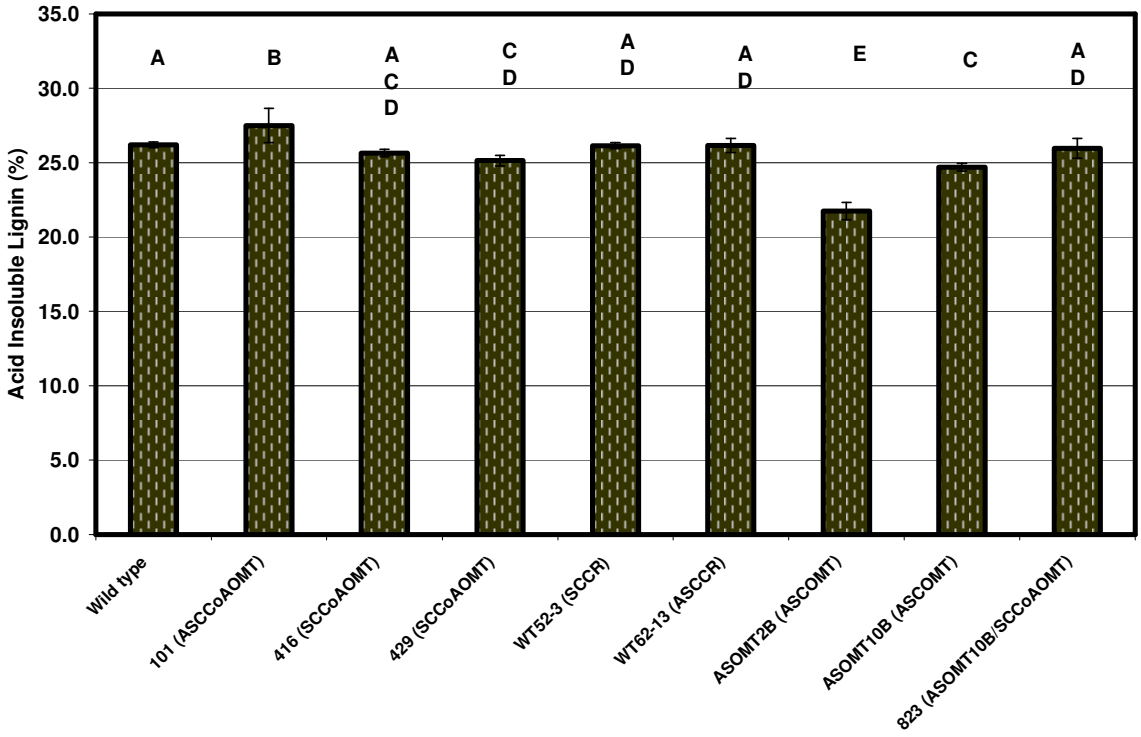

Figure 6: AIL after AAS pretreatment (n=3) where each letter signifies a significant difference (P <0.5)

Dilute acid pretreatment had minimal impact on the AIL content of the samples as compared to the untreated samples (Figure 7). Mosier et al (2005), suggests dilute acid pretreatment alters the structure of lignin but does not remove the lignin from the pretreated feedstock. Increasing the duration and amount of washing following dilute acid pretreatment has little impact on lignin removal (Hsu and Nguyen, 1995). Delignification following dilute acid pretreatment was relatively unsuccessful, averaging less than 5% removal which may be attributed to the partial hydrolyzation of polysaccharides.

The AAS-HP pretreatment resulted in AIL contents ranging from 20-25%, slightly less than the 22-28% seen in AAS pretreated samples. The AAS-HP of ASOMT10B(ASCOMT) resulted in a higher AIL content as compared to the wild type. COMT enzyme modification in transgenic poplar plants subjected to Kraft pulping resulted in a higher AIL content over the wild type variety (Baucher et al., 2003), suggesting the modification could be more resistant to delignification.

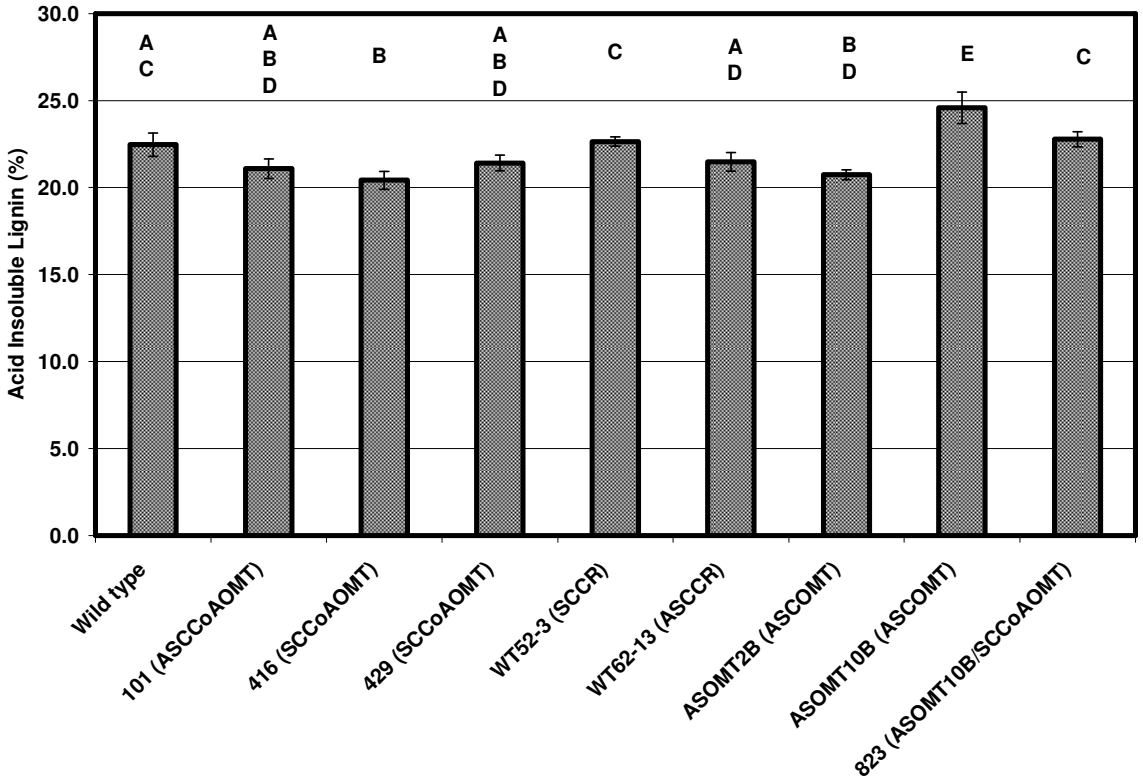

Figure 7: AIL after AAS-HP pretreatment (n=3) where each letter signifies a significant difference (P <0.5)

Organosolv pretreatment of wild type and transgenic samples resulted in lignin contents ranging from 15-21%. Modifications to the CCoAOMT enzyme pathway resulted in the consistent delignification throughout the four samples with this modification. The CCoAOMT enzyme modification in transgenic poplar plants subjected to Kraft pulping resulted in a lower AIL content than the wild type (Baucher et al., 2003). Organosolv pretreatment was most successful in delignification for the wild-type and ASOMT2B(ASCOMT) variety. COMT had a negative effect on Kraft pulping (Baucher et al., 2003) but appears to have a positive effect in organosolv pretreatment.

