DEVELOPMENT AND OPTIMIZATION OF PRESSURIZED BATCH HOT WATER PRETREATMENT AND COMPARISON OF WARM SEASON GRASSES FOR BIOCONVERSION TO ETHANOL

by

SARAH KATHERINE BRANDON

(Under the Direction of Joy Doran-Peterson)

ABSTRACT

Efficient conversion of lignocellulosic biomass to ethanol is required to make the overall process more economically feasible. Developing new and more effective pretreatments to disrupt lignocellulosic biomass prior to enzymatic saccharification and fermentation is one approach to meet this end. A pressurized batch hot water (PBHW) reactor was designed and tested as an autohydrolytic pretreatment for grass biomass. Pretreatment of whole biomass solids at 230° for two minutes was found to be the optimal operating parameters for the pretreatment to disrupt the biomass and remove hemicellulose. Pressure and solids loading during pretreatment had negligible effects on pretreatment efficacy. Tifton 85 bermudagrass, ADEL switchgrass, and Merkeron napiergrass were evaluated for bioconversion to ethanol using PBHW pretreatment, with pretreated napiergrass and bermudagrass producing the highest ethanol yield of 22.45 g/L and 21.3 g/L respectively. Inhibitors were also evaluated during the bioconversion process, and PBHW pretreatment results in removal of fermentation and enzyme inhibitors prior to fermentation.

INDEX WORDS: lignocellulose, biomass, pretreatment, pressurized batch hot water, grass, inhibitors

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DEDICATION

I would like to dedicate this work to my parents, Warren and Linda Brandon. Without their love and unfailing belief in me, I would not have been able to make it here. Thank you and I love you.

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CHAPTER 1

INTRODUCTION

1.1 Purpose

As of September 2008, 58.2% of the oil consumed in the United States was imported (http://www.eia.doe.gov). Due to an increased emphasis on domestic energy security and the acknowledgement of the impact of petrochemical use on the environment, the Federal Energy Regulatory Committee passed the US Energy Policy Act of 2005 (http://www.ferc.gov/) which requires the incorporation of 7.5 billion gallons of renewable fuels into gasoline by 2012. As of 2007, the US produces over 6.5 billion gallons of ethanol yearly, most of that from corn (http://www.ethanolrfa.org). However, corn supply is limited due to its demand as human and animal food, so alternative sources of biomass are needed to meet the growing need for ethanol. The US produces over a billion tons of lignocellulosic biomass annually, which makes it the next reasonable substrate for ethanol production (73).

Compared to corn, which is composed mainly of starch, lignocellulosic biomass is far more complex. It contains from 40-50% cellulose, 25-35% hemicellulose, and 15-20% lignin, depending on the source (38). Ethanol production from corn requires relatively simple pretreatments, generally an initial grinding step followed by a cooking step, addition of only two classes of enzymes (α-amylase and glucoamylase), and then fermentation by *Saccharomyces cerevisiae*. However, because of the complexity of lignocellulose, novel and more complicated processes are required to convert it to ethanol. These include pretreatments to make the biomass more available for enzymatic digestion, more and different enzymes to saccharify the pretreated

biomass (depending on biomass composition), and fermentation by microorganisms, yeast or bacterial, to produce ethanol. This introduction will outline the research and current processes used in lignocellulosic ethanol production, specifically focusing on the feedstocks available for conversion and the pretreatment methods being investigated.

1.2 Lignocellulosic Biomass

The availability of corn to convert to fuel ethanol is limited due to two main reasons. First, corn requires nutrient rich soil (or large amounts of fertilizers) and comparatively large amounts of water to grow successfully, limiting the areas in the US where it can be grown. Secondly, there is a worldwide demand for it as a feedstock for humans and animals. Lignocellulosic material, generally referred to as biomass, is a viable option as a substrate for ethanol conversion. Issued in 2005 by the United States Department of Agriculture and the Department of Energy, the "Billion Ton Report" estimated that the US has the ability to produce more than a billion tons of biomass annually, part from forest resources and the remainder from agricultural resources. These sources range from dedicated crops to waste residues from existing industries (73).

Lignocellulosic biomass generally contains cellulose, hemicellulose, lignin, and varying levels of pectin, protein, and ash. Cellulose (Figure 1.1) is the predominant polymer in plant cell walls, consisting of repeating units of β-1,4-linked glucose dimers, called cellobiose. These homopolymers are highly hydrogen bonded to each other, forming crystalline microfibrils, but they also exist in an amorphous form (20).

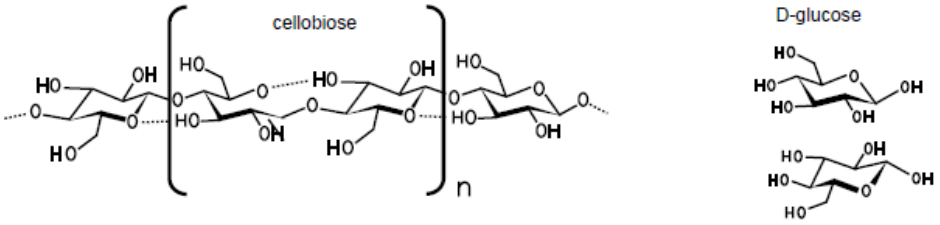

Figure 1.1 Structure of cellulose, cellobiose, and glucose (28).

Around the cellulose microfibrils are hemicellulose polymers. There are two major classes of hemicelluloses, each occurring predominantly in different types of primary cell walls. In type I cell walls, found in dicots and non-commelinoid monocots, the majority of the hemicellulose is xyloglucan. Arabinoxylan is the predominant hemicellulose polymer in commelinoid monocot cell walls, which are type II. There are also glucomannan, galactomannan, and several other combinations and linkages of five- and six-carbon sugars (Figure 1.2) (18, 19, 72).

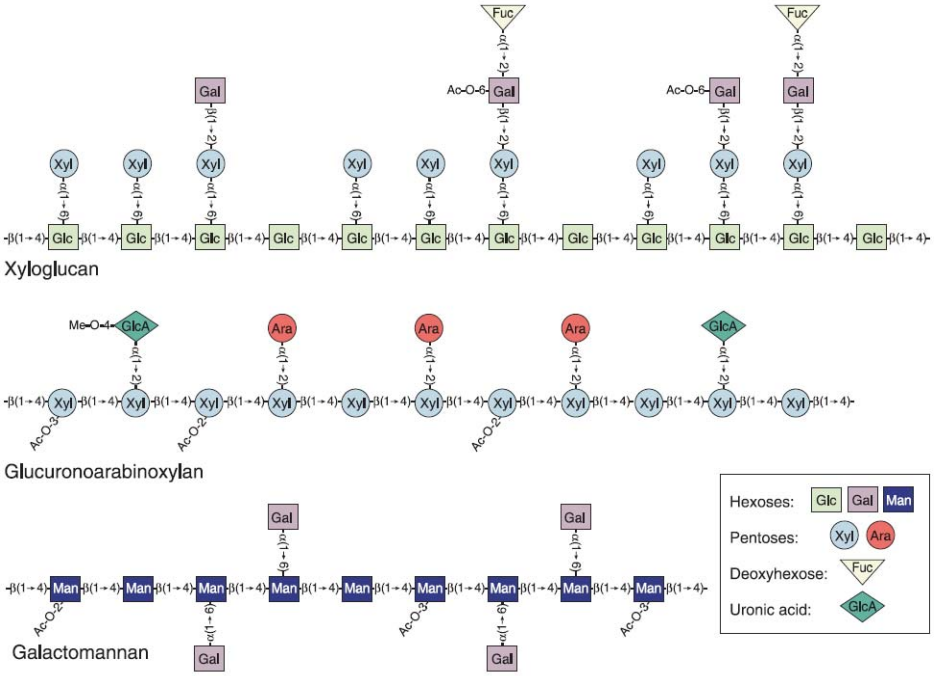

Figure 1.2 Common hemicellulose structures (72).

Lignin (Figure 1.3) is also one of the major components of plant cell walls. This polymer, unlike cellulose and hemicellulose, is non-fermentable. It consists of polymerized phenylpropanoid groups and other aromatic compounds. The three main monomeric components are *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol; all three are *p*-hydroxycinnamyl alcohols. These monomers are oxidatively coupled to one another, forming a variety of polymers that are covalently linked to the hemicellulose (21, 76).

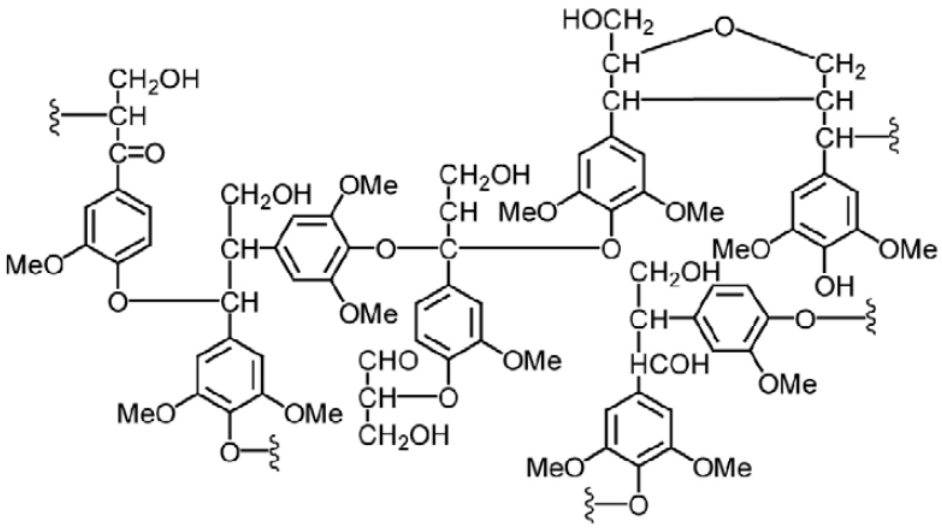

Figure 1.3 General structure of lignin (21).

In addition to these three major polymers, plant cell walls can also contain pectin, which is a methylated polygalacturonic acid polymer, as well as protein and ash. These polymers interact to protect one another and provide structure to the plant cell wall, as seen in Figure 1.4.

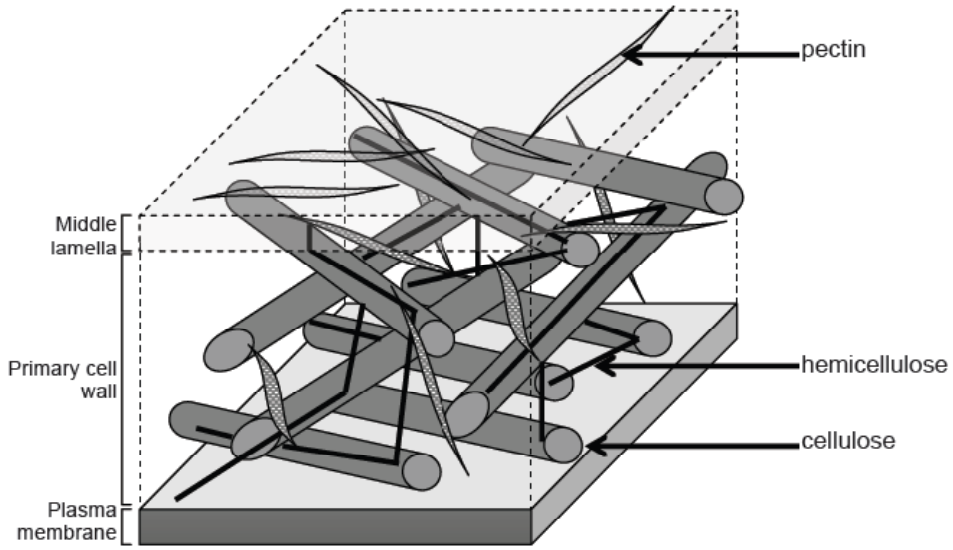

Figure 1.4 Generic structure of a plant cell wall. Lignin is omitted for visual clarity (43).

1.3 Pretreatment Methods

Lignocellulosic biomass is significantly more complex and recalcitrant than starch-based feedstocks. To increase accessibility to the carbohydrate polymers during the enzymatic saccharification step, a variety of pretreatment methods have been developed to disrupt the biomass. Ultimately, the aim is to expose the cellulose fibers by removing the hemicellulose and lignin.

Physical Pretreatments. The most basic pretreatment is particle size reduction. This can be achieved through cutting, shredding, or milling, in a ball or hammer mill. Physical pretreatments are needed for later steps in bioconversion to ethanol, specifically to increase the surface area to allow greater accessibility to hydrolytic enzymes and the fermenting organism (62).

Another relatively simple method of pretreatment is called autohydrolysis, which refers to using the innate properties of the biomass to assist in its breakdown and disruption. There are several ways to achieve this: uncatalyzed steam explosion and liquid hot water extraction are two examples. Uncatalyzed steam explosion heats biomass rapidly with high-pressure steam. The

temperature and pressure are held for a specific amount of time and then explosively decompressed (12). Liquid hot water extraction uses pressure as well, but maintains super-heated water in the liquid state (2, 50, 64). The biomass is then exposed to this superheated liquid water in one of four ways: a biomass-water slurry is passed through the reactor, being heated and cooled at the same time (co-current); the biomass and the water move opposite one another during heating (counter-current); stationary biomass has hot water passed over it (flow-through); or biomass soaking in water is heated, with biomass and water both remaining stationary, and then separated at the end of the process (batch). Both steam explosion and liquid hot water extraction methods work on the principle that water, when super-heated, acts like an acid (2, 93). This allows the liberation of the acetyl groups from the hemicellulose, promoting further depolymerization of the hemicellulose by increasing the concentration of acidic groups. In addition, the rapid depressurization that occurs in steam explosion actually expands and disrupts the remaining structure of the biomass to allow more accessibility to the carbohydrate polymers (13). One of the most notable benefits of physical pretreatments is that only water is used; there are no other chemicals, and thus there are fewer concerns for safety and nothing to dispose.

Acid Pretreatment. An array of acids have been used to pretreat lignocellulosic biomass: both concentrated and dilute sulfuric acid, hydrochloric acid (36), nitric acid (11, 47), and phosphoric acid (47) have all been used to dissolve the hemicellulose, though dilute sulfuric acid has been the most widely used (39, 55, 84). The hemicellulose is hydrolyzed and removed by the acid, thus allowing greater accessibility to the cellulose structure (12). To enhance the action of an acid, it can be coupled with both uncatalyzed steam explosion and liquid hot water extraction to result in a physiochemical pretreatment (11). However, acid treatments such as dilute acid hydrolysis (DAH) have been used industrially for decades to produce furfural, the degradation

product of pentose sugar (31). Furfural is a known fermentation inhibitor, so a detoxification step must be added after acid pretreatment to protect the fermenting organism. This step is referred to as "over-liming" and involves the addition of a strong base to degrade the furfural and 5-hydroxymethyl furfural (39, 58, 59). Detoxification can also be conducted using ion exchange resins; cation exchange chromatography can be used to remove aromatic compounds and mixed bed ion (anion/cation) resins can partially remove acetic acid as well as sugar and lignin degradation products (69). Another method is using another microorganism to convert the inhibitors to less or non-toxic compounds. This has been extensively investigated with white-rot fungi, which degrade lignin and its components with enzymes like laccases and peroxidases (4). Acid pretreatment, regardless of the acid, involves added costs for the chemicals used, for disposal of those chemicals, and additional costs for the non-reactive materials that are needed for construction of the pretreatment apparatus.