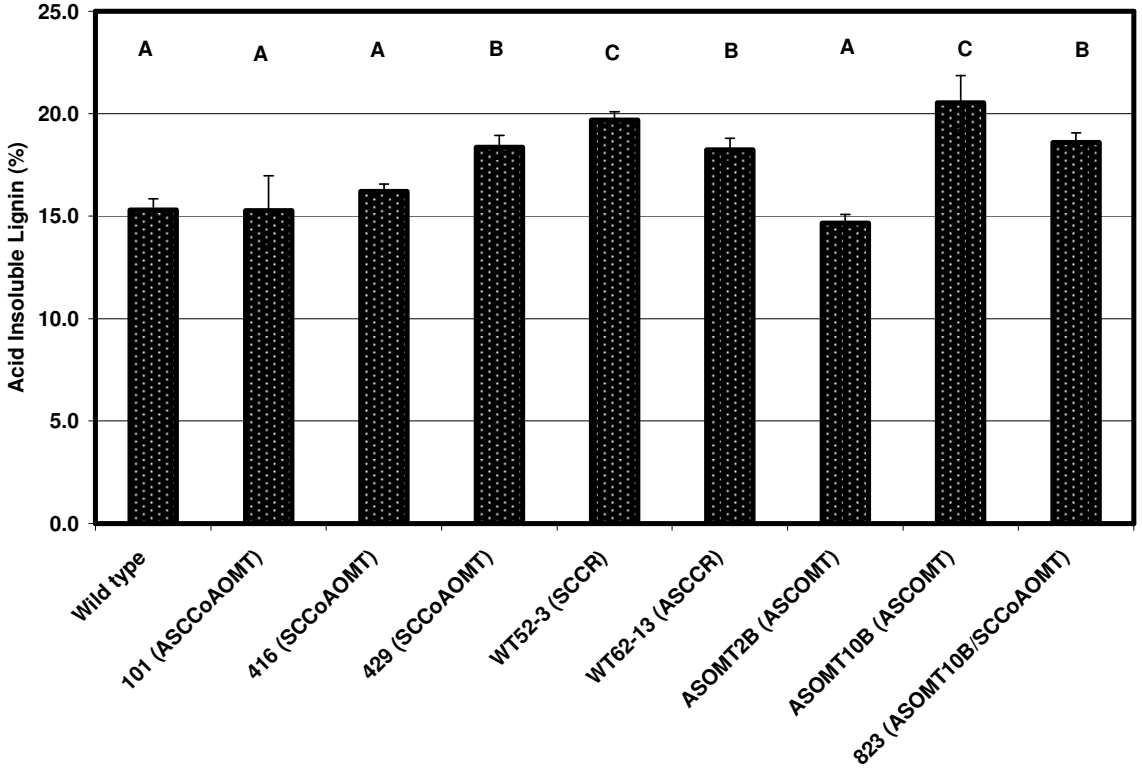

Figure 7: AIL following organosolv pretreatment (n=3) where each letter signifies a significant difference (P <0.5)

Overall, pretreatment of ASOMT2B(ASCOMT) was the most successful transgenic plant variety with regards to delignification, achieving 22, 55, and 68% removal of ASL with AAS, AAS-HP, and organosolv pretreatments, respectively. Similar, AIL delignification of this mutant was 28, 32, and 51% for AAS, AAS-HP, and organosolv pretreatments, respectively. The other COMT enzyme modification, ASOMT10B(ASCOMT), did not perform as well, especially with respect to AIL content following AAS-HP and organosolv pretreatment, in which the wild type resulted in a higher delignification.

Conclusions

Pretreatment was significant to transgenic plant varieties and lignin removal. Some transgenic plant variety resulted in slightly less ASL than the wild type but no difference among AIL lignin was found throughout the samples without pretreatment. AAS pretreatment was successful in removing a fraction of approximately 15% of ASL and AIL. Dilute acid pretreatment removed ASL but had only a small effect on AIL. AAS-HP was successful in removing ASL and AIL, particularly in the case of plants with modifications to that CCoAOMT enzyme. Organosolv pretreatment was the most successful in removing lignin throughout the wild type and transgenic plants. Among the eight transgenic lines evaluated, ASOMT2B(ASCOMT) was the most successful variety with regards to delignification across the range of pretreatments in this study.

Acknowledgements

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CHAPTER 4. AQUEOUS AMMONIA SOAKING FOLLOWED BY ANAEROBIC DIGESTION: ENERGY YIELD FROM A BENCH-SCALE BIOCHEMICAL METHANE POTENTIAL STUDY

A paper to be submitted to *Biomass and Bioenergy*

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Abstract

This paper reviews the biochemical methane potential (BMP) production from anaerobic digestion of switchgrass pretreated with aqueous ammonia soaking (AAS) and AAS pretreated switchgrass hydrolyzed under various enzyme loadings. Triplicate BMP's were performed on: untreated switchgrass, AAS-pretreated switchgrass soaked in 29.5% reagent-grade aqueous ammonia at 5 L/kg switchgrass for 5 d, and AAS-pretreated switchgrass at 62.5 filter paper units (FPU) enzyme/ g volatile solids (VS) loading. Biogas and methane production were measured daily in all treatments for 21 d. Both biogas and methane production varied significantly among treatments, especially during the first 7 d of incubation. Overall methane yields were compared over the course of the experiment: After 2 d, the highest enzyme loadings produced 17-25 x more methane than the untreated switchgrass, but this difference decreased to a factor of 2-7 x at 14 d, and 3-5 x at 21 d. The energy content of the biogas was compared to the energy content in ethanol produced from simultaneous saccharification and fermentation of the same material in previous work by our group, suggesting that between 50 and 100% more energy could be extracted at the highest enzyme loading rates. However, this analysis excluded separation energy costs and residue

energy returns from the ethanol process. Overall, the addition of enzymes to AAS-pretreated switchgrass greatly accelerated the rate of methane production over the untreated switchgrass and AAS-pretreated switchgrass without enzymes. Further work is needed to determine whether pretreating switchgrass with aqueous ammonia and/or enzymes before anaerobic digestion (AD) is economically advantageous.

Introduction

Current schemes for biofuel production generally focus on liquid transportation fuels like ethanol and biodiesel. Each has its own challenges – ethanol in part because of the energy intensive distillation step (Ragauskas et al., 2006), and biodiesel because of its relatively low energy per unit cropped area (Pimentel and Patzek, 2005). A biofuel derived from a high-yielding lignocellulosic feedstock that does not require significant processing energy inputs is an attractive target. One alternative is biogas, which self-separates from the aqueous reactor contents and which has been proven as a viable transportation fuel in Northern Europe with largest production currently in Sweden (Svensson et al., 2006; Auer et al., 2006). Biogas is mainly of methane and carbon dioxide and is produced through the anaerobic digestion (AD) of a variety of biomass substrates including lignocellulosic material. In addition to the low energy investment required to produce biogas from biomass, methane is an attractive vehicle fuel from an end-use air-quality standpoint: one commercially available compressed-natural-gas powered vehicle is certified as a partial-zero emission vehicle (Ridlington and Davis, 2005).

Lignocellulosic material is the most abundant organic resource on earth and is thus a promising raw material for bioenergy production (Lynd and Wang, 2004). Gunaseelan (1997), Chynoweth (1993), and Smith et al. (1992) provide extensive reviews of AD of

various feedstocks, including lignocellulosic material for methane production. Lignocellulosic feedstocks, such as corn stover and wheat straw, were identified to be substrates with excellent methane potential, yielding 0.360 to 0.383 m^3/kg volatile solids (VS) during a 60-d biochemical methane potential (BMP) trials (Gunaseelan 1997). More recently, Labatut and Scott (2008) explored the co-digestion of 30 substrates including food residues, lignocellulosic material, and combinations of manure. The BMP trials of switchgrass yielded about 0.12 L CH_4/g VS added and corn silage yielded 0.30 L CH_4/g VS added during 60-d digestions (Labatut and Scott, 2008). Switchgrass was the lowest yielding of the 30 substrates tested and achieved only 24% of theoretical yield, leaving great room for improvement of this recalcitrant biomass. The BMP assay was developed as a standardized method to determine the biodegradability and associated methane yield during anaerobic methanogenic fermentation of organic material (Speece, 1996). A modified method based on the procedure outlined by Owen et al. (1979) involves batch incubation of substrates under conditions ideal for anaerobic decomposition to evaluate the digestibility and biogas production. This BMP procedure provides a valuable and inexpensive method to determine the potential extent and rate of conversion of candidate feedstocks.

Lignin has been shown to severely hinder cellulose decomposition under anaerobic conditions in lignocellulosic biomass (Stinson and Ham, 1995) with methane yields inversely related to lignin content (Smith et al., 1992). Pretreatment of lignocellulosic material modifies the lignin bonds, freeing cellulose and hemicellulose, enhancing the biodegradability, and possibly increasing biogas production (Yadvika et al., 2004). Alkaline pretreatment at ambient temperature has been proposed as a chemical pretreatment process compatible with AD because of the desirable alkalinity (Neves et al., 2005). In a 50 d

experiment, AD of alkali pretreated wheat straw produced 37 to 100% more methane than the untreated wheat straw (Pavlostathis and Gossett 1985). He et al. (2008) found rice straw pretreated with 6% sodium hydroxide increased biogas yield by 27.3-64.5% in a 21 d study. However, pretreating winter rye, oilseed rape, and fava beans with Na_2CO_3 at 195^{o}C and 12 bar for 15 minutes failed to significantly increase methane production in a 50-d trial, possibly due to an inhibitor toxic to microorganisms produced during high temperature, high pressure pretreatment (Petersson et al., 2007). Low temperature, low pressure AAS pretreatment appears to be an attractive pretreatment method for AD.

The AD of lignocellulosic biomass is a slow process, generally accomplished at hydraulic retention times (HRT) of 30-50 d in industrial facilities, and therefore requiring large reactor volumes (Yadvika et al., 2004). In contrast, AD of simple substrates can be extremely rapid requiring HRT from 1–3 d for readily degradable food wastes (e.g., Moody and Raman, 2001). Cellulosic material is converted to simple substrates by hydrolysis, which is the rate-determining step in the conversion process of lignocellulosic material (Adney et al., 1991). Accelerating hydrolysis with a combination of pretreatment and added hydrolytic enzymes (as opposed to the endogenous hydrolytic enzymes produced by the AD microbial consortia, e.g., Lynd, 2002) during AD can shorten the HRT, allowing for smaller reactor volumes, and possibly improving overall process economics. Accordingly, the objective of this study was to examine the effect of AAS pretreatment, and of AAS-pretreatment plus enzymes, on the performance of AD of switchgrass. Specifically, by determining and comparing daily biogas production (cc), methane content of biogas (%), methane yields (m^3 CH_4/kg VS fed), and theoretical yields of the treatments to the AD of untreated switchgrass.

In addition, energy yields (MJ/kg VS) of the AD process were compared to the energy yield of ethanol production from the same AAS-pretreated switchgrass.

Materials and Methods

Raw Materials

Switchgrass was collected from mature, 4 year old stands of *Cave-in-Rock* cultivar in mid-October 2007 at the Iowa State University Agronomy and Agricultural Engineering Farm near Ames, IA (42º 00'N, 93º 50'W; elevation 341 m above sea level). The stand was established in late summer and autumn of 2003 and was fertilized at 140 kg/ha N as ammonium nitrate. Switchgrass was harvested above a 5 cm height following killing frost. Dry switchgrass was ground to a size of 5-6 mm at the Biomass Energy Conversion Center, BECON, Nevada, IA using a hammer mill Grinder (Model 400430, Art's Way, Armstrong, IA). Composition of the switchgrass was determined by the Iowa State University Department of Agronomy using the ANKOM method (ANKOM Technol. Corp., Fairport, N.Y.) as described by Vogel et al. (1999). Klason lignin was determined as described by Crawford and Pometto (1988), slightly modified by Isci et al. (2007). Untreated switchgrass contained 41% cellulose, 32% hemicellulose, 7% acid detergent lignin, 19% Klason lignin, and 0.7% ash.