Basic Pretreatment. Another chemical pretreatment involves the addition of a base to the biomass. Though this pretreatment also removes some of the acidic substitutions on the hemicellulose (mainly acetyl and uronyl groups), the main action of alkali pretreatment is lignin removal (22). Pretreatment using lime can be carried out at ambient temperatures but will proceed faster at higher temperatures (66). Alkali pretreatment has been used on many types of biomass, originally having been employed in Kraft pulping for paper production from wood.

Basic pretreatment can also be conducted using ammonia. Ammonia freeze explosion (AFEX) or ammonia recycled percolation (ARP) both use liquid ammonia at elevated pressures to soak lignocellulosic biomass. Soaking can be done at ambient or elevated temperatures, with the elevated temperature reducing the soak time. At the end of the soaking step, the system is allowed to rapidly depressurize. The soaking step allows for delignification just as lime

pretreatment does, but the rapid depressurization disrupts the crystalline cellulose. Ammonia also causes the cellulose to swell, allowing increased accessibility to enzymes (44). However, the cost of the ammonia makes this process expensive (44, 45).

1.4 Bioconversion to Ethanol

Enzymatic Saccharification. Although enzymatic saccharification serves as a pretreatment of the biomass prior to fermentation, it is generally considered a separate process from the pretreatments mentioned above. The goal of enzymatic preincubation is to convert lignocellulosic biomass to monomeric sugars that the fermenting organism can use. Due to the complex composition of lignocellulosic biomass, and the difference in lignocellulose between biomass sources, it requires a far greater variety of enzymes to hydrolyze it, than does starchy biomass.

To degrade hemicellulose, many more enzymes are required due to the heterogeneity of sugars, acidic groups, and bonds. For xylan, the main hemicellulose that is found in hardwoods and herbaceous biomass like grasses, xylanases are used to break the xylose backbone, cleaving the β-1,4 bonds, into smaller oligomers of xylose. Xylosidase is then used to cleave the shorter oligomers into monomeric xylose. The xylose backbone is also substituted with chains of sugars and acidic groups. Ferulic esterases and acetyl esterases cleave the ester-bonds that link ferulic acid and acetic acid from the sugars to which they are attached. Arabinofuranosidases cleave arabinose side-chains from the xylose backbone. Lastly, glucuronidases cleave glucuronic acid groups (80).

Substituted β-mannan is the dominant hemicellulose polymer found in softwoods. β-mannanases cleave the mannose backbone at the β-1,4 bonds, which are further depolymerized

into mannose monomers by β-mannosidases. Galactosidases and glucosidases cleave the side chains, galactose and glucose respectively, from the substituted mannose backbone (80).

The second set of enzymes used to hydrolyze lignocellulose is cellulases. These are generally used after the hemicellulose is hydrolyzed since the hemicellulose blocks access to the cellulose fibrils. To degrade cellulose into its basic unit, cellobiose, an endo-glucanase and a cellobiohydrolase (exo-glucanase) are required. These two classes of enzymes cleave the glycosidic bonds in the middle and at the ends of the cellulose polymers, respectively. Also, a third class of enzymes, β-glucosidases, are used to cleave cellobiose into glucose monomers (32, 33).

Another strategy that is being studied is the degradation of biomass by organisms that produce cellulosomes. Cellulosomes are multienzyme complexes in which cellulases and other glycosyl hydrolases are bound to a scaffoldin domain that is attached to the surface of the cell as well as the substrate that is being degraded by a carbohydrate binding module (CBM) (82, 94). These extracellular organelles are found in anaerobes, both thermophilic and mesophilic, and have been described in several *Clostria* species. There are several benefits of the cellulosome in comparison to free cellulase systems. First, the cellulosome brings the cellulosic substrate in very close proximity to the bacterium, preventing the loss of the monomeric sugars that the cellulosome frees by ensuring they are near the bacterium for transport into the cell. Also, the cellulosome shows a high amount of synergism between the different glycosyl hydrolases attached to the scaffoldin, making it a very efficient system (27). Research is being conducted to determine how these cellulosome systems are regulated with the aim of possibly being able to engineer custom cellulosomes for biomass in the future.

Fermentative Organisms. There are several desirable characteristics for development of a robust ethanologen. Ideally, it should be an organism that has few growth requirements and can utilize both hexose and pentose sugars. It should have both high ethanol yield and tolerance, and it should produce ethanol quickly. An increased resistance to inhibitors would be beneficial, since many inhibitors are produced from pretreatment as well as enzymatic saccharification. Also, an ethanologen should be easily genetically modified and have heterologous gene expression.

S. cerevisiae, better known as Baker's yeast, has been used industrially for hundreds of years for the production of ethanol, the majority for human consumption. This robust eukaryotic organism has high ethanol production and tolerance, but can only use monomeric hexose sugars, which means that the hemicellulose component of lignocellulose goes unutilized during bioconversion (67). Research in recent years has attempted to integrate the ability to use pentose sugars and cellobiose in addition to hexoses. Several strains have been developed that have heterologous expression of β-glucosidases to allow the breakdown of cellobiose (89). Another approach is to engineer new metabolic pathways into *S. cerevisiae* to allow utilization of other sugars. Strains have been developed to utilize xylose by integrating xylose isomerase to convert xylose to xylulose , or by integrating the xylose-fermenting pathway from *Pichia stipitis* (51, 52). Arabinose fermentation has been achieved roughly the same way by introducing bacterial and fungal pathways (7, 77). Manipulation of expression of some of *S. cerevisiae*'s native galactose utilization genes has increased galactose uptake and conversion (70). However, *S. cerevisiae* preferentially uses glucose over any other sugar, slowing the rate of ethanol production (67).

Zymomonas mobilis is a gram negative bacterium with a homoethanol pathway that has a significantly higher ethanol productivity than *S. cerevisiae* (26, 78). *Z. mobilis* utilizes the Entner-Dourdoroff (ED) pathway instead of the Embden-Meyerhoff-Parnas (EMP) pathway during anaerobic fermentation, which produces half the ATP per glucose compared to the EMP pathway. This results in a larger portion of the carbon entering the cell being funneled into fermentation products instead of biomass (83). However, *Z. mobilis* can only use glucose, sucrose, and fructose to produce ethanol. Strains have since been developed to ferment xylose and arabinose by inserting *Escherichia coli* genes into the chromosome (25, 101). Genes for both of these fermentation pathways were integrated into the chromosome in *Zymomonas mobilis* strain AX101. This strain can completely ferment glucose and xylose, but leaves some arabinose untouched (54).

Escherichia coli has been investigated as a possible ethanologen. The advantages of using this organism include its ability to ferment a wide variety of sugars, easy growth and management, and a wealth of experience in industrial applications. Unfortunately, it has a narrow pH range for growth and produces mixed acids in addition to ethanol from fermentation. In addition, there are negative perceptions of *E. coli* in the media and the public because of pathogenic strains. *E. coli* strain KO11 (Figure 1.5) was created by integrating the PET (production of ethanol) operon containing pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh II*) genes from *Z. mobilis* into the *E. coli* chromosome, resulting in almost exclusive production of ethanol in that strain. It was inserted at the pyruvate formate lyase (*pfl*) gene, which eliminated the competing *E. coli* pathway for ethanol production. Also, fumarate reductase (*frd*) was knocked out to prevent succinate production (46). After a long-term adaption

study with *E. coli* KO11, the ethanol tolerance was increased by 10%, fermentation time was reduced, and tolerance to inhibitors was increased, creating *E. coli* LY01(97).

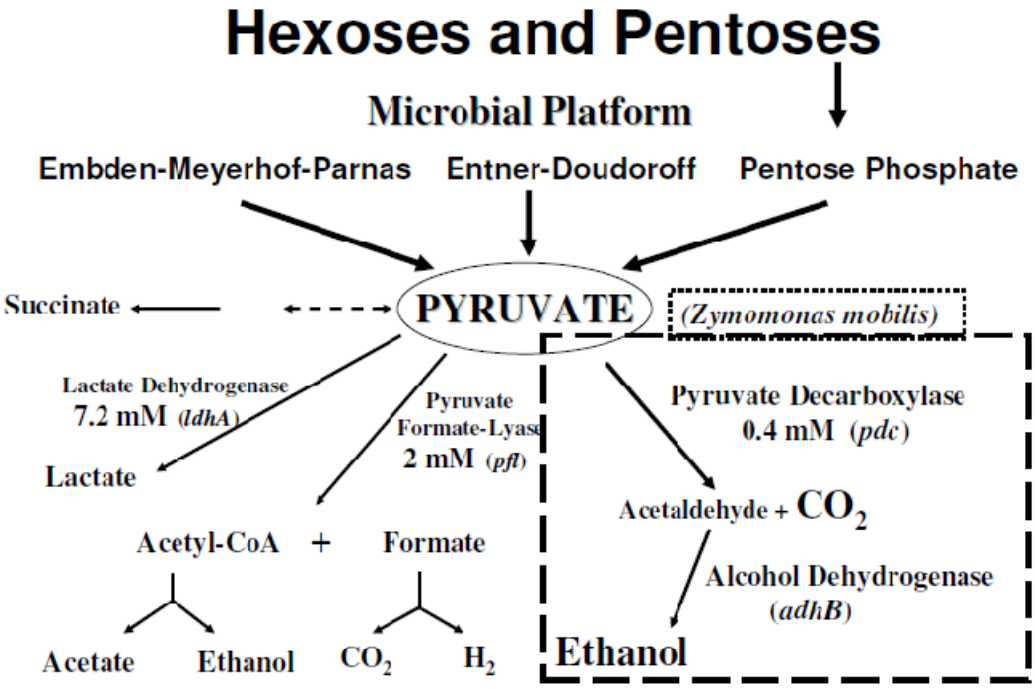

Figure 1.5 Schematic for conversion of hexose and pentose sugars by recombinant *E. coli* with the *Z. mobilis* ethanol pathway. Dotted lines indicate *Z. mobilis* gene activity(74).

Another approach has been taken to engineer *E. coli* with a native homoethanol pathway in an *E. coli* B background. Instead of adding in foreign genes, native genes encoding pathways for competing fermentation products (products other than ethanol) were knocked out in *E. coli* SZ420. Also, the pyruvate dehydrogenase complex was highly expressed under an anaerobic induced promoter, resulting in a 90% conversion of glucose and xylose (102).

Fermentation Processes. Several processes can be used after pretreatment to conduct the enzymatic saccharification and fermentation, including separate hydrolysis followed by fermentation, simultaneous saccharification and fermentation, partial saccharification and co-fermentation, and consolidated bioprocessing.

Separate Hydrolysis and Fermentation (SHF). In SHF, all the steps to conversion to ethanol occur discretely. First is a physical/thermo chemical pretreatment, then enzymatic saccharification, producing a sugar stream which is then fermented separately with the ethanologen (29). With this process it is possible to use any type of enzyme (generally fungal) and any fermenting organism, fungal or bacterial, at its respective optima since the steps are separate. However, creating a concentrated sugar stream during the enzymatic preincubation that must then be transferred to a fermentation tank increases the risk of contamination by other organisms and osmotic stress in the ethanologen.

Simultaneous Saccharification and Fermentation (SSF). In SSF, the enzymatic saccharification and fermentation are conducted concurrently in the same vessel. This allows the monomeric sugars to be consumed at approximately the same rate as they are produced, reducing the risk of contamination as well as osmotic stress. Also, sugar consumption relieves any enzyme inhibition that can occur as the enzyme products, sugars, build up (95). This process was patented in the 1970s by Gulf Oil Chemicals Company and reduced the complexity of bioconversion of ethanol in addition to reducing cost (35, 85). However, one is limited to using an organism and enzymes that have approximately the same temperature and pH optima, which generally limits the fermenting organism to a yeast such as *S. cerevisiae* or *P. stipitis.*

Partial Saccharification and Co-Fermentation (PSCF). PSCF is a hybrid between SHF and SSF. An enzymatic preincubation is conducted for a discrete amount of time at the pH and temperature optima for the fungal enzymes. The temperature and pH are changed to the optima for the ethanologen. However, the enzymes remain active, though at a reduced rate, throughout the course of the fermentation (29). This can result in some initial enzyme inhibition as the monomeric sugar concentration increases during the enzymatic preincubation, but inhibition is

relieved when the ethanologen is added. Also, the fermenting organism receives a constant sugar feed from the continued enzyme activity. Again, the process is simpler than SHF since it occurs in one vessel, and the risk of contamination is reduced since the enzymatic preincubation product need not be transferred to a new tank.

Consolidated Bioprocessing (CBP). CBP is a completely different approach from the three discussed previously. In this process, an organism would produce enzymes required to hydrolyze lignocellulose and ferment all the sugars released, both hexoses and pentoses. CBP is being approached two different ways: increasing the ethanol production capability of strains with high enzyme activity, or increasing the enzyme expression in highly fermentative strains (57). The first strategy, also called the native cellulolytic strategy, has been focusing on thermophilic anaerobes like *Clostridium thermocellum* and *Clostridium cellulolyticum.* Ethanol production in *C. cellulolyticum* was increased by a little over 50% when the *pdc* and *adhII* from *Z. mobilis* were integrated into the organism (40). This and other work has proven promising, and projects to eliminate other fermentation products as well as increase ethanol tolerance are underway (57).

The second approach, called the recombinant cellulolytic strategy, has been attempted with Gram-type negative organisms and *S. cerevisiae*. Cellulolytic enzymes have been successfully added to *E. coli, Z. mobilis, Klebsiella oxytoca, and S. cerevisiae.* Also, integrating the ability to utilize non-native carbon sources has been investigated and successfully done, as discussed previously. Either of these strategies results in an organism that does not need added enzymes to break down biomass, and thus a simpler process overall. However, there are currently no organisms that can do this efficiently enough to stand alone (57).

1.5 Objectives

Attempting to reduce the cost and increase the efficiency of enzymatic saccharification and fermentation of lignocellulosic biomass, this study aimed at developing and optimizing a pretreatment method, pressurized batch hot water, for the pretreatment of, specifically, warm season grasses. This pretreatment is gentle yet effective, requiring no harsh chemicals. It also significantly increases the efficiency of the enzyme saccharification step. Warm season grasses were chosen as a substrate for these studies since they were bred for, and grow well in, the southeastern United States.

Chapter 2 provides the design and proof of concept for this pretreatment through the pretreatment and fermentation of Tifton 85 bermudagrass. Chapter 3 describes the optimization through particle size and solids loading of PBHW pretreatment, again using Tifton 85 bermudagrass. Chapter 4 provides a comparison of Tifton 85 bermudagrass, ADEL switchgrass, and Merkeron napiergrass for conversion to ethanol, looking at the fermentation of untreated and PBHW pretreated material as well as changes in composition and inhibitors through the process. Lastly, Chapter 5 summarizes all the findings from this study.

CHAPTER 2

HYDROLYSIS OF TIFTON 85 BERMUDAGRASS IN A PRESSURIZED BATCH HOT WATER REACTOR[1]

[1]Brandon, Sarah Katherine, Mark A. Eiteman, Krishna Patel, Michelle M. Richbourg, David J. Miller, William F. Anderson, and Joy Doran Peterson. 2008. Hydrolysis of Tifton 85 bermudagrass in a pressurized batch hot water reactor. J. Chem. Technol. Biotechnol., 83: 505-512. Reprinted here with permission of the publisher.

2.1 Abstract

Background: Ethanol production from grass is desirable due to the large amount of biomass it produces. However, a pretreatment is necessary before fermentation to increase ethanol yield. Tifton 85 bermudagrass was treated with a newly designed pressurized batch hot water reactor. Multiple temperatures, pressures, and reaction times were evaluated, and reducing sugars liberated during enzymatic hydrolysis were determined.