Pretreatment

Based on previous work by our group (Isci et al., 2007), forty grams of dry switchgrass was soaked in reagent-grade 29.5 wt% aqueous ammonium hydroxide (Fisher Scientific) in 1.0-l high-density polyethylene bottles at room temperature without agitation for 5 d. Following pretreatment, the biomass was washed *in situ* with 12 L of deionized (DI) water using the custom fluidized bed-biomass washing system (Isci et al., 2007). AAS

pretreatment removed an average of 35% of Klason lignin and 41% hemicellulose, resulting in approximately 56% cellulose in the pretreated material.

Enzyme

Spezyme CP, a cellulase enzyme produced by Genencor (Palo Alto, CA, Lot # 301-05330-206), was selected to be consistent with previous switchgrass-to-fuel studies by our group (Isci et al., 2007). The measurement of the cellulase enzyme activity was determined by the DNS method according to Adney and Baker (1996). Measured activity level was 55 filter paper units (FPU)/ml enzyme.

Treatments

Eight treatments were evaluated, as listed in Table 1. The untreated switchgrass was a baseline and enabled comparison to previous literature, while the mixed pentose/hexose control allowed assessment of the microbial community's ability to handle these hydrolysis by-products. The AAS-pretreated switchgrass was examined without enzyme, and at four non-zero enzyme loading rates ranging 10-fold. An inoculum-to-feed ratio of 1:2 (VS basis) was used in this study (Labatut and Scott, 2008)

Table 2: Description of the seven treatments

Treatment Number	Treatment substrate
1	Untreated Switchgrass
2	AAS-pretreated switchgrass
3	AAS-pretreated switchgrass + 12.5 FPU enzyme/g VS
3	AAS-pretreated switchgrass + 25 FPU enzyme/g VS
4	AAS-pretreated switchgrass + 62.5 FPU enzyme/g VS
5	AAS-pretreated switchgrass + 125 FPU enzyme/g VS
6	Mixture of 60% glucose, 40 % xylose
7	Inoculant control

Biochemical Methane Potential (BMP) Assay

An aliquot of substrate was added to a 250-ml serum bottle along with 83 ml of inoculum. The substrate mass was such that the inoculum-to-feed VS ratio was 1:2. Inoculum was obtained from a 60-l mesophilic (35^{o}C) continuous stirred tank reactor (CSTR), fed daily at a loading rated of 2 g VS/l/d (Wu-Haan et al., 2008). The inoculum concentration was 0.0024 g/l VS. The headspace in the serum bottle was purged with 30% CO_2 in 70% N_2 at a flow rate of approximately 0.5 L/min for 5 min and then sealed. The serum bottles were then placed in a shaker rotating at approximately 150 rpm and incubated at 35^{o}C (Wu-Haan et al., 2008). Each assay was performed in triplicate.

Each day, vials were depressurized and biogas was collected by inserting a hypodermic needle connected to a 50-mL graduated glass syringe through the serum cap. The biogas composition was measured daily using a nondispersive infrared sensor, the NDIR-CH_4 gas-analyzer (Institute of Agricultural Process Engineering, University Kiel, 08/003). Calibration with 60% CH_4 in 40% CO_2 and 30% CO_2 in 70% N_2 for 3 min at a 0.3-0.4 L/min was performed weekly and control checks with 60% CH_4 in CO_2 were performed prior to daily measurement (NDIR-CH_4 Gas-Analyzer User Manual). Reported results are average values of the triplicate samples.

Results and Discussion

Daily biogas production varied significantly between treatments (Figure 1). On day one Mass Electric, the sugar control produced the most biogas: more than 90 cc, presumably due to the availability of simple sugars utilized for immediate digestion. The two high enzyme treatments produced 75 and 100 cc of biogas, respectively, after 2 d of incubation (Figure 1). At 2 d, the biogas production rates peaked in all treatments. Peak gas production varied

directly with enzyme loading level with even the no-enzyme AAS-pretreated switchgrass producing twice as much biogas as the untreated switchgrass. Following 6 d of incubation, the biogas production in all treatments decreased to below 20 cc/d and remained at low levels for the remainder of the study. Variability within treatments was modest: less than 8% of the daily biogas production data had a coefficient of variance greater than 25%, the majority of which were from the low-yielding untreated and no-enzyme AAS-pretreated switchgrass samples.

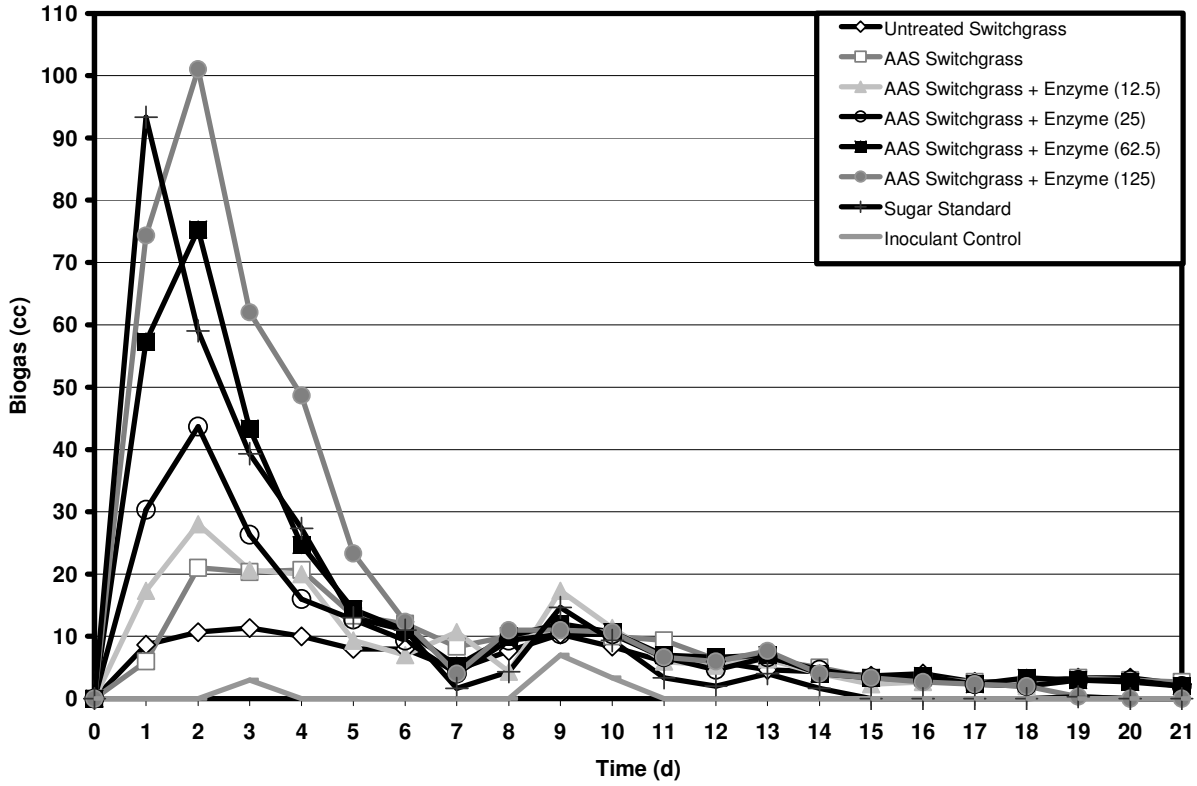

Figure 8: Daily biogas production (cc) obtained for each treatment as outlined in Table 1 (n=3).

Biogas composition varied significantly during the first 12 days of incubation (Figure 2), but stabilized at 40–58% methane after that time. Biogas from the two high-enzyme

treatments and the sugar control reached the highest methane concentrations (50 to 58%), which is within the expected range of methane content (50 to 70%) for biogas (Speece, 1996). These treatments with high steady-state methane content were also those which had the most rapid rise in methane content (Figure 2). As with the biogas production data, variability of composition within treatments was modest: 6.5% of the biogas composition data set had a coefficient of variance greater than 5%.

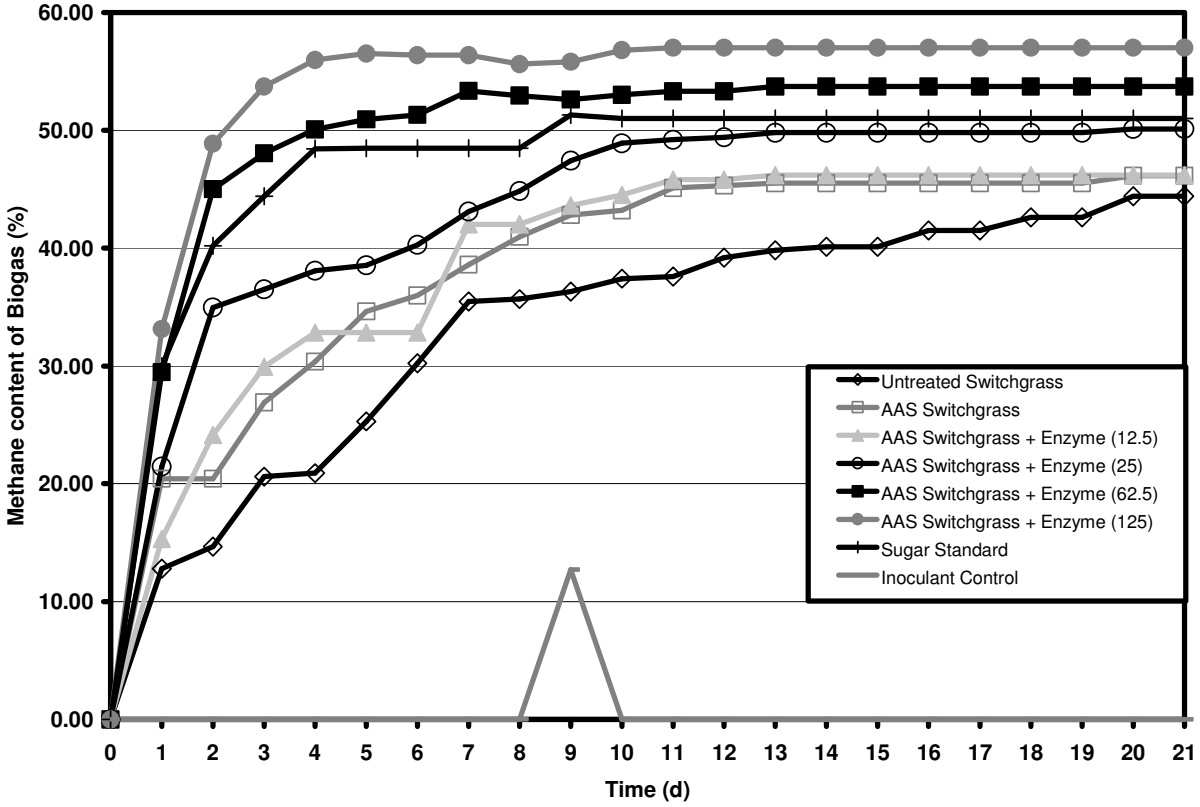

Figure 9: Methane composition of biogas (%) obtained for each treatment as outlined in Table 1 (n=3).

Cumulative methane yield, determined from daily biogas production and methane content data, is shown in Figure 3. The cumulative methane yield at 21-d ranged from 0.09 to 0.49 m^3/kg VS added, corresponding to 20–98% of theoretical production. As expected, the AAS-pretreated material produced significantly more methane than the untreated

switchgrass, presumably due to the removal of lignin by the pretreatment. Based on prior work by our group (Isci et al., 2007), an estimated 35% of the lignin was removed during AAS pretreatment, freeing the cellulose and hemicellulose and making them more readily available to enzymes and to microorganisms. The methane yield from the lowest enzyme loading treatment of AAS-pretreated switchgrass with 12.5 FPU/g VS enzyme was not significantly different than the untreated switchgrass suggesting the enzyme loading was too low to effectively hydrolyze the cellulose.