Results: Pressure had a negligible effect on digestibility of the grass, and a reaction temperature of 230°C for two minutes was the most effective in releasing reducing sugars. Fermentations were conducted with untreated grass and with grass treated for two minutes at 200°C or 230°C to confirm that the increase in reducing sugar concentration resulted in an increased ethanol yield. Following hydrolysis with 2 filter paper units (FPU) of a mixed cellulase enzyme cocktail per gram dry weight of grass, fermentations were performed with engineered *Escherichia coli* strain LY01. Grass treated at 230°C produced 14.7 g/L of ethanol, which was significantly higher than 200°C treated grass (11.0 g/L) and untreated grass (9.0 g/L). Ferulic and *para*-coumaric acids were also released during the fermentations.

Conclusion: Pressurized batch hot water reactor pretreatment is effective in increasing ethanol yield of grass in fermentations.

2.2 Introduction

Renewed interest in alternatives to petroleum products, especially for liquid transportation fuels, has increased demand for ethanol. Producing fuel from renewable resources such as grasses is desirable because of the large quantities of biomass available(23). Established forage grass crops, such as switchgrass, bermudagrass, and napiergrass initially were bred for increased biomass production as animal feedstocks, but this characteristic is amenable for ethanol

production. Bermudagrass (*Cynodon dactylon*) is grown on 10-15 million acres in the southern United States. Tifton 85 (T85) is a hybrid between Tifton 68 and PI 290884 from South Africa. This grass is hardy and produces significantly more dry matter than other bermudagrass cultivars (17).

Efficient conversion of plant material to ethanol requires a pretreatment prior to enzymatic hydrolysis, making the substrate more available for enzymatic action. Once the hemicellulose and cellulose are converted to monomeric sugars by enzymatic hydrolysis, these sugars can be fermented by microorganisms to produce ethanol. In addition to lignified cell walls, grasses have concentrations of low molecular weight phenolic acids ester-linked to arabinose (42). These compounds also occur in grasses in non-lignified parts of the cell walls (18). Treatments designed to separate the fermentable sugars from the aromatic constituents could enhance fermentation yields and provide a valuable co-product.

Liquid hot water (LHW) extraction of biomass provides an effective way to pretreat cellulosic material by beginning disruption of hemicellulose prior to enzymatic hydrolysis. This treatment consists of exposing biomass to highly pressurized water at high temperatures. Liquid water at 220°C has a pH of approximately 5.5 as a result of an ion product of 10^{-11} (2). Exposure of biomass to LHW causes liberation of acetyl groups from hemicellulose and increased depolymerization. These reactions decrease the pH of the solution further, mimicking very dilute acid hydrolysis (DAH), a common technique which uses low concentrations of acid in hot water to break down hemicellulose (41). When LHW was applied to sugarcane bagasse and leaves, all hemicellulose and more than 60% of the lignin was hydrolyzed with little loss of cellulose (2). A similar study with alfalfa fiber resulted in hydrolysis of almost 90% of hemicellulose, 24% of cellulose and 6% of lignin (50). In this paper, we examine the effects of a high temperature

pressurized water reactor for hydrolysis of T85 bermudagrass and the subsequent effect of this pretreatment on ethanol production.

2.3 Materials and Methods

Pressurized Batch Hot Water (PBHW) Hydrolysis Reaction

Tifton 85 bermudagrass obtained from the USDA-ARS Coastal Plain Experiment Station (Tifton, GA) was used for all hydrolysis studies. The bermudagrass was harvested at 4 weeks and dried in the field in bales for an additional week. The moisture content of the grass was determined to be 6.5% by drying at 110°C for 1 h.

PBHW hydrolysis was examined in a 2-liter pressure vessel (Model 4600 Parr Instrument Co., Moline, IL) surrounded by retractable ceramic heaters (Figure 2.1). Approximately 15g of unprocessed grass was placed in a 500μm (35 mesh) stainless steel basket and then immersed in 1450 mL of deionized water in the vessel for a final solids concentration of 1% w/v. Prior to reaction cycles, the head plate was secured and the headspace purged with nitrogen via two ports. The vessel was filled with nitrogen at room temperature to achieve a target pressure at the set point temperature. Heating, release of vessel contents and collection of time, temperature and pressure data were measured via a datalogger and associated software (Model 21X micrologger, Campbell Scientific, Inc., Logan UT). The reaction cycle began by heating the vessel to a set point temperature. The *reaction time* was the time set to elapse from the moment the contents of the reactor first reached the set point temperature to the moment the outlet valve automatically opened. The *reaction temperature* and *reaction pressure* were calculated as the mean of each variable recorded at 15 second intervals during the reaction time. After the reaction time elapsed at this set point temperature, an 80 psi pneumatically actuated ball valve released the liquid

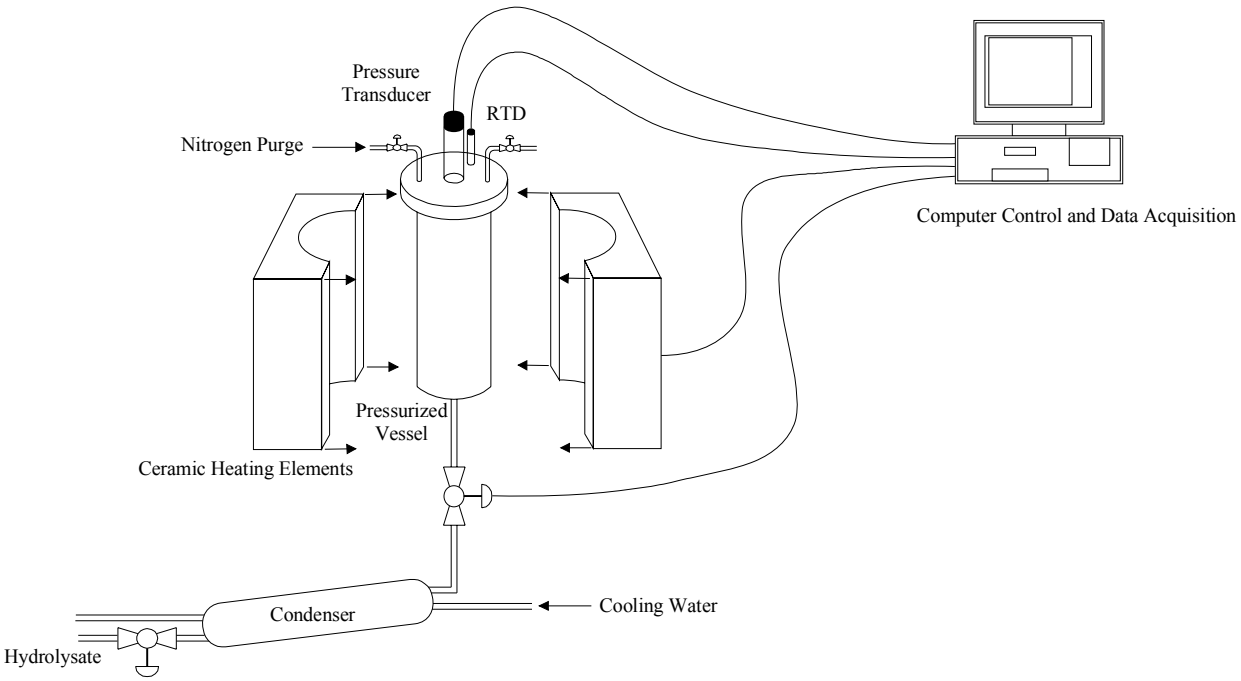

Figure 2.1 Schematic diagram of pressurized hot water hydrolysis system.

hot liquid was cooled to less than 50°C and the system depressurized to less than 40 psi in roughly ten seconds. The hydrolyzed solids (wet but no longer pressurized) remained in the basket to cool. As a safety precaution, a low pressure switch at the water inlet required a minimum pressure of 10 psi to actuate the pneumatic valve and to allow the ball valve to release the hydrolysate into the condenser. A manual ball valve to release the condensate and a 50 psi pressure relief valve were located at the outlet of the condenser. The hydrolyzed solids were then removed from the vessel and dried at 40°C for 90 minutes using a fluidized bed dryer (Endecott FBD2000, London, UK). Liquid and dried samples were stored (at –20°C and 4°C, respectively) for subsequent enzyme and fermentation studies.

Temperature was monitored inside the vessel using two 1.5 mm platinum resistance temperature detectors (RTDs, Model PR11, Omega Engineering, Inc, Stamford, CT). One RTD was connected to the process controller (CN8200 Series, Omega Engineering, Inc., Stamford,

CT) for the system. The remaining RTD was connected to the datalogger. A pressure transducer (PX02 Series, Omegadyne, Inc., Sunbury, OH) occupied a port on the reactor head plate. The vessel, valves and sensors were designed to withstand operating conditions of 350°C and 1000 psi, and the maximum operating conditions used in this study were 230°C and 700 psi (5 MPa).

Post-PBHW Enzymatic Hydrolysis

After PBHW pretreatment, dried bermudagrass samples were ground in a mill (Cyclotech Model Sample mill, Foss, Tecator, AB Hognas, Sweden) and further hydrolyzed enzymatically for later statistical analysis. The enzyme reaction was conducted for 48h at 40°C in a 0.05 M citrate buffer solution pH 4.5 with a 5% (w/v) solids load. Sodium azide was added at 0.15% (w/v) to inhibit microbial contamination. Celluclast 1.5 FG containing approximately 102 filter paper units (FPU) /mL and Novozyme 431 containing approximately 250 cellobiase units (CBU)/mL (both from Novozymes, Franklinton, NC) were loaded at a rate of 4.5 FPU and 44.3 CBU per gram of dry weight of bermudagrass. Samples were boiled for 15 minutes to terminate enzymatic hydrolysis (34).

Statistical Analysis of Enzymatic Hydrolysis

Reducing sugar concentrations of the hot water hydrolysate and the enzyme hydrolysate were measured using the dinitrosalicylic acid assay with glucose as the standard (60). Glucose and xylose concentrations of the hot water hydrolysate were determined by HPLC (63). These values were then applied to a Box-Behnken response surface statistical design (Design-Expert software, Stat-Ease, Inc., Minneapolis, MN, USA) to evaluate the performance of the pressurized water vessel and the effect of temperature (targeted as 200-230°C), pressure (targeted to be in range 315-700 psia) and reaction time (2-8 minutes) on four hydrolysis dependent variables: glucose dissolution, xylose dissolution, total reducing sugars dissolution, and the enzymatic digestibility

of the remaining solid material. The actual recorded reaction temperature and pressure, rather than the target values, were used in the statistical analysis. The saturated model was fit for each of these variables according to the constraints of the design (the model with linear terms, two-way interaction effects plus quadratic effects), and eliminated the non-significant terms to yield a reduced model. The values for the pressure were constrained by the vapor pressure of water at the reaction temperature (minimum) and by the permissible pressure in the condenser (maximum), therefore additional values for this parameter were used in the statistical analysis.

PBHW Hydrolysis for Fermentation

T85 bermudagrass was treated by PBHW at 200°C and 230°C at 1% w/v solids to generate enough material for two fermentations of each treatment. The hydrolysate collected was analyzed for sugars, furfural, 5-hydroxymethylfurfural (5-HMF), *p*-coumaric acid, and ferulic acid using HPLC. Treated grass was dried as described previously. Following drying, grass samples with the same treatment were combined, ground twice in a Fritsch Pulverisette 25 (6.0 grill) (Laval Labs, Laval (Quebec) Canada), and ground in a coffee grinder (IDS77 Mr. Coffee, Inc., Bedford Heights, OH). Final particle size varied between 0.1 mm and 3 mm.. Percent moisture was determined by drying a sample of each condition overnight in a drying oven at 100°C. Grass samples were then analyzed for neutral detergent fiber (NDF), acid detergent fiber (ADF), lignin (ADL), and protein NIR at the Feed and Environmental Water Lab (FEW-AESL, University of Georgia, Athens, GA), according to standard protocols (68).

Fermentations

Fermentations were conducted at 10% w/v solids. Grass and dH_2O were added to equal 100 mL and autoclaved. Subsequently, 190 mL of 2x Luria Bertani medium (LB, Fisher, Fair Lawn, NJ) was added. Novozymes (Franklinton, NC) Batch NS50012 (23 FPU /ml, 443 IU/ml

xylanase, and 3497 polygalacturonase units (PGU)/ml) and Batch NS50013 (57 FPU/ml, 4049 IU/ml xylanase, and 12 PGU/ml) were filter sterilized, added to 2x LB, and then added to the fermentors for a final concentration of 2 FPU/g dry wt substrate. The pH was adjusted to 4.5 with 2N HCl. These mixtures were incubated in a 45°C circulating water bath with stirring for 22h.

Escherichia coli strain LY01 (37, 97) was inoculated from glycerol stocks and incubated at 37°C for 18 hours in LB containing 50 g glucose and 40 mg chloramphenicol. Fermentors were inoculated for a starting OD_{550} of 1. The pH was adjusted to 5.5 with KOH, and water temperature bath decreased to 35°C. Samples were taken every 24 h for 120 h. Samples were filtered (Corning Spin-X® Centrifuge Tube Filter 0.22 μm, Sigma-Aldrich, St. Louis, MO), stored in O-ring microfuge tubes and frozen at -80°C. Reducing sugars were determined as described previously (60). Filtered samples were analyzed for ethanol by gas chromatography (Shimadzu GC-8A, Columbia, MD) as previously described(30) using a flame ionization detector and the parameters: injector/detector temperature of 250°C, column temperature of 65°C, 0.53 mm ID × 30 m column with 3 μm film. They were also analyzed for phenolic acids by HPLC and for sugars by GC.

Analysis of Soluble Carbohydrates

25 μL of filtered liquid sample was blown to dryness by nitrogen after adding 50 μL of MeOH containing 91 μg of phenyl glucose as the internal standard. One-two drops of acetonitrile were also added to dried samples and then blown to dryness again. Silylation was performed by adding 50 μL of both trimethylsilane (TMS) and N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) to dried samples followed by incubation at 75°C for 30 minutes. Arabinose, xylose, and glucose, both α and β conformations, were determined for 1 μL aliquots of silylated sugar

derivatives by gas chromatography (model 5890, Hewlett Packard Inc., Atlanta, GA) using J&W DB-5 capillary column (30 M x 0.25 mm I.D.) (Agilent, Wilmington, DE). The temperature program started at 155°C, and increased to 215°C at a rate of 1.3°C/ min. The temperature then increased to a final temperature of 320°C at a rate of 5°C/min. Injector temperature was 250°C and detector temperature was 350°C.

Phenolic Quantification

This procedure was adapted from a chlorogenic acid quantification protocol (75). 100 µL of sample was diluted with 100 µL dH_2O. 50 µL of MeOH containing 0.0403 mg of chrysin was added as an internal standard. 3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid (ferulic) and 3-(4-hydroxyphenyl)-2-propenoic (*p*-coumaric) acid concentrations were determined for 20 µL aliquots of the solution by reverse-phase HPLC (model 1050, Hewlett Packard Inc., Atlanta, GA) using an H_2O/ MeOH linear gradient from 10% to 100% MeOH in 35 minutes and a flow rate of 1 mL/ min. The column was a 250 mm × 4.6 mm ID, 5 µm Ultrasphere C18 (Beckmann Instruments Inc., Norcross, GA). The detector was a diode array system and 340 nm was used for further analysis. Each solvent contained 0.1% H_3PO_4. Response factors were determined with pure authentic compounds (Sigma-Aldrich Co., St. Louis, MO). Quantification of ferulic and *p*-coumaric acid was based on the internal standard (chrysin) and peak identification was based on co-chromatography (spiking) and spectral analysis.