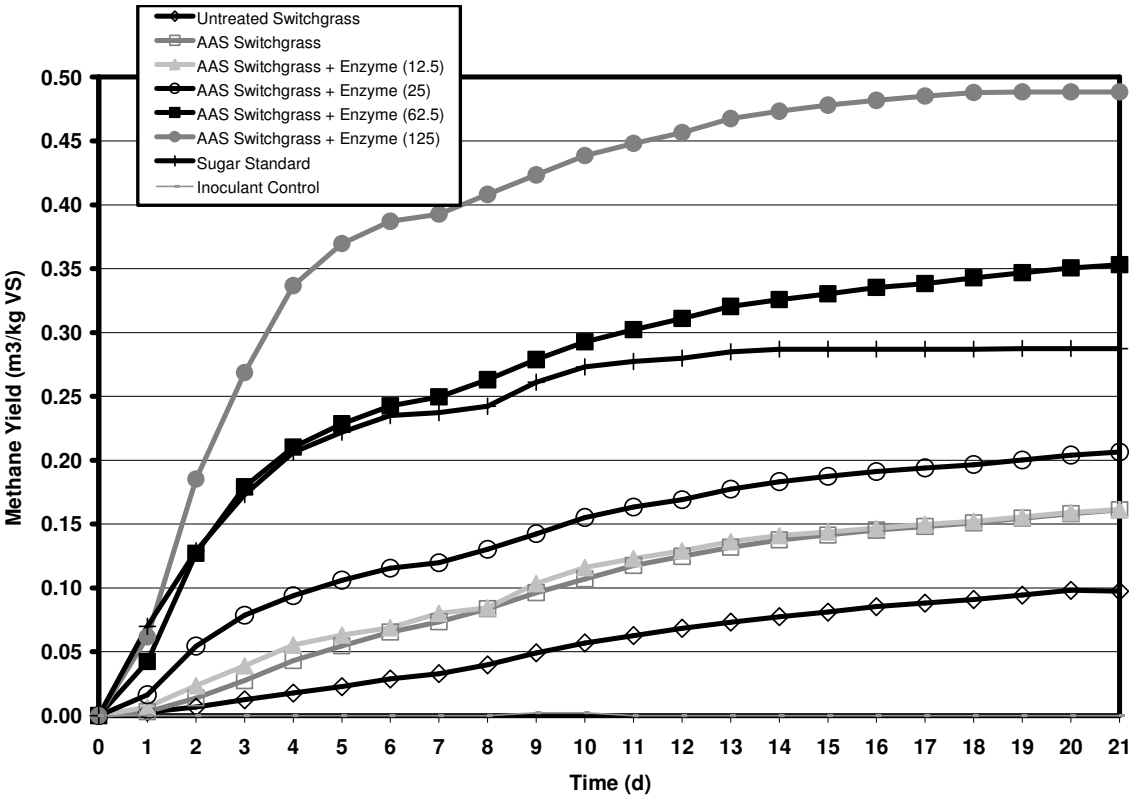

Figure 10: Methane yield (mL/g VS) during the first 21 d obtained for each treatment as outline in Table 1. Note that no correction for the biogas that could be produced from the degradation of the enzyme solution is included here (n=3).

This result contrasts with our experiences with low-enzyme loading ethanol fermentations (Isci et al., 2007), and suggests that significant enzyme inhibition may be occurring in the

AD process. Enzymatic inhibition could be reduced by 1) incrementally adding enzyme, 2) hydroylzing biomass for 1 d prior to AD, or 3) selecting hydrolytic enzymes better suited to AD conditions (e.g., elevated pH). The average starting pH of the BMP assays was 7.4 and the average final pH of the BMP assays was 7.1. Optimal enzymatic hydrolysis for Spezyme CP occurs at a pH of approximately 5.

At 21 d, the 25 FPU/g VS treatment produced 40% of theoretical yield based on switchgrass volatile solids, while the 62.5 FPU/g VS treatment reached 70% and the 125 FPU/g VS treatment reached nearly 98%. Near optimal yield promoted the idea that degradation of the enzymes could be occurring and contributing to biogas yield. This is not accounted for in Figure 3; however, it is addressed later.

To better visualize the temporal variation in benefits, Figure 4 displays a ratio of methane yield as compared with the untreated switchgrass for each treatment, on a daily basis. After 2 d, the 62.5 and 125 FPU/g VS treatments produced 18 and 27 times more methane, respectively, than the untreated switchgrass. The various pretreatments stabilized after 10 d producing between 2 and 7 times more methane than the untreated switchgrass. Although the dramatic differences between treatments seen early in Figure 4 decrease over time, they never disappear completely.

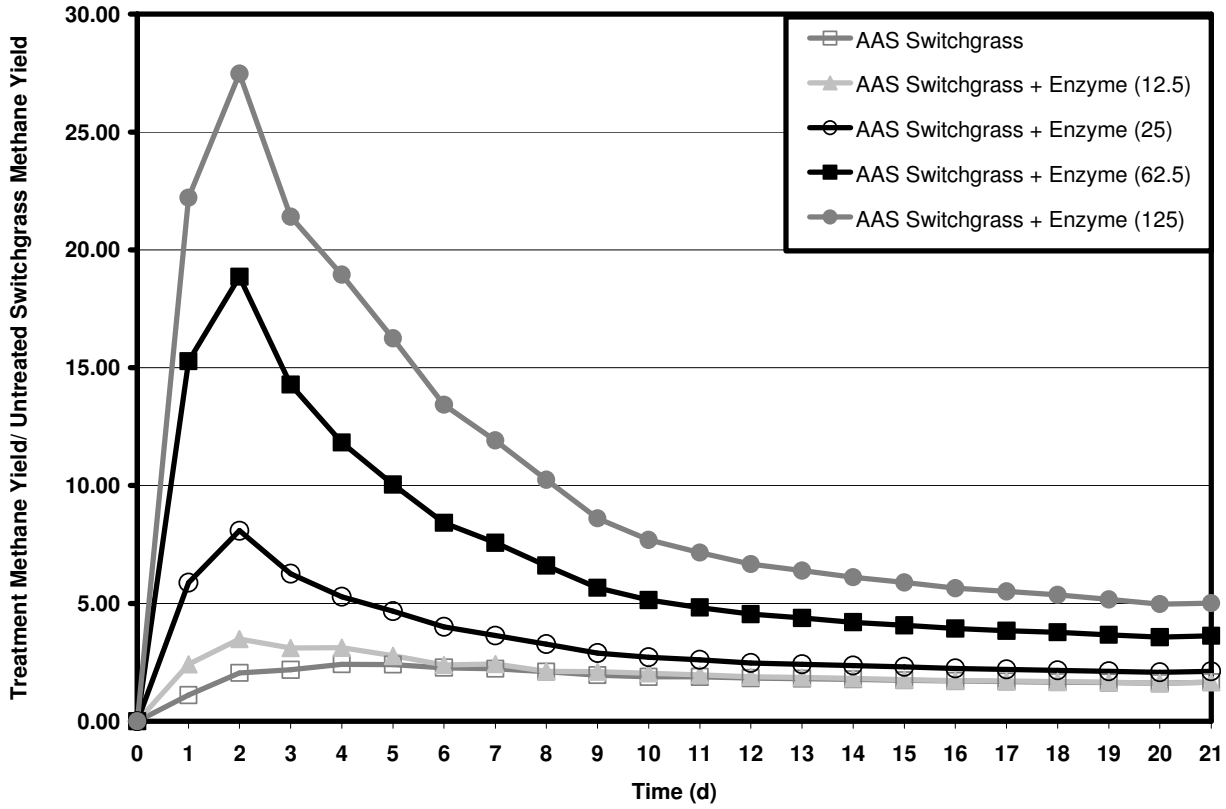

Figure 11: Ratio of methane yield (treatment methane yield/untreated switchgrass methane yield) (n=3)

After 7 d of incubation, the treatments were compared to determine how each treatment increased the methane rate of production (Table 2). AAS-pretreated switchgrass yielded 2.24 times more methane than untreated switchgrass. With the addition of enzymes methane production increased yield 2 to nearly 12 times compared to untreated switchgrass. Comparisons were also made among enzyme treatments. Doubling the enzyme load from 12.5 to 25 FPU/g VS increased methane yield by 50%, while doubling the load at high doses, from 62.5 to 125 FPU/g VS increased methane yield by 57%. Overall, the 10-fold increase from the low to high enzyme loading increased the rate of methane production by a factor of 4.9.