2.4 Results

A series of 25 experiments were performed using three variables: reaction time (2 minutes, 5 minutes, and 8 minutes), reaction temperature (200°C, 215°C, 230°C) and reaction pressure (range of 315 – 700 psia). Table 2.1 depicts both the set values for temperature and pressure and the actual values obtained during the reactor runs. Figure 2.2 shows the temperature and

Table 2.1. The effect of reaction time, temperature, and pressure on hydrolysis of Tifton 85 bermudagrass in a pressurized batch hot water reactor.

Set temperature (°C)	Real temperature values (°C)	Time (min)	Initial pressure (psig)	Set pressure (psig)	Real pressure values (psig)
200	205.4	2	35	315	352.7
200	205.2	2	80	400	436.5
200	201.7	2	140	525	519.6
200	206.1	5	35	315	342
200	201.8	5	140	525	532.9
200	202.3	8	35	315	310.9
200	202.1	8	80	400	415.4
200	201.1	8	140	525	522
215	215.8	2	37	400	372.5
215	216.3	2	140	625	620.8
215	215.7	5	105	550	548.9
215	224.9	5	105	550	610.9
215	216.9	5	105	550	548.4
215	215.4	5	105	550	544.4
215	216.1	5	105	550	534
215	212.1	8	37	400	378.4
215	215.5	8	140	625	627.8
230	231.1	2	0	415	418.2
230	231.7	2	57	550	560
230	230	2	120	700	696.8
230	231.2	5	0	415	409.5
230	230.2	5	120	700	644.7
230	229.1	8	0	415	408.5
230	230.4	8	57	550	526.8
230	230.5	8	120	700	682.2

pressure profile during a typical run. The vessel was heated from ambient to 100°C in 8 minutes, at which point data collection began, and from 100°C to a set point (e.g., 200°C) in another 15 minutes. The temperature increased linearly to the set point, and then commonly exceeded the set point by 2-3°C, before decreasing slightly during the reaction time. The release of the hot water reduced the pressure immediately to less than 50 psia, but the temperature of the grass remaining in the vessel typically decreased only 10-15°C immediately, and then slowly over 20-30 minutes to 100°C. In general, the actual temperature deviated less than 5°C from the targeted temperature (mean deviation was 2.6°C), while the actual pressure generally deviated less than 40 psia from the targeted pressure (19 psia mean deviation). The rapid heating (6.67°C/min average) and

cooling (4°C/min average) of the reactor justifies using the two minute temperature plateau region as the reaction time.

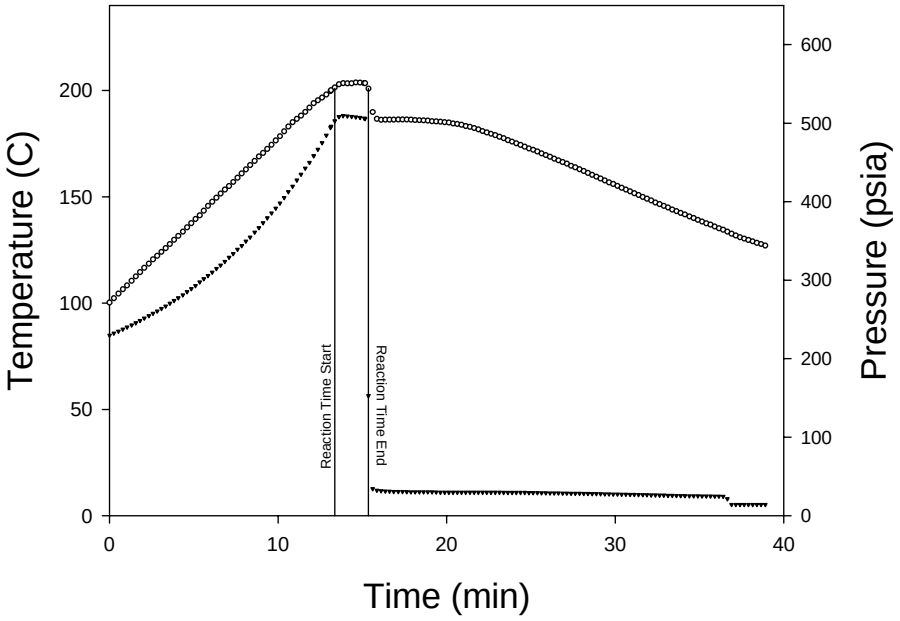

Figure 2.2 Temperature and pressure profile for a typical hydrolysis experiment. For this particular experiment the reaction time was 2 minutes and the set point temperature was 200°C**.**

Of the four hydrolysis dependent variables studied, three quantified the effect of the physical parameters of the reactor (time, temperature, and pressure) on the dissolution of simple sugars in liquid hydrolysate. Pressure did not significantly affect any of the four measured variables. The mass of glucose dissolved over the range of temperature and time studied did not correlate with either of these two factors. However, the mass of xylose and the total mass of reducing sugar both correlated linearly with the time and temperature, increasing as either variable increased, but with the temperature having a slightly greater effect. The mass of xylose dissolved was determined to be described by the following model (33 degrees of freedom, F-test = 29.67, $R^2 = 0.657$, $P<0.0001$):

$$\textit{Xylose dissolved (mg)} = 62.6 + 25.63 \times \textit{time} + 50.76 \times \textit{temperature}$$

In this model, both the time (-1 = 2 min, 0 = 5 min, +1 = 8 min) and temperature (-1 = 200°C, 0 = 215°C; +1 = 230°C) are represented as coded variables. Similarly, the mass of reducing sugar was described by the following coded model (35 degrees of freedom, F-test = 26.96, R^2 = 0.620, P<0.0001):

$$\textit{Reducing sugar dissolved (mg)} = 1225.0 + 312.6 \times \textit{time} + 418.3 \times \textit{temperature}$$

The other variable studied was the digestibility of the solid grass which was calculated by determining the sugar yield, defined as the mass of reducing sugar hydrolyzed in the enzymatic reaction after hot water treatment per mass of sample. Both temperature and time significantly affected this sugar yield. Specifically, the sugar yield was described by a (coded) model which included a quadratic term and an interaction term (24 degrees of freedom, F-test=27.81, R^2 = 0.848, P<0.0001):

$$\textit{Sugar yield (mg/mg)} = 0.4074 + 0.0304 \times \textit{time} + 0.0896 \times \textit{temperature}$$
$$- 0.0448 \times \textit{temperature}^2 - 0.0442 \times \textit{temperature} \times \textit{time}$$

Although sugar yield increased linearly with both time and temperature, the presence of the negative interaction term caused the optimal time to be lower the greater the temperature. Moreover, the maximum sugar yield within the range studied occurred at the highest temperature (230°C) and lowest time (2 min.), while the minimum occurred at the lowest temperature (200°C) and time (2 min.). This phenomenon for a two minute hydrolysis time is shown in Figure 2.3.

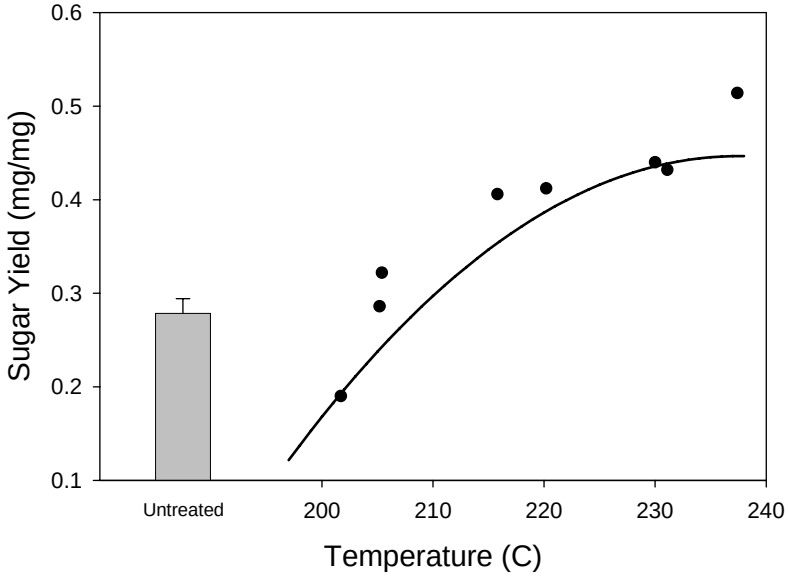

Figure 2.3 Enzyme digestibility of bermudagrass following 2 minute hydrolysis. The curve depicts the model prediction of sugar yield resulting from statistical analysis and the data points represent observed sugar yields as a function of reaction temperature (at a variety of reaction pressures). Values are corrected for contribution of DNS-reactive stabilizers in the enzyme mixtures. The bar represents the sugar yield of a sample of untreated bermudagrass.

By combining net weight loss data with the NIR data, the percent dissolution of cellulose, hemicellulose and lignin were estimated. There is a significant increase in the dissolution of hemicellulose for the 230°C pretreatment (54 %) over the 200°C pretreatment (21 %). A modest increase in cellulose dissolution from 6% at 200°C to 11% at 230°C was observed. Lignin dissolution increased from 0% to 5% at the higher temperature.

To confirm that increase in digestibility would correlate in increased ethanol yield, a series of partial saccharification and co-fermentation experiments (PSCF) were conducted using three conditions: untreated T85, 200°C (2 min.) treated T85, and 230°C (2 min.) treated T85. There was no furfural or 5-HMF present in the hydrolysate following the pretreatments (data not shown). Table 2.2 outlines the profile of sugars released by the PBHW pretreatments as well as by the 24 hour enzymatic hydrolysis. Minimal sugars were released by the PBHW pretreatment

alone. More arabinose and xylose were released from the 230°C treated solids than the 200°C or untreated solids which corresponded well with the increased dissolution of hemicellulose that occurred in the 230°C treated grass (Table 2.2). At time zero there is more glucose liberated in the untreated grass, perhaps because autoclaving liberated the easily released sugars and these sugars had already been released in the PBHW pretreated samples. However, after 24h of enzymatic hydrolysis, the glucose released from either pretreatment of the grass solids is very similar and higher than the untreated grass, presumably due to enhanced accessibility of the cellulose. Ethanol production and reducing sugar levels over the course of the fermentations are shown in Figure 2.4. As expected from the preliminary reducing sugar analyses, the 230°C pretreated grass resulted in an increase in ethanol production of roughly 4.5 g/L over the 200°C pretreated grass. Untreated grass produced the least amount of ethanol of the experiment (9 g/L).

Table 2.2. Sugars released from treated and untreated Tifton 85 bermudagrass by 24 hour enzymatic hydrolysis.

	Arabinose (mg g^{-1} grass)			Xylose (mg g^{-1} grass)			Glucose (mg g^{-1} grass)		
Treatment	Hydrolysate	0 h	24 h	Hydrolysate	0 h	24 h	Hydrolysate	0 h	24 h
Untreated	n/a	48.91	60.45	n/a	14.79	29.88	n/a	371.69	541.63
200 °C Treated	0	56.28	69.23	0	60.03	133.15	0.69	244.09	640.34
230 °C Treated	0.25	79.39	76.75	0	129.49	282.36	0.31	209.5	635.32

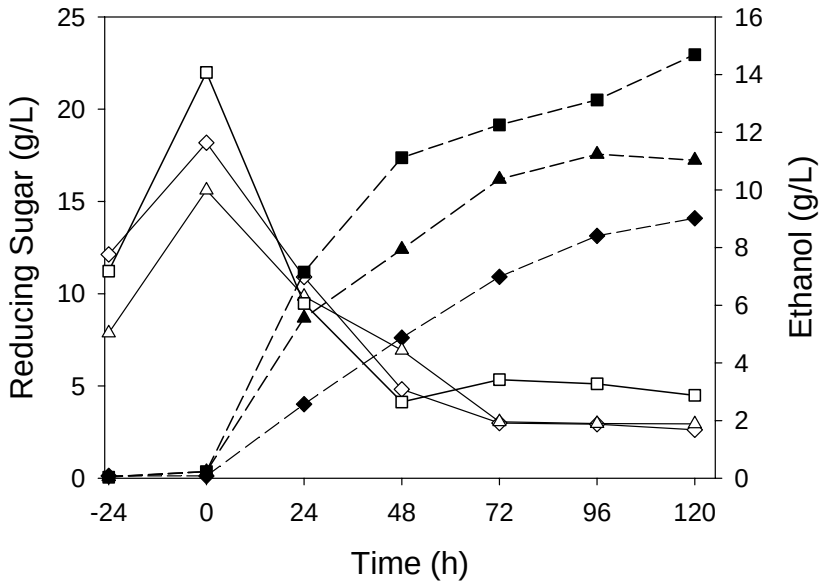

Figure 2.4 Average reducing sugar concentration and ethanol production over the course of fermentations of untreated, 200°C, and 230°C treated T85. Solid lines correspond with ethanol concentrations and dotted lines correspond with sugar concentrations. Symbols are as follows: diamond, untreated; triangle, 200°C treated; square, 230°C treated. Fermentations were conducted at 35°C in an immersion circulator for five days at 10% solids. The -24 hour time point corresponds with the beginning of the 24 hour enzymatic hydrolysis (at 45°C). The actual fermentation began (bacterial inoculation) at time 0 h. Reducing sugars are removed at the same rate that ethanol is produced.

Phenolic acids, *p*-coumaric acid and ferulic acid, were also released during the fermentations. There were small amounts of these compounds in the hydrolysate from each of the pretreatment conditions. Following enzyme addition and inoculation of the fermentations, both *p*-coumaric and ferulic acid levels increased over the 120 hours. Of the three conditions, the levels of both compounds were highest in the untreated grass (data not shown). Hydrolysate samples from the PBHW pretreatment were also analyzed for furfural and 5-hydroxymethylfurfural (5-HMF), neither of which was present.

2.5 Discussion

PBHW pretreatment is a promising option for grass biomass. Our non-flow-through PBHW reactor is reliable and effective; pressure and temperature were held constant over the reaction time, and significant dissolution of complex carbohydrates occurred as measured by enzymatic hydrolysis. The first objective was to evaluate the effectiveness of enzymatic hydrolysis of PBHW pretreated T85 bermudagrass compared to untreated grass samples. Cellulase and cellobiase enzymes used for this aspect of our studies have been used previously to determine the effectiveness of cellulose degradation from pretreated biomass (24, 65, 98) The later enzymatic hydrolysis reactions and subsequent fermentations were conducted in order to correlate digestibility with fermentability of PBHW pretreated bermudagrass. Several different commercial enzyme combinations were compared for their ability to liberate sugars from cellulose and hemicellulose in the course of this study. Although all performed well, the Novozyme batch preparations used during the fermentation study performed the best for our current protocol (data not shown). We reduced the FPU enzyme load in the fermentations in order to better observe differences in the pretreatment conditions. Future studies will optimize the enzyme loading for maximum ethanol production. The ethanologen LY01 was selected as the biocatalyst because it is more resistant than many other ethanol producing organisms to potential fermentation inhibitors such as furfural, HMF, and phenolic compounds (64, 99, 100).

Pressure had a negligible effect on sugar yield from T85, which agrees with prior research on other cellulosic materials (2, 64, 90). Although the heating and cooling took longer than the specified reaction time, the rates were rapid enough that the plateau region is justified as the actual reaction time (Figure 2.2). After determining the sugar yield of the reactions in Table 1, the data was fit to a graph (Figure 2.3). Based on the data presented in Figure 2.3, we determined

the optimal reaction time of 2 minutes and temperature of 230°C, which agrees with previous findings of other groups (2, 50, 53, 64). We conducted the pretreatment at 1% w/v solids concentration since low solids concentrations may also be critical to effective pressurized batch hot water pretreatment (53).

An advantage of this pretreatment is the absence of a required strong base or acid used respectively in ammonia fiber explosion (AFEX) and DAH pretreatments (66). Not only does this remove the additional cost of these reagents, but it eliminates the expense for their subsequent safe removal and disposal. van Walsum and colleagues compared DAH, steam explosion, and LHW pretreatments for effectiveness based on several criteria, from fiber reactivity to construction materials. Though they are all effective pretreatments, LWH resulted in high pentosan recovery and was less costly than the more researched DAH, which also requires particle size reduction, unlike LHW (92).

Grass particle size and enzyme loading were not optimized for fermentation in these experiments. Mosier and colleagues determined that biomass undergoing LHW does not need to have particle size reduction due to the physical properties of the treatment (71). This conclusion should apply to our PBHW pretreatment; however, this was not investigated as our reactor design does not currently permit processing of very small particles. In order to standardize the enzymatic hydrolysis of the pretreated grasses, particle size was reduced prior to enzymatic digestion. Even with low enzyme loading, the 230°C pretreatment was effective in making the grass more available for enzymatic attack.

Inhibitors are often produced by biomass degradation during pretreatment and hydrolysis steps and include phenolics from lignin degradation and furfural and 5-HMF produced when monomeric sugars are degraded into aldehydes or reactive acids. One study found that these are

produced by LHW pretreatment when O-acetyl and uronic groups from hemicellulose are cleaved and become reactive acids (48). The high temperatures and pressures in LHW pretreatments accelerate this acid-catalyzed degradation of monomeric sugars by decreasing the pH as organic acids are formed. This is a result of the pretreatment as well as the substrate that is being treated. Weil and colleagues found that controlling the pH of yellow poplar wood sawdust, which reached a pH between 2.8 and 3, during a LHW pretreatment by adding base prevented the formation of inhibitors (92). The pH of the liquid hydrolysate from our reactor ranged from 4.2 to 4.8 and may not have been low enough to promote significant formation of inhibitors. The short residence time of the pretreatment likely prevented the formation of inhibitors as well. The absence of these compounds in this study is promising for future applications of our PBHW system specifically for bermudagrass.

Samples after pretreatment, at the beginning, and at the end of the fermentations were also analyzed for phenolic acids, specifically *p*-coumaric and ferulic. These compounds are released from grasses during hydrolysis and are inhibitory to fermentation (71). Ferulic acid and its related compounds possess potent antioxidant properties and may have applications in disease prevention and treatment (48). Extraction of these compounds prior to fermentation could be pursued further and may serve as a potential source of value-added by-product from ethanol production in addition to increasing ethanol yields.

PBHW is an effective and gentle pretreatment resulting in greater enzymatic digestibility of T85 bermudagrass. For our reactor, 230°C is the most efficient temperature for increasing the digestibility without producing detrimental concentrations of inhibitors. The increased digestibility directly resulted in an increased ethanol yield from fermentations using *E. coli*

LY01. The results of this study warrant further research to determine the efficacy PBHW pretreatment for other biomass sources and possibly use on a larger scale.

2.6 Acknowledgments

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Krishna Patel performed the enzymatic hydrolyses for the statistical models and the Eiteman lab (Michelle M. Richbourg and David J. Miller) performed the HPLC analysis and statistical modeling of the reaction parameters.

CHAPTER 3

EFFECTS OF SOLID LOADING AND PARTICLE SIZE ON PRESSURIZED BATCH HOT WATER PRETREATMENT OF TIFTON 85 BERMUDAGRASS[1]

[1]Brandon, S. K., L. N. Sharma, C. K. Chambliss, J. Doran-Peterson. Effects of solids loading and particle size during pressurized batch hot water pretreatment of Tifton 85 bermudagrass.2008. Submitted to Bioresource Technology, 11/20/2008.

3.1 Abstract

Pretreatment of grasses is required to optimize the maximum ethanol yield during fermentation. Pressurized batch hot water (PBHW) pretreatment has been shown to be effective in increasing the ethanol yield of fermented Tifton 85 (T85) bermudagrass (*Cynodon* spp.) when using an engineered *Escherichia coli* as the biocatalyst. Effects of solids loading and particle size variation during the PBHW pretreatment were evaluated in this study. Particle size reduction of grass, prior to PBHW pretreatment, dramatically reduced ethanol yields during partial saccharification and co-fermentation. Increased conversion efficiencies were observed with increased degradation product concentrations in liquid hydrolysates from pretreatment, suggesting that inhibitory compounds were liberated into hydrolysates and removed from whole grass versus reduced particle size grass during PBHW pretreatment. Some potential inhibitors are value-added compounds that could be recovered and/or removed as they are liberated during saccharification to increase enzymatic digestion, ethanol yields, and make the conversion process more economically feasible.

Keywords: pretreatment, pressurized batch hot water, inhibitors, lignocellulose

3.2 Introduction

With renewed concerns over global warming and energy security, alternatives to petroleum-based fuels are being investigated as possible solutions to both problems. In 2007, the US produced approximately 6.5 billion gallons of ethanol, replacing a little over 2.5% of fuel petroleum usage in the United States (http://www.ethanolrfa.org). The majority of this ethanol was produced from "first generation" processes, or corn-to-ethanol. To meet the current and ever-increasing needs of America's petroleum use, it is necessary to consider lignocellulosic ethanol as a substitute for petroleum based fuels. The USDA and DOE, in a joint report released

in 2005, determined that the US has over a billion tons of lignocellulosic biomass available for conversion to ethanol (73). In the southeastern United States, promising sources of lignocellulosic biomass for conversion to ethanol are warm season grasses. These grasses are grown on marginal lands, as part of crop rotation patterns, and as feed stocks for animals. Tifton 85 (T85) bermudagrass is a hybrid cultivar developed at the USDA-Tifton, GA campus to address the need for a more nutritive feedstock for cattle. Currently being grown on 10-15 million acres in the southeast, T85 produces more biomass than other bermudagrass cultivars and is more easily digested by ruminants. This digestibility, which is a result of more ester-linked than ether-linked phenolic acids in the lignin and also in non-lignified cell walls, corresponds to faster and better conversion to ethanol by bacteria (15, 17).

Converting lignocellulosic biomass to ethanol is a four step process: 1) pretreatment to disrupt the biomass structure, 2) enzymatic preincubation to convert the carbohydrate polymers to monomeric sugars, 3) fermentation of the monomeric sugars by a microorganism to make ethanol, and 4) recovery of ethanol from the fermentation beer. Pressurized batch hot water (PBHW) pretreatment was shown to be effective in increasing enzymatic digestibility of plant cell walls in a previous study (10). This method of autohydrolysis liberates acetyl groups from the hemicellulose, promoting further depolymerization without producing high levels of inhibitors generated when compared to acid catalysts such as sulfuric and hydrochloric are used. This study evaluates the effects of particle size and solids loading during the PBHW pretreatment. Potential inhibitors of fermentation and enzymatic hydrolysis were identified and monitored during pretreatment.

3.3 Pressurized Batch Hot Water Loading Study

A loading study was conducted to evaluate the effects of particle size and solids loading on the efficacy of PBHW pretreatment (Figure 1). In order to investigate the roles that these two variables would play in ethanol yield, the biomass receptacle for the PBHW reactor was redesigned to accommodate the reduced particle size material. A 100 µm stainless steel mesh basket and lid of the same material were built to prevent the reduced particle size biomass solids from floating out during the pretreatment. Tifton 85 bermudagrass was subjected to PBHW (10) pretreatment at 230°C for 2 minutes using the following four conditions (on a dry matter basis): 1% w/v reduced particle size solids, 1% w/v whole grass solids, 5% w/v reduced particle size solids, and 5% w/v whole grass solids. Reduced particle size solids were generated by grinding grass in a Wiley® Mill (Thomas Scientific, Swedesboro, NJ) with a 2mm mesh size screen. Whole grass after PBHW pretreatment was dried for 6 hours at 80°C and then ground as described above.

All enzyme digestions and fermentations were performed essentially as described previously (10) at 10% w/v solids concentration in the bioreactor. Enzymatic preincubation was conducted for 24 h at 45°C and pH 4.5 with an enzyme loading of 15 FPU cellulase (Batch NS50013, Novozymes, Franklinton, NC) and 60 IU cellobiase (Novo 188, Novozymes, Franklinton, NC) per g dry wt. grass and the fermentation was conducted at 35°C and pH 5.5 with *Escherichia coli* LY01 (97). Ethanol and reducing sugar concentrations were tracked over the course of the fermentations (10, 60). After 96 h of fermentation, the 1% and 5% w/v whole grass solids resulted in 20.3 g/L and 19.8 g/L of ethanol, respectively. The reduced particle size grass, at 1% and 5% w/v solids, exhibited yields of 9.6 g/L and 9.2 g/L, respectively. Reducing sugar concentrations for both whole grass solids after 24 h of enzymatic preincubation were roughly

2.5 to 3 times that of the reduced particle size grass solids at either loading (Fig. 1). A sugar analysis (NREL standard protocol LAP-014) was conducted on the hydrolysate samples (Table 1). At 1% solids loading in the reactor, the reduced particle size solids released 26.3 mg sugar/g grass compared to the whole solids at 79.8 mg sugar/g grass, a 3-fold increase. At 5% solids loading, the reduced particle size solids released 18.3 mg sugar/g grass versus a 25.7 mg sugar/ g grass release from the whole grass solids, a 1.4-fold increase.

Table 3.1 Analysis of the PBHW hydrolysate for inhibitors and sugars released (mg/g grass). Samples were pooled prior to analysis (n=3).

Hydrolysate	Sugars Liberated [a] (mg/g grass)	Sugar Degradation Products [b] (mg/g grass)	Lignin Degradation Products [b] (mg/ g grass)	Aliphatic Acids [b] (mg/g grass)	% Material Solublized from Hemicellulose[c]
1% w/v Reduced Particle Size Grass Solids PBHW Hydrolysate	26.3	4.7	2.3	9.8	10.7
1% w/v Whole Grass Solids PBHW Hydrolysate	79.8	9.4	3.6	10.3	25.6
5% w/v Reduced Particle Size Grass Solids PBHW Hydrolysate	18.3	1.0	0.7	2.5	5.6
5% w/v Whole Grass Solids PBHW Hydrolysate	25.7	4.0	0.9	3.9	8.6

[a] Calculated from sugar analysis (NREL LAP-014).
[b] Calculated from inhibitor analysis (81).
[c] Calculated by dividing the sum of the values in this table by the % hemicellulose in each treatment (from NIR analysis (68), data not shown) . On a per gram grass basis.

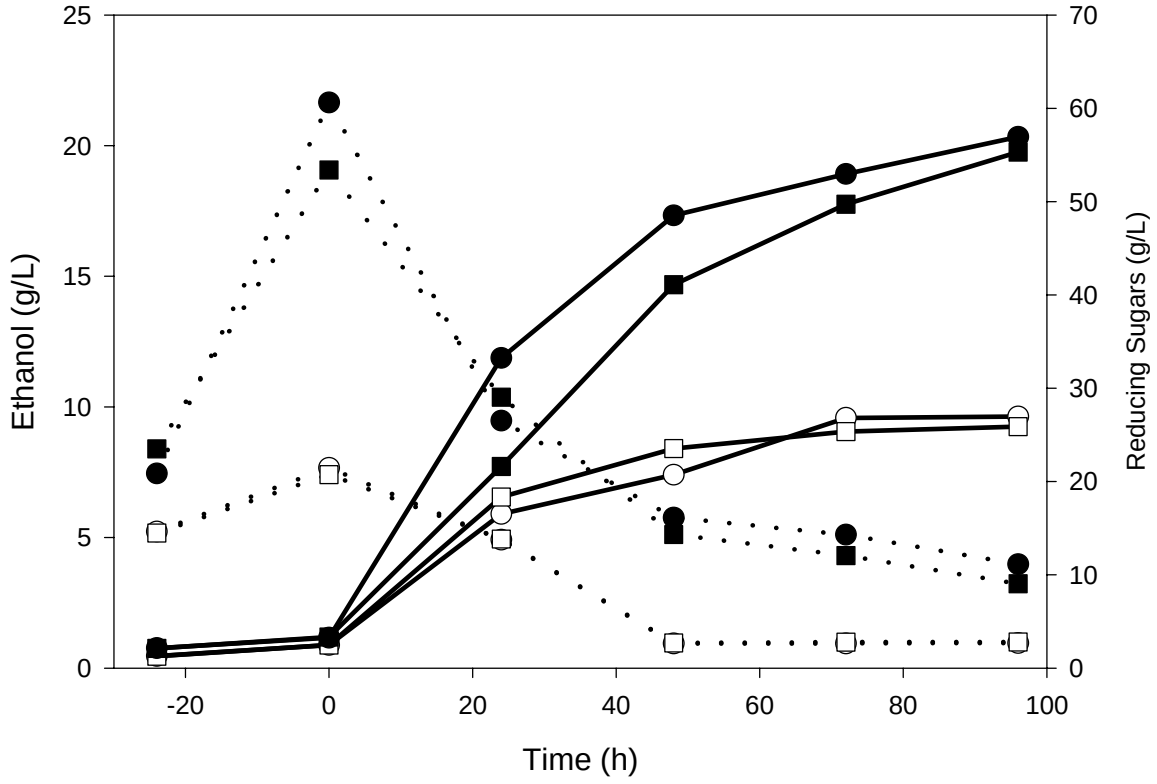

Figure 3.1 Effect of Solids Loading and Particle Size on Pressurized Batch Hot Water Efficacy. Average ethanol production and reducing sugar levels during fermentation of PBHW-pretreated Tifton 85 bermudagrass. Solid lines correspond to ethanol concentration and the dotted lines correspond to reducing sugar concentrations. Symbols are as follows: filled square, 5% w/ v whole grass solids; filled circle, 1% w/v whole grass solids; open square, 5% w/ v reduced particle size grass solids; open circle, 1% w/ v reduced particle size grass solids. The -24 h time point corresponds to the beginning of the 24 h enzymatic preincubation. Bacterial inoculation occurred at time 0 h. Reducing sugars are removed at the same rate ethanol is produced. Fermentations were performed in duplicate.

All PBHW hydrolysate samples were analyzed for 42 potential inhibitors (81), released from the grasses during pretreatment. These compounds can be classified as sugar degradation products, lignin degradation products, or aliphatic acids, which may or may not be lignin degradation products (56). For both solids loadings (1% or 5%) during pretreatment, the whole

grass solids showed an increase over reduced particle size solids in dissolution of all classes of degradation products into the hydrolysate (Table 1).

Percent solubilization of hemicellulose in the pretreated grass is shown in Table 1. The most hemicellulose was solublized in the 1% solids loading at 10.7% and 25.6% for the reduced particle size and whole solids, respectively. The 5% solids loading resulted in 5.6% and 8.6% solubilization of the reduced particle and whole solids, respectively.

3.4 Discussion

In a prior study, PBHW pretreatment was shown to be an effective pretreatment for Tifton 85 bermudagrass; however, grass particle size and solids loading were not investigated or optimized (10). Our current study found that the solids loading during pretreatment had no effect on ethanol yield; particle size was the determining factor. The lower level of reducing sugars seen after enzymatic digestion of reduced particle size PBHW pretreated solids could indicate either very efficient hydrolysis of the hemicellulose into the liquid hydrolysate (which is not fermented) during pretreatment or higher levels of enzyme inhibitors present during the enzymatic digestion (Fig. 1). If the hemicellulose sugars were liberated into the liquid hydrolysate and removed, the total sugars available for release during enzymatic digestion of the solids would be effectively reduced. If sugars are retained in the solids, but not released during enzymatic digestion, this would suggest the presence of inhibitory compounds interfering with digestion. When sugar levels in the hydrolysate samples were evaluated, the higher concentration of sugars from whole grass solids over reduced particle size solids did not support an increase in hemicellulose dissolution into the hydrolysate from the reduced particle size grass. The increased release of potential inhibitors into hydrolysate from whole grass solids removed these compounds from the solids before enzymatic digestion and fermentation.