Table 3: Factor increase across various treatments based on Day 7 cumulative methane yields

Treatment	AAS	AAS + E (12.5)	AAS + E (25)	AAS + E (62.5)	AAS + E (125)
Untreated	2.24	2.42	3.64	7.58	11.91
AAS		1.08	1.62	3.38	5.31
AAS + E (12.5)			1.50	3.125	4.91
AAS + E (25)				2.08	3.28
AAS + E (62.5)					1.57

Table 3 reviews the treatment comparison following 21 d of incubation. At this time, AAS-pretreated switchgrass yielded 1.66 times more methane as untreated switchgrass. Adding enzymes increased methane production yield from 1.66 to nearly 5 times the untreated switchgrass. Doubling the enzyme load at the low doses increased methane yield by 28%, while doubling the load at high doses increased methane yield by 38%. Comparing Table 2 and 3 suggest that greater differences in treatments are seen at shorter durations.

Table 4: Factor increase across various treatments based on Day 21 cumulative methane yields

Treatment	AAS	AAS + E (12.5)	AAS + E (25)	AAS + E (62.5)	AAS + E (125)
Untreated	1.66	1.66	2.12	3.64	5.03
AAS		1.00	1.28	2.19	3.03
AAS + E (12.5)			1.28	2.19	3.03
AAS + E (25)				1.71	2.37
AAS + E (62.5)					1.38

Figure 5 depicts the gross energy yield (MJ/kg switchgrass dry-basis added) at 2, 7, 14, and 21 d. A reference line depicted at 7.0 MJ/kg switchgrass represents the maximum gross fuel energy yield observed from the SSF of AAS-pretreated switchgrass (1:5 5d with enzyme loading rate of 77 FPU/g and 3% cellulose) in previous work by our group (Isci et al., 2007). Furthermore, the complete conversion of hydrolyzed cellulose and hemicellulose to ethanol would yield 11.7 MJ/kg switchgrass. Energy yields associated with AAS-

pretreated switchgrass plus enzyme conditions were adjusted based on a first approximation of enzyme protein content of 116 mg/ml for Spezyme CP (Coward-Kelley et al., 2002), as we are waiting on protein content of the lot number used in this study. The 16.8 MJ/kg energy content of the protein used to adjust energy yields of AAS-pretreated switchgrass plus enzyme by 0.195-1.95 MJ, depending on the enzyme loading. This assumed all energy available in the enzyme was used during the AD. At day 2, none of the biogas systems produced as much energy as the ethanol fermentation. However, at longer retention times and high enzyme loadings, significantly more energy was produced by AD, with the highest enzyme loading system producing 15.5 MJ/kg switchgrass after 14 d, nearly 2.5 times more than the C6-utilizing ethanol system, but at a much longer retention time.

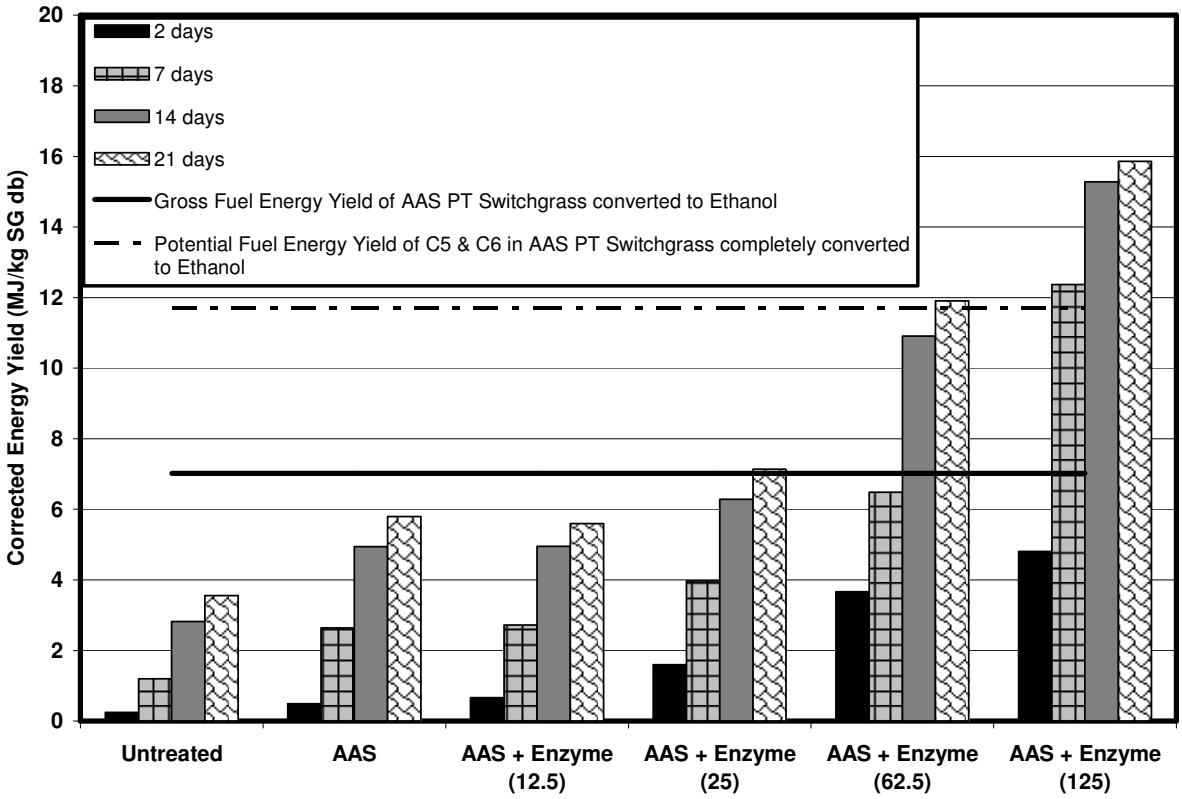

Figure 12: Gross energy yield of treatments compared to gross energy yield of AAS-pretreated switchgrass converted to ethanol via SSF at 2, 7, 14, and 21 d corrected assuming for energy yield of protein in enzyme (n=3)

The results show that a significant amount of energy can be harvested from AAS-pretreated switchgrass and AAS-pretreated switchgrass with enzyme, as compared to untreated switchgrass. However, the effectiveness of any pretreatment and addition of hydrolytic enzymes must be balanced against the cost of these additions. Without system optimization and scale-up of this bench-scale process, an economic analysis is premature.

Conclusions

Aqueous ammonia steeping is a relatively simple delignification pretreatment method for biomass that significantly increases biogas energy production from the anaerobic digestion of switchgrass. After 21 d of incubation, AAS-pretreated switchgrass produced

65% more methane than the untreated switchgrass. The addition of sufficient commercially available hydrolytic enzymes greatly increased biogas yields, methane concentration, and total methane yields. At 21 d, the lowest enzyme treatment (12.5 FPU/g VS) was not significantly different than the non-enzyme AAS pretreated switchgrass. However, relative to the no-enzyme treatment the AAS pretreated switchgrass with 25, 62.5, and 125 FPU/g produced 130, 227, and 325% more methane, respectively. AAS-pretreated switchgrass at 125 FPU/g VS reached 98% of theoretical methane yield on a switchgrass volatile solids basis and 50% more energy yield that available from the carbohydrate fraction of the switchgrass. At the highest enzyme loading, gross energy production from AD was well over twice the gross energy production from ethanol fermentation of the same material, and this energy difference would be expected to grow when the separation energy requirements of ethanol are included. However, the AD approach does not produce a liquid transportation fuel, and requires significantly longer retention times (21 d vs. ~2 d) to extract this excess energy. Other factors such as residue use and fuel value must be considered in determining the merits of this AD approach relative to cellulosic ethanol systems. However, these preliminary results suggest that further work on the enzyme enhanced AD of pretreated biomass is justified.

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CHAPTER 5. GENERAL CONCLUSION

General Discussion

Biomass derived transportation fuels promise to provide a renewable energy source, but also reduce dependence on foreign oil supplies and reduce greenhouse gas emissions. Pretreatment is one of the most important steps in converting lignocellulosic biomass to biofuels, and in this thesis, aqueous ammonia soaking (AAS) was used as a pretreatment method in a variety of settings. Several key conclusions were drawn from this work. In Chapter 2, *"Design and Testing of a Pilot Scale Aqueous Ammonia Soaking Biomass Pretreatment System,"* a method for generating kilogram-quantities of AAS-pretreated biomass was developed and demonstrated at pilot-scale, with relatively inexpensive equipment. Based on economic, safety and convenience factors, a 75-L soaking vessel was selected and shown to be effective in pretreating 4 kg of switchgrass per run with 20-L of aqueous ammonia. This pilot-scale pretreatment system increased cellulose content and decreased hemicellulose and Klason lignin content in the remaining solids in a similar manner as observed in bench-scale experiments. To our knowledge, this is the first description of a pilot-scale AAS biomass pretreatment system. Key challenges overcome in our effort included the handling of multi-liter quantities of aqueous ammonia, the separation of biomass from rinsate, and the disposal of over 1000-L of ammonia-enriched rinsate. Large-scale application of the AAS method will need to address safety, separation, and ammonia recycling issues that were encountered here.

Chapter 3, "*Aqueous Ammonia Soaking and Other Pretreatment Methods of Poplar"* described the use of AAS, dilute acid, AAS with hydrogen peroxide supplement, and

organosolv, as pretreatments of transgenic varieties of poplar (*Populus* spp) that had modifications to enzymes in the lignin biosynthesis pathway. All pretreatment techniques were successful in removing a fraction of both acid soluble lignin (ASL) and acid insoluble lignin (AIL) from the transgenic varieties. The AAS pretreatment was successful in removing approximately 15% of ASL and AIL. Dilute acid pretreatment removed 30-60% of ASL but less than 5% of AIL. The aqueous ammonia soaking with hydrogen peroxide(AAS-HP) was successful in reducing ASL by 43-52% ASL and AIL by 18-30%, particularly in the case of plants with modifications to the CCoAOMT enzyme. Organosolv pretreatment was the most effective in removing lignin throughout the wild type and transgenic plants varieties. Among the eight transgenic lines evaluated, ASOMT2B(ASCOMT) was the variety most susceptible to delignification across the board of pretreatments with 22-68% removal of ASL and 6-52% removal AIL. Overall, modifications made to the lignin biosynthesis pathway in transgenic poplar plants improved the delignification ability of the various pretreatment methods.

Chapter 4, *"Energy Yield from the Anaerobic Digestion of Aqueous Ammonia Steeped Switchgrass: A Bench-Scale Biochemical Methane Potential Study"* explained that AAS-pretreated switchgrass significantly increases biogas energy production from the anaerobic digestion (AD) of switchgrass. The AAS-pretreated switchgrass produced 65% more methane than untreated switchgrass after 21 d. The addition of sufficient commercially available hydrolytic enzymes greatly increased biogas yields, methane concentration, and total methane yields. Specifically, AAS-pretreated switchgrass with 25, 62.5, and 125 FPU/g produced 130, 227, and 325% more methane, respectively, relative to the no-enzyme treatment. At the highest enzyme loading, gross energy production from AD was over twice the gross energy production from ethanol fermentation of the same material.

Future Work

In order to be a viable pretreatment technology, the economics associated with AAS pretreatment must improve. Optimizations of the soaking time and solid to liquid ratio for AAS have important economic implications in proposed commercial-scale lignocellulosic ethanol production. Furthermore, a method of separating and purifying phenolic compounds from the AAS waste stream could provide potential co-products which would increase the economic feasibility of this pretreatment method.

Developing a method to screen and match transgenic plants to different pretreatment characteristics would be beneficial in analyzing transgenic plants for improve lignocellulosic ethanol production characteristics. An assay that would rapidly and easily predict the delignification and fermentability of transgenic plant varieties would be advantageous in selecting varieties ideal for biofuels production.

Further work on the enzyme enhanced anaerobic digestion of pretreated biomass is justified. First, it is important to reduce enzymatic inhibition in the experiment presented. Potentially, this could be done by 1) incrementally adding enzyme, 2) hydrolyzing biomass for 1 d prior to AD, or 3) selecting hydrolytic enzymes or other microbes better suited to AD conditions. Furthermore, it is important to consider the processing economics associated with AD of AAS-pretreated switchgrass. Biomass pretreatment and the addition of hydrolytic enzymes introduce significant additional costs and the paybacks may not justify there investments. Also, AD does not produce a liquid transportation fuel, and requires significantly longer retention times as compared to ethanol fermentations to extract this excess energy, therefore, performing a biogas feasibility study is also important. Considering

AD of AAS-pretreated switchgrass as a potential on-farm conversion technology would also be interesting.

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