This study supports prior findings that determined particle size reduction is not needed for liquid hot water pretreatments (66), but also builds on them by demonstrating that particle size reduction before pretreatment can be detrimental to ethanol yield. Previously, PBHW pretreatment was proven to be an effective way to dissolve the hemicellulose of biomass, making the cellulose more available to enzymatic digestion and fermentation. It can now also be viewed as a method of enzyme and fermentation inhibitors removal, increasing fermentation efficiency.

Inhibitors are formed and work in several ways. Sugar degradation products include furfural from pentose sugars and 5-hydroxymethylfurfural from hexoses. These two compounds inhibit fermentation by affecting growth rates in addition to inhibiting glycolytic enzymes and alcohol dehydrogenase (71). Formic and levulinic acid are both formed when 5-HMF is further degraded and furfural forms formic acid and 2-furoic acid when it breaks down (31, 56, 88). Lignin degradation products are generally aromatic compounds that contain methoxy-substituted benzene or phenol rings. Some of these compounds, such as *p*-coumaric, ferulic, and sinapic acids, are powerful antioxidants (48) and preservatives, and other compounds, like vanillin, have applications in the food industry as flavorings. Regardless of their commercial uses, phenolics inhibit microbial growth in bacteria and eukaryotes (3, 71) and are ubiquitous in the lignin of grasses (1). Also, even in very small amounts, ferulic acid can decimate the activity of commercially available cellulases (96). Aliphatic acids can cross the plasma membrane into cells, and due to dissociation, decrease the pH through anion accumulation, and reduce ATP production through uncoupling (79). If these or similar compounds are liberated during pretreatment and subsequently removed before enzymatic preincubation and fermentation, it could be possible to increase the final ethanol yield by preventing inhibition.

When bermudagrass is pretreated in the reactor, acetate is liberated from the hemicellulose along with sugars, and sugar and lignin degradation products, including phenolic acids, are formed. All of these solublized compounds collect in the liquid hydrolysate which is evacuated into the condenser and discarded, removing any inhibitors that were released into the liquid from the solids and serving as a wash for the biomass. This study found that 1% w/v whole grass solids loading resulted in the highest percent hemicellulose dissolution; however, the authors recommend a solids loading of 5% whole grass to prevent loss of fermentable sugars as well as optimizing the time, energy, and resources that are involved in the pretreatment step. Technologies for separation and recovery of components in the hydrolysate, including sugars and alternative degradation products, could provide an additional revenue stream from value-added co-products as many of the "inhibitors" have desirable traits outside the fermentation process. Ion exchange resins have been employed in the past, and membrane extraction may also be an option. Nylon membranes have been shown to be effective for removing phenolics, specifically ferulic acid, from aqueous solutions (unpublished data in collaboration with R. A. Holser). Recovering the sugars from the hydrolysate could increase the total ethanol yield from grass biomass, although this gain in ethanol may not be cost effective without recovery of the additional value-added co-products due to the low concentration of hemicellulose sugars in the hydrolysate.

3.5 Acknowledgements

We would like to thank Dr. Bruce Dien (NCAUR-USDA, Peoria, IL) for his help with sugar analyses. Dr. William F. Anderson (USDA ARS, Tifton, GA) kindly provided Tifton 85 bermudagrass and compositional analyses.

CHAPTER 4

ETHANOL AND CO-PRODUCT GENERATION FROM PRESSURIZED BATCH HOT WATER PRETREATED BERMUDAGRASS, SWITCHGRASS, AND NAPIERGRASS USING RECOMBINANT *ESCHERICHIA COLI* AS BIOCATALYST[1]

[1]Brandon, Sarah Katherine, Matt Hawkins, Lekh Sharma, Kevin C. Chambliss, William F. Anderson, and Joy Doran Peterson. 2008. Ethanol and Co-product Generation from Pressurized Batch Hot Water Pretreated Bermudagrass, Napiergrass, and Switchgrass using Recombinant *Escherichia coli* as Biocatalyst. To be submitted to Biomass and Bioenergy.

4.1 Abstract

Warm season grasses are a viable biomass source for bioconversion to ethanol, especially in the southeastern United States. Pretreatment of grasses is required to maximize the ethanol yield during fermentation. A study was performed to test pressurized batch hot water (PBHW) pretreatment, which has been shown to be effective in increasing the ethanol yield of fermented Tifton 85 (T85) bermudagrass (*Cynodon dactylon* L.). T85 bermudagrass and Merkeron napiergrass (*Pennisetum purpureum* Schumach.) were either left untreated or were PBHW pretreated for 2 minutes at 230°C at 5% w/v whole grass solids loading. Following a 24 h enzymatic digestion, untreated and PBHW pretreated grasses were evaluated for ethanol production, potential inhibitors, and value-added co-product generation. Fermentation of PBHW pretreated grasses with *Escherichia coli* LY01 produced twice the ethanol of their untreated counterparts. PBHW pretreated Merkeron napiergrass produced 224.5 mg/g grass ethanol (72.8% maximum theoretical yield) and PBHW pretreated T85 bermudagrass reached 213 mg/ g grass (69.5% maximum theoretical ethanol yield). Pretreatment by PBHW resulted in increased solubilization of hemicellulose and potential inhibitors. Despite some of these potential inhibitors remaining with the solids after PBHW pretreatment, there was more efficient hydrolysis of the cellulose and remaining hemicellulose during the enzymatic digestion of the grasses prior to fermentation when compared to the untreated grasses. This increase in digestibility observed with enzymes prior to fermentation and in traditional forage digestibility assays, resulted in increased ethanol yields.

Keywords: biomass, grass, pretreatment, digestibility

4.2 Introduction

The Energy Independence and Security Act of 2007 requires the incorporation of 16 billion gallons of cellulosic ethanol into gasoline by 2022. Lignocellulosic biomass sugars can be converted to ethanol in a similar manner as starch; however, liberating these sugars is more complex than liberating glucose from starch. First, the lignocellulosic biomass is pretreated to allow greater access for hydrolytic enzymes. Second, the pretreated biomass is then saccharified using hydrolytic enzymes to convert the structural polymers to monomeric sugars. Last, the monomeric sugars are fermented by a biocatalyst to make ethanol.

For the southeastern United States, warm season grasses are a viable option. Having been used for centuries as feed for ruminants, there is an extensive knowledge base in breeding and propagation. Grasses are monocotyledons, whose primary cell walls are constructed of cellulose microfibrils surrounded by hemicellulose; the major type of hemicellulose found in grasses is arabinoxylan (18). In the secondary cell walls, cellulose and hemicellulose are also present, but there added complexity with the addition of lignin, a polymer of *p*-hydroxylcinnamic alcohol monomers (72, 76). In addition to serving as a structural component in the cell wall, lignin also provides resistance to pathogens. Warm season grasses are unique because they contain non-lignified cell walls that are very resistant to biodegradation (4). This recalcitrance is due to arabinose in the hemicellulose having ester- and ether-linkages to ferulic and *p*-coumaric acids. These ferulate compounds, also found in lignin, form dimers with each other, linking hemicellulose to hemicellulose and hemicellulose to lignin. This crosslinking of the polymers of the grass cell walls lead to decreased accessibility, and even masking, of fermentable materials in the cell wall (1, 42).

There has been an extensive breeding program at the USDA-ARS in Tifton, GA for the last 70 years, resulting in improved cultivars of warm season grasses for both animal feed and bioenergy (4). Two cultivars developed by Glenn Burton (USDA-ARS Tifton) have proven to be very promising as lignocellulosic feedstocks. Tifton 85 bermudagrass (*Cynodon dactylon* L.) was created as a hybrid between Tifton 68 bermudagrass and South African PI 290884 to generate a very digestible grass that resulted in a significant weight gain over existing cultivars in cattle grazing studies (15, 16). Merkeron napiergrass (*Pennisetum purpureum* Schumach.) was also developed by Burton, by hybridizing dwarf no. 208 and tall selection no. 1, to create a new cultivar with very high biomass production (14). To determine growth characteristics and biomass production per hectare, Tifton 85 bermudagrass, Merkeron napiergrass, and Alamo switchgrass (parent of ADEL) were planted in three locations across Georgia. Averaging yields for 6 years, Alamo and Tifton 85 performed similarly, producing between 16,000 and 17,500 kg/ha annually. However, Merkeron produced almost double that, with its highest yield being over 28,000 kg/ha (9).

When evaluating possible feedstocks for bioconversion, it is also important to evaluate the potential inhibitors native to the biomass as well as inhibitors produced during the conversion process that may have an effect on enzymes or fermenting organisms. The liberation of lignin and low molecular weight phenolic acids from the cell walls of warm season grasses by pretreatment or enzymes can inhibit the enzymes used in saccharifying the biomass and/or the biocatalyst. For example, ferulic and *p*-coumaric acids that are ester-linked to arabinose in non-lignified cell walls can be released by esterases commonly found in industrial enzyme preparations. These compounds are highly toxic to fermenting organisms (8, 86) and inhibitory to enzymes (71, 96). Also, pretreatments to make biomass more amenable to saccharification and

fermentation can produce inhibitors. These inhibitors include aldehydes from sugar degradation (100), lignin constituents, and acidic compounds released from the hemicellulose, like acetic acid (49, 98). Production of these inhibitors depends on the biomass being pretreated and the severity of the pretreatment. Some of these inhibitors may be value-added co-products which could be recovered, thus increasing the valuable products generated from the grass biomass.

This study evaluates potential biomass feedstocks Tifton 85 bermudagrass, ADEL switchgrass, and Merkeron napiergrass using pressurized batch hot water (PBHW) pretreatment and fermentation to measure and compare ethanol production and release of potential inhibitors. Previously, PBHW pretreatment was shown to be an effective method for the pretreatment of Tifton 85 bermudagrass, producing minimal inhibitors while increasing the effectiveness of enzymatic digestion and subsequent fermentation of grass biomass (10).

4.3 Materials and Methods

Warm Season Grass Cultivars

Tifton 85 bermudagrass and Merkeron napiergrass were harvested as described previously (6). The 4-wk-old second harvest was used for both grasses in this study.

PBHW Pretreatment of Warm Season Grass Cultivars

Both cultivars were treated by pressurized batch hot water (PBHW) (10) at 230°C for 2 minutes at 5% w/v whole grass solids. PBHW pretreated solids were dried for 6 hours at 80°C and then ground in a Wiley® Mill (Thomas Scientific, Swedesboro, NJ) with a 2mm screen. Liquid hydrolysate was also collected for later analysis.

Enzymatic Digestion and Fermentation of PBHW Pretreated Grass Solids

A series of partial saccharification and co-fermentation experiments were conducted on the following materials: untreated T85, PBHW pretreated T85 solids, untreated Merkeron, and

PBHW pretreated Merkeron solids. Fermentations were conducted at 10% w/v solids on a dry matter basis as previously described (10). Moisture content of the PBHW pretreated grasses was determined by an automated infrared moisture analyzer (IR-35 Moisture Analyzer, Denver Instrument, Denver, CO). All components were either autoclaved or filter sterilized separately. Bioreactors were loaded with grass, dH_2O, Luria-Bertani medium (LB) (Fisher, Fair Lawn, NJ), Novo 188 cellobiase (Novozymes, Franklinton, NC), and Batch NS50013 (Novozymes, Franklinton, NC). Final enzyme concentrations were 15 Filter Paper Units (FPU) cellulase and 60 IU cellobiase per g dry wt. grass. A sample was removed after combining autoclaved and filter-sterilized components to determine the amount of sugar and inhibitors present at the start of enzymatic digestion. Enzymes were allowed to digest the grasses for 24 h at pH 4.5 and 45°C with stirring. *Escherichia coli* strain LY01 (37, 97) was inoculated from glycerol stocks and incubated at 37°C for 18 hours in LB containing 50 g glucose and 40 mg chloramphenicol per liter. The pH was adjusted to 5.5 with KOH, and water bath temperature decreased to 35°C. Bioreactors were inoculated for a starting OD_{550} of 1. Samples were taken every 24 h, filtered (Corning Spin-X® Centrifuge Tube Filter 0.22 μm, Sigma-Aldrich, St. Louis, MO), and stored in O-ring microfuge tubes at -20°C until analysis. A 24h enzymatic digestion and sampling regime was used for convenience.

Analytical Methods

In vitro dry material digestibility (IVDMD) as well as NDF, ADF, and ADL analyses were conducted on untreated grasses, PBHW pretreated grasses, and both untreated and PBHW pretreated grasses after enzymatic digestion following standard protocol (87, 91).Percent fermentable carbohydrates as well as cellulose and hemicellulose dissolution were calculated on a dry matter basis by using the NDF, ADF and ADL results to estimate the cellulose (NDF-ADF)

and hemicellulose (ADF-ADL) values. Percent fermentable carbohydrates were the sum of the estimated cellulose and hemicellulose values. Percent maximum theoretical yield was calculated from the total fermentable carbohydrate x dry weight of grass in fermentor x 0.53 (molecular ratio of ethanol/ polymer carbohydrate) x 0.9 or 0.85 for conversion efficiency of 6C and 5C sugars, respectively. Untreated grass values were used as the baseline for calculating percent dissolution from the PBHW pretreated solids and enzyme digested untreated grasses. PBHW pretreated grass values were used as the baseline for calculating percent dissolution from enzyme digested PBHW pretreated grass. Reducing sugars were determined by dinitrosalicylic acid assay (61). Filtered samples from the enzymatic digestion and fermentations were analyzed for ethanol by gas chromatography (Shimadzu GC-8A, Columbia, MD) as previously described (30) using a flame ionization detector and the parameters: injector/detector temperature of 250°C, column temperature of 65°C, 0.53 mm ID × 30 m column with 3 μm film. Soluble sugar composition during the course of the fermentations was determined by gas chromatography, as previously described (10). Liquid hydrolysate from PBHW pretreatment, as well as the enzyme digested PBHW pretreated and untreated grass samples were analyzed for a suite of 42 inhibitors by HPLC-MS-MS (81). These were grouped into sugar degradation products (furfural, hydroxymethylfurfural, formic acid, levulinic acid, 2-furoic acid), lignin degradation products (adipic acid, 3,4-dihydroxybenzoic acid, 3,5-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, salicylic acid, 4-hydroxybenzeldehyde, vanillic acid, homovanillic acid, 4-hydroxyacetophenone, caffeic acid, syringic acid, vanillin, 4-

hydroxybenzoic acid, benzoic acid, syringaldehyde, 4-hydroxycoumaric acid, ferulic acid, sinapic acid, 3-hydroxy-4-methoxycinnamic acid, 4-hydroxycoumarin, ortho-toluic acid, para-toluic acid), and aliphatic acids (malonic acid, lactic acid, maleic acid, acetic acid, *cis*-

aconitic acid, methylmalonic acid, succinic acid, fumaric acid, *trans*-aconitic acid, glutaric acid, itaconic acid, 2-hydroxy-2-methylbutyric acid, gallic acid). Total inhibitors are the sum of the sugar degradation products, lignin degradation products, and aliphatic acids.

4.4 Results

Total sugar concentrations from untreated grass and pretreated grass before and after the 24h enzymatic digestion are shown in Figure 4.1. PBHW pretreated Merkeron and PBHW pretreated T85 liberated the most sugar per gram of grass after enzymatic hydrolysis. PBHW pretreated grasses nearly doubled the amount of sugar initially present in the fermentor. Untreated T85 bermudagrass and untreated Merkeron napiergrass both doubled total sugar

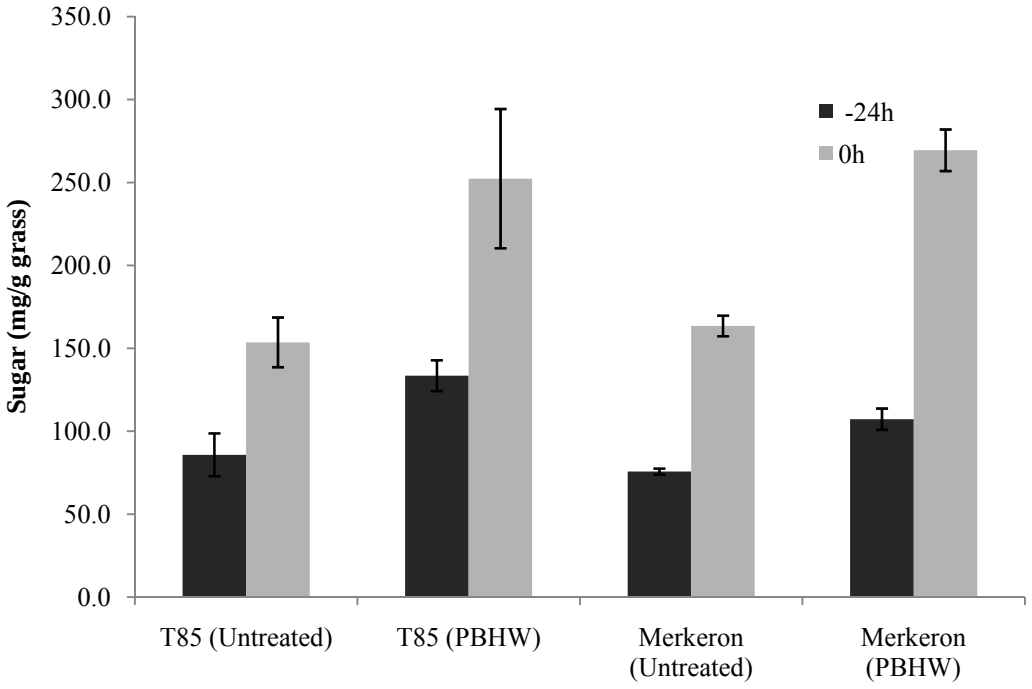

Figure 4.1 Comparison of total sugar released from untreated grasses and PBHW pretreated grass solids at the beginning (-24h) and end (0h) of enzymatic digestion. Experiments were conducted in triplicate and all values are mg sugar/g grass.

content after 24 h enzymatic digestion (Figure 4.1). Negligible sugars were released into the liquid hydrolysate for both grasses (data not shown). PBHW liquid hydrolysate was evacuated from the reactor and the solids were not washed prior to drying, thus retaining some of the hydrolysate compounds in the solid fraction.

Ethanol yield and reducing sugars were traced over the course of the fermentations, as seen in Figure 4.2. PBHW pretreated grasses increased ethanol yields over the untreated grasses for both cultivars. PBHW pretreated Merkeron napiergrass and PBHW pretreated T85 produced 224.5 mg ethanol/g grass and 213.0 mg/g grass, respectively. Ethanol concentrations are presented as a percent of the maximum theoretically possible based on the cellulose and hemicellulose content in Table 4.1. PBHW pretreated Merkeron and PBHW pretreated T85 reached 73% and 70% of their respective maximum theoretical yields, respectively. Both untreated Merkeron and T85 reached 36% of their theoretical maxima.

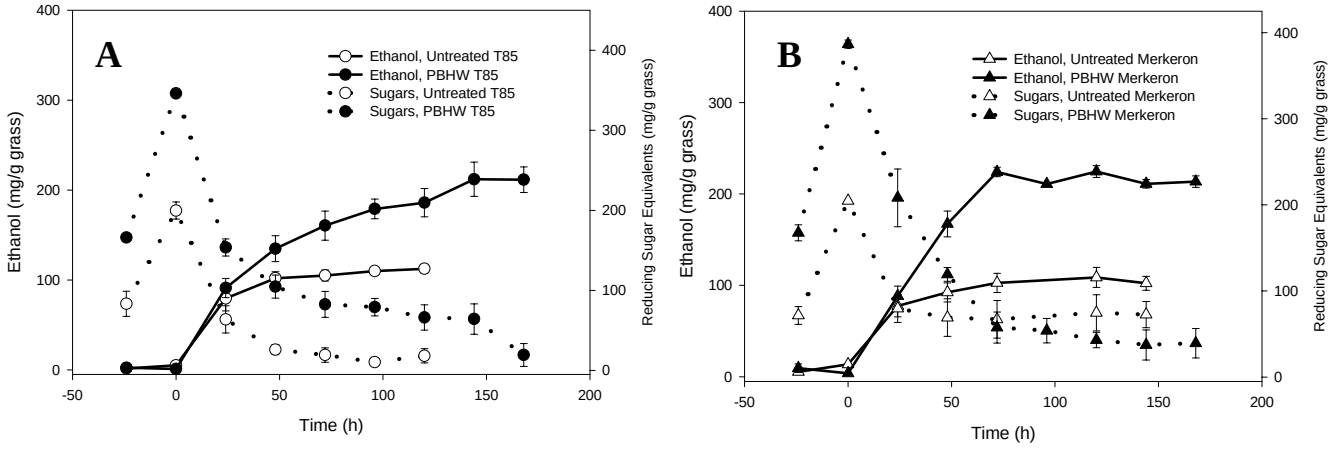

Figure 4.2 Average ethanol production and reducing sugar concentrations during fermentation of untreated and PBHW-pretreated warm season grasses. All PBHW- pretreatments were carried out at 5% w/v solids in the reactor at 230°C for two minutes. The -24 hour time point corresponds to the beginning of the 24 hour enzymatic digestion. Bacterial inoculation occurred at time 0 hour. Reducing sugars are removed at approximately the same rate ethanol is produced. Fermentations were performed in triplicate.

Table 4.1 Maximum ethanol yields and percent theoretical yields from untreated and PBHW pretreated warm season grasses. Experiments were conducted in triplicate.

Fermentation Substrate	Percent Cellulose and Hemicellulose (dry matter basis)	Maximum Ethanol Concentration (mg/g grass)	Percent Theoretical Yield
T85(PBHW)	65.6	213.0 ± 19.2	70
T85 (Untreated)	66.7	112.5 ± 4.7	36
Merkeron (PBHW)	65.4	224.5 ± 11.2	73
Merkeron (Untreated)	65.4	108.7 ± 10.9	36

IVDMD, NDF, ADF, and ADL analyses were conducted on the grass solids before and after PBHW pretreatment, and after enzymatic digestion of untreated and PBHW pretreated grasses (Table 4.2). In PBHW pretreated solids, there was no solubilization of the cellulose. However, 24 and 30 percent of the hemicellulose fraction was solubilized in the PBHW pretreated T85 and Merkeron solids, respectively. For both T85 and Merkeron, after PBHW pretreatment, cellulose dissolution increased to over 40% during enzymatic digestion. As expected, further hemicellulose solubilization occurred during enzymatic digestion of PBHW pretreated solids. Untreated Merkeron resulted in the highest dissolution of fermentable material after enzymatic pretreatment at 61% compared to 52% for T85. For enzyme digested pretreated material, the sum of hemicellulose and cellulose dissolution was 85% for Merkeron and 76% for T85.

During hemicellulose dissolution, carbohydrates and other water soluble compounds are liberated. Some of these compounds are potential inhibitors of enzymes and fermenting organisms. Analysis of potential inhibitors was conducted on the liquid hydrolysate from PBHW pretreatment of the grasses as well as the liquid phase after 24h enzymatic digestion of untreated and PBHW pretreated grasses (Figure 4.3). Overall total inhibitor concentrations were similar for

enzymatically digested PBHW pretreated solids for both grasses. Upon examination of the corresponding liquid hydrolysate from these PBHW pretreatments, a difference in total inhibitor concentration was observed ranging from 8.6 mg/g grass for Merkeron and 14.7 mg/g T85. Enzyme digested untreated grasses were also evaluated and Merkeron released more inhibitors than T85 (Figure 4.3).

Table 4.2 NDF, ADF, ADL, and IVDMD in untreated and PBHW pretreated and enzyme digested solids. Samples were pooled prior to analysis (n=3). Enzyme digested solids were evaluated after 24h of enzymatic digestion.

Substrate	NDF	ADF	ADL	IVDMD
Untreated T85	69.7	38.0	3.0	55.2
PBHW T85	71.7	47.5	6.0	54.3
Enzyme Digested Untreated T85	53.7	32.8	4.0	56.4
Enzyme Digested PBHW T85	52.7	31.0	6.8	54.5
Untreated Merkeron	67.4	41.6	2.4	62.6
PBHW Merkeron	70.4	52.3	5.0	57.0
Enzyme Digested Untreated Merkeron	47.2	27.8	2.9	67.6
Enzyme Digested PBHW Merkeron	50.3	34.2	7.5	53.6

4.5 Discussion

The goal of this study was to evaluate two different cultivars of warm season grasses for conversion to ethanol with and without PBHW pretreatment. PBHW pretreatment dramatically increased the ethanol yields for all three cultivars over their untreated counterparts (Table 4.1 and Figure 4.2). This was achieved by increasing sugars liberated from the grass during the enzymatic digestion (Figure 4.1 and 4.2). PBHW pretreated Merkeron and PBHW pretreated

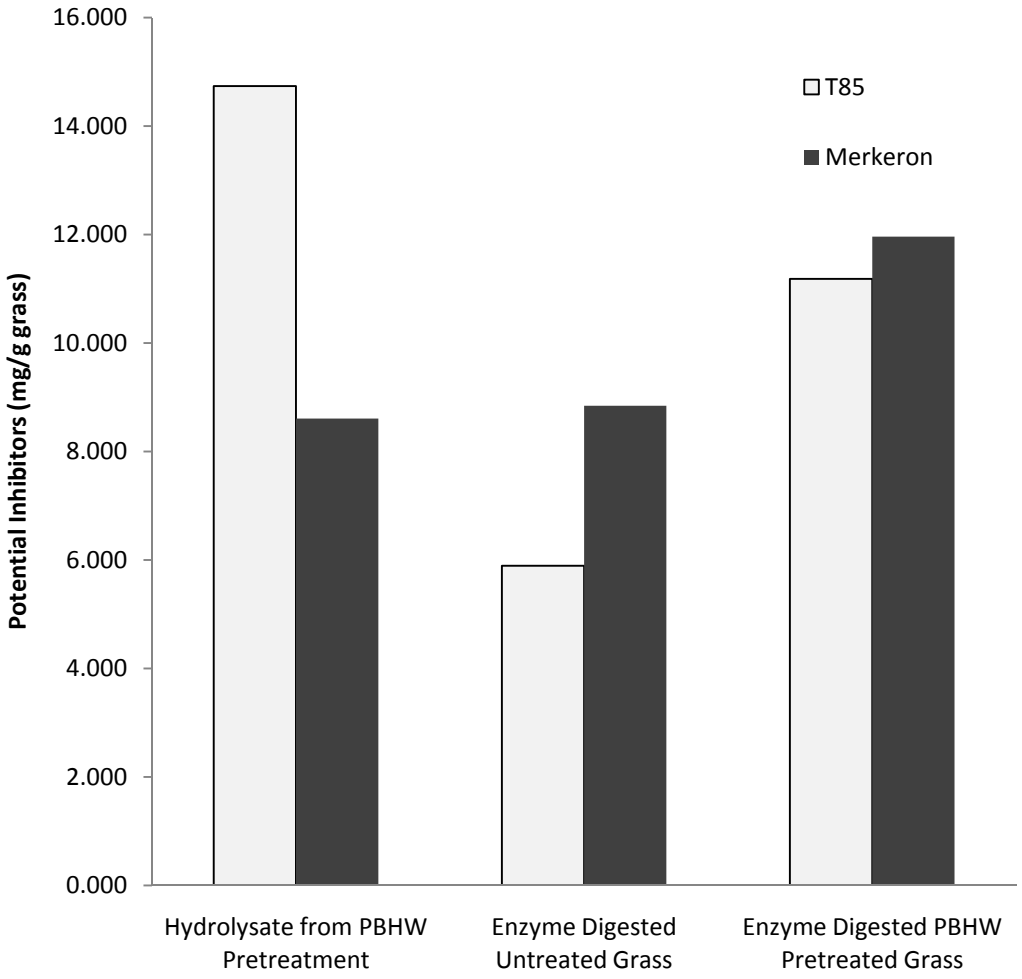

Figure 4.3 Comparison of inhibitor levels in process hydrolysates of PBHW pretreated and enzyme digested solids. Samples were pooled prior to analysis (n=3). Enzyme digested solids were evaluated after 24h.

T85 reached the highest ethanol concentrations and the highest percent of maximum theoretical ethanol yields, which were based on ethanol production and IVDMD analyses for composition. Previous studies compared T85 and Merkeron for bioconversion to ethanol. Anderson *et al.* used IVDMD analysis of the separate leaves and stems to determine that 4 week and 8 week old Merkeron were more highly digestible than T85, regardless of the part of the grass analyzed or age at harvest (Anderson 2005). A similar study in 2007 found that the leaves of mature Merkeron were more digestible than 12 wk old T85, but not the stem (Anderson, 2007). The

result of this study, using primarily leaf material of Merkeron, generally agreed with previous research. Both napiergrass and bermudagrass used in this study were harvested at 4 weeks and therefore were immature and primarily leaf material, and thus more digestible than older grass (5, 6).

The presence of inhibitors is one of the main causes of suboptimal ethanol production (49, 71). In this study, the total level of potential inhibitors measured at the beginning of fermentation for PBHW pretreated grasses was similar for both grasses, and there were no significant differences in solubilization or ethanol production. It may be possible that one or more compounds we did not measure in this study are affecting either the enzyme digestion and/or the fermentation of the grasses, preventing the conversion process from reaching the theoretical maximum ethanol yield. Although we cannot confirm or reject that potential inhibitors investigated in this study are actually inhibiting our conversion process, it may still be of interested to utilize an additional separation step to collect these compounds. A solid state extraction procedure, such as ion exchange chromatography or membrane filtration could be utilized after enzymatic digestion to produce a concentrated sugar stream that could subsequently be fermented while collecting these potential inhibitors. Many of these compounds have commercial applications currently and could serve as a value-added co-product stream (69). For example, furfural is produced industrially for use in resins and as a solvent (31). Also, phenolic acids such as ferulic and *para*-coumaric acid, are potent antioxidants used in food and cosmetics (48). Collecting and selling these value-added co-products would mimic the current model for corn ethanol production, which produces several other valuable products such as Distillers Dry Grains with Solubles (DDGS) and wet distillers grains, both of which are valuable animal feeds. These “waste” streams from corn ethanol production make the overall economics for the

conversion process favorable; collection and commercialization of potential inhibitors as value-added co-products would do the same for lignocellulosic ethanol.

PBHW pretreatment is an ideal pretreatment for grassy biomass; by liberating the acetyl groups from the hemicellulose, the hemicellulose is partially solublized through a very mild acidic autohydrolysis (10, 66), exposing the remaining cellulose for enzymatic digestion. This was confirmed in this study through the dissolution of hemicellulose but lack of dissolution of cellulose in the PBHW pretreated solids (Table 4.2). Merkeron napiergrass and T85 bermudagrass hold promise as potential biomass feedstocks due to their high ethanol yields. Both of these PBHW pretreated grasses showed increased solubilization of hemicellulose and cellulose after enzymatic digestion over their untreated counterparts as well as either untreated or PBHW pretreated GA-993. Despite the presence of solublized inhibitors, Merkeron reached 73% and T85 reached 69% of their maximum theoretical ethanol yield which would translate to approximately 80 gallons per dry ton of PBHW pretreated T85 or Merkeron could be produced based on the process as described in this study. Regardless of this, it is of great interest to evaluate the two cultivars described here, as well as others, at varying harvest times. The data presented here represent a snapshot of the grasses at the 4 wk harvest age. Investigating grasses at varying stages of maturity in our process would allow the optimization of harvest parameters for feedstocks, making the overall bioconversion process more efficient.

4.6 Acknowledgements

The authors would like to thank Dr. Maurice Snook, USDA-ARS, Athens, GA, for his kind help with the analytical analysis of sugars.

IVDMD, NDF, ADF, and ADL analyses were conducted by William Anderson's lab (UDSA, Tifton, GA). Inhibitor analyses were conducted by Lekh Sharma (Baylor University, Waco, TX).

CHAPTER 5

CONCLUSIONS

Our need to convert the current petroleum-driven economy of the world to one based on renewable fuels grows every day. In order to make bioethanol an economically feasible and efficient process, pretreatments to reduce biomass recalcitrance and improve enzymatic hydrolysis are necessary. Pressurized batch hot water pretreatment was developed as a method of autohydrolysis to gently pretreat biomass without the need for harsh chemicals.

Initially tested on Tifton 85 bermudagrass, PBHW pretreatment at 1% w/v solids in the reactor was found to promote the release of the most sugars after enzymatic hydrolysis when T85 was pretreated at a higher temperature for a shorter time, specifically 230°C for 2 minutes (Chapter 2). When PBHW pretreated T85 was fermented with *E. coli* LY01, grass pretreated at 230°C for two minutes performed better than 200°C pretreated grass or untreated grass.

However, particle size and solids loading were not initially investigated in order to optimize PBHW pretreatment. Reducing the particle size of the grass by grinding prior to PBHW pretreatment resulted in a dramatic decrease in ethanol yield after fermentation than whole grass that had been pretreated (Chapter 3). Solids loading during pretreatment had a negligible effect on performance during fermentation. PBHW pretreatment not only hydrolyzed the hemicellulose, allowing greater accessibility to the cellulose, but it removes inhibitors that would otherwise have been present during fermentation.

With these optimized parameters for PBHW pretreatment, three cultivars of warm season grasses were compared for bioethanol production. T85 bermudagrass, ADEL switchgrass, and

Merkeron napiergrass were either pretreated or left untreated and subsequently fermented by *E. coli* LY01. PBHW pretreated grasses produced more ethanol than their untreated counterparts; PBHW pretreated Merkeron produced the most ethanol, but still had the highest level of inhibitors at the beginning of fermentation. It also showed the highest percent dissolution of cellulose and hemicellulose after enzymatic preincubation. This liberation of monomeric sugars then resulted in the higher ethanol yield. PBHW pretreated ADEL produced the least ethanol and showed no increase in dissolution of hemicellulose and a smaller dissolution of cellulose after enzymatic preincubation. With similar levels of inhibitors at inoculation, the lack of increase in cell wall component dissolution appears to be the major factor preventing efficient fermentation.

It should be mentioned that in Chapter 2, the maximum ethanol yield observed was for PBHW pretreated T85 bermudagrass that had been pretreated for 2 minutes at 230°C at 1% whole solids (14.7 g/L) after an enzymatic digestion with 2 Filter Paper Units (FPU)/ g dry weight of grass. In Chapter 3, T85 that has been pretreated identically, but that was digested with 15 FPU/g dry weight grass and 60 CBU/g dry weight grass, produced 21 g/L of ethanol. One would expect that such an increase in enzyme loading would increase ethanol production more than 7 g/L. However, enzyme preparations used for enzymatic preincubation are concentrated culture supernatants from hyper-secreting fungal strains and generally contain multiple enzyme activities. The enzyme mixes used in Chapter 2 did contain other activities, but we chose to express their activity with the known and accepted FPU, even though they contained xylanases, β-glucanse, and pectinase activities as well. These various other activities may have aided in the hydrolysis of T85 in the study in Chapter 2.

The recalcitrance of biomass to conversion to bioethanol needs to be addressed in several ways, including crop breeding and improved biocatalysts. New pretreatments and the application

of current pretreatments to a wider variety of biomass must also be pursued. PBHW pretreatment is an effective and efficient pretreatment developed and optimized for grassy biomass.

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APPENDIX A

RAW DATA FROM "EFFECTS OF SOLIDS LOADING AND PARTICLE SIZE DURING PRESSURIZED BATCH HOT WATER (PBHW) PRETREATMENT OF TIFTON 85 BERMUDAGRASS"

Table A.1. Raw inhibitor data from PBHW loading study hydrolysate. Conducted by Lekh Sharma (Baylor University, Waco, TX).

	Compound (mg/g grass)	1% w/v Reduced Particle Size Grass Solids PBHW Hydrolysate	1% w/v Whole Grass Solids PBHW Hydrolysate	5% w/v Reduced Particle Size Grass Solids PBHW Hydrolysate	5% w/v Whole Grass Solids PBHW Hydrolysate
Aliphatic Acids	malonic acid	4.107	4.658	0.835	1.773
	acetic acid	0.109	0.109	0.198	0.027
	lactic acid	4.233	4.192	0.713	1.756
	maleic acid	0.176	0.133	0.028	0.041
	cis-aconitic acid	0.018	0.041	0.030	0.003
	methylmalonic acid	0.001	0.001	0.001	0.000
	succinic acid	0.459	0.436	0.441	0.105
	fumaric acid	0.397	0.375	0.127	0.118
	trans-aconitic acid	0.008	0.005	0.017	0.001
	glutaric acid	0.036	0.055	0.026	0.011
	itaconic acid	0.210	0.241	0.039	0.068
	2-hydroxy-2-methylbutyric acid	0.010	0.014	0.007	0.003
	adipic acid	0.000	0.001	0.000	0.000
Sugar Degradation Products	levulinic acid	0.096	0.107	0.019	0.026
	formic acid	3.261	5.558	0.871	2.692
	2-furoic acid	0.001	0.001	0.000	0.000
	5-hydroxymethylfurfural	0.248	0.670	0.061	0.087
	furfural	0.142	3.798	0.050	2.107
Lignin Degradation Products	gallic acid	0.103	0.150	0.019	0.031
	3,4-dihydroxybenzoic acid	0.019	0.022	0.013	0.004
	3,5-dihydroxybenzoic acid	0.003	0.012	0.007	0.003
	2,5-dihydroxybenzoic acid	0.025	0.040	0.017	0.006
	3,4-dihydroxybenzaldehyde	0.059	0.167	0.009	0.040
	salicylic acid	0.050	0.053	0.041	0.010
	4-hydroxybenzeldehyde	0.138	0.211	0.064	0.034
	vanillic acid	0.072	0.104	0.075	0.018
	homovanillic acid	0.011	0.021	0.006	0.004
	4-hydroxyacetophenone	0.018	0.028	0.006	0.005
	caffeic acid	0.007	0.015	0.006	0.003
	syringic acid	0.058	0.078	0.037	0.014
	vanillin	0.142	0.324	0.073	0.047
	4-hydroxybenzoic acid	0.020	0.023	0.014	0.005
	benzoic acid	0.039	0.040	0.017	0.008
	syringaldehyde	0.047	0.066	0.017	0.012

	4-hydroxycoumaric acid	0.601	1.421	0.091	0.130
	ferulic acid	0.160	0.627	0.146	0.123
	sinapic Acid	0.732	0.288	0.064	0.430
	3-hydroxy-4-methoxycinnamic acid	0.003	0.011	0.005	0.002
	4-hydroxycoumarin	0.000	0.001	0.000	0.000
	ortho-toluic acid	0.003	0.003	0.000	0.001
	para-toluic acid	0.071	0.080	0.008	0.016

Table A.2. Sugar data from PBHW loading study hydrolysate. Conducted by Dr. Bruce Dien's laboratory (USDA, Peoria, IL)

Hydrolysate Sample	Glucose	Xylose	Galactose	Arabinose	Fructose	Totals
	mg/g	mg/g	mg/g	mg/g	mg/g	mg/g
1% Reduced Particle Size Solids	18.979	0.000	0.000	2.711	4.648	26.339
1% Whole Solids	66.187	1.947	0.000	5.451	6.229	79.813
5% Reduced Particle Size Solids	6.232	1.181	0.525	3.608	6.757	18.302
5% Whole Solids	10.980	0.366	1.318	0.586	12.444	25.693

Table A.3. NDF, ADF, and ADL data of untreated and PBHW pretreated solids. Conducted in Bill Anderson's lab (USDA, Tifton, GA).

Treatment	NDF	ADF	ADL
5% PBHW Whole Solids	75.7	45.8	5.8
1% PBHW Whole Solids	78.1	49.1	6.0
5% PBHW Reduced Particle Size Solids	80.6	42.7	7.1
1% PBHW Reduced Particle Size Solids	80.4	46.0	4.3
Untreated T85 Bermudagrass	71.3	36.5	3.2

APPENDIX B

RAW DATA FROM "ETHANOL AND CO-PRODUCT GENERATION FROM PRESSURIZED BATCH HOT WATER PRETREATED BERMUDAGRASS AND NAPIERGRASS USING RECOMBINANT *ESCHERICHIA COLI* AS A BIOCATALYST" AND APPENDIX C

Table B.1. Raw sugar data of PBHW hydrolysate and enzyme digested untreated and PBHW pretreated T85, ADEL, and Merkeron grasses.

	Arabinose (mg/ g grass)			Xylose (mg/ g grass)			Glucose (mg/ g grass)		
Treatment	**Hydrolysate**	**0 h**	**24 h**	**Hydrolysate**	**0 h**	**24 h**	**Hydrolysate**	**0 h**	**24 h**
T85 (Untreated)	N/A	1.555 ± 0.237	0.746 ± 0.085	N/A	1.439 ± 0.719	9.038 ± 1.506	N/A	59.969 ± 7.294	129.814 ± 7.695
T85 (PBHW)	1.1408	4.844 ± 0.324	6.205 ± 0.440	0.8172	26.913 ± 2.019	36.571 ± 3.296	4.1184	83.000 ± 6.896	177.267 ± 41.975
ADEL (Untreated)	N/A	0.616 ± 0.094	0.512 ± 0.031	N/A	2.363 ± 0.133	3.625 ± 0.171	N/A	41.925 ± 6.147	43.967 ± 2.318
ADEL (PBHW)	1.119	0.790 ± 0.041	0.828 ± 0.098	0.980	15.143 ± 0.122	16.222 ± 1.544	1.946	26.776 ± 1.441	77.322 ± 7.242
Merkeron (Untreated)	N/A	0.394 ± 0.022	0.791 ± 0.029	0.128	8.689 ± 0.443	24.489 ± 1.813	0.14	61.601 ± 1.654	129.998 ± 4.501
Merkeron (PBHW)	0.163	1.404 ± 0.673	1.084 ± 0.042	N/A	17.258 ± 4.634	42.645 ± 7.018	N/A	63.547 ± 5.191	200.110 ± 10.141

	Mannose (mg/ g grass)			Cellobiose (mg/g grass)			Total Sugars (mg/ g grass)		
Treatment	**Hydrolysate**	**0 h**	**24 h**	**Hydrolysate**	**0 h**	**24 h**	**Hydrolysate**	**0 h**	**24 h**
T85 (Untreated)	N/A	21.835 ± 4.616	12.821 ± 6.229	N/A	0.965 ± 0.062	1.143 ± 0.042	N/A	85.763 ± 12.916	153.562 ± 14.973
T85 (PBHW)	1.9396	6.277 ± 0.696	20.902 ± 6.730	0	12.479 ± 0.954	11.396 ± 2.139	8.016	133.513 ± 9.266	252.341 ± 41.975
ADEL (Untreated)	N/A	7.803 ± 0.631	3.377 ± 0.558	N/A	0.991 ± 0.052	0.955 ± 0.082	N/A	53.699 ± 5.731	52.435 ± 2.835
ADEL (PBHW)	0.725	3.006 ± 0.378	3.516 ± 0.663	0	9.580 ± 2.105	7.421 ± 0.766	4.77	55.296 ± 3.979	105.309 ± 7.499
Merkeron (Untreated)	0.119	2.430 ± 0.636	2.175 ± 0.191	0	2.555 ± 0.421	6.109 ± 0.795	N/A	75.668 ± 1.766	163.526 ± 6.217
Merkeron (PBHW)	N/A	1.281 ± 0.196	1.291 ± 0.155	N/A	23.834 ± 2.387	24.318 ± 2.858	0.550	107.325 ± 6.407	269.449 ± 12.528

Table B.2. Raw inhibitor data of PBHW hydrolysate and enzyme digested untreated and PBHW pretreated T85, ADEL, and Merkeron grasses. Conducted by Lekh Sharma (Baylor University, Waco, TX).

	Compound (mg/g grass)	Hydrolysate from PBHW T85	Enzyme Digested Untreated T85	Enzyme Digested PBHW T85	Hydrolysate from PBHW ADEL	Enzyme Digested Untreated ADEL	Enzyme Digested PBHW ADEL	Hydrolysate from PBHW Merkeron	Enzyme Digested Untreated Merkeron	Enzyme Digested PBHW Merkeron
Aliphatic Acids	malonic acid	0.054	0.162	0.021	0.036	0.076	0.023	0.039	0.231	0.042
	lactic acid	0.499	0.549	0.634	0.194	0.300	0.606	0.260	0.816	1.011
	maleic acid	0.036	0.001	0.006	0.004	0.002	0.005	0.003	0.002	0.081
	acetic acid	8.957	3.590	7.750	6.137	1.756	4.347	4.006	4.784	3.317
	cis-aconitic acid	0.005	0.012	0.003	0.000	0.010	0.002	0.004	0.006	0.007
	methylmalonic acid	ND	0.008	0.005	0.000	0.001	0.001	0.001	0.001	0.001
	succinic acid	0.136	0.247	0.206	0.035	0.045	0.133	0.258	0.678	0.753
	fumaric acid	0.064	0.024	0.036	0.011	0.028	0.017	0.350	0.845	0.281
	trans-aconitic acid	0.028	0.049	0.022	0.010	0.033	0.018	0.053	0.083	0.034
	glutaric acid	0.013	0.004	0.012	0.004	0.007	0.006	0.009	0.018	0.019
	itaconic acid	0.039	0.003	0.013	0.007	0.001	0.004	0.010	0.002	0.012
	2-hydroxy-2-methylbutyric acid	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.000
	gallic acid	0.001	0.000	0.000	0.004	0.001	0.003	0.000	0.000	0.000
Sugar Degradation Products	levulinic acid	0.019	0.044	0.033	0.013	0.015	0.014	0.015	0.031	0.033
	formic acid	2.892	0.500	1.198	1.366	0.310	4.782	1.969	0.544	5.105
	2-furoic acid	0.050	0.007	0.059	0.019	0.006	0.021	0.014	0.004	0.056
	5-hydroxymethylfurfural	0.103	0.015	0.106	0.129	0.002	0.042	0.004	0.001	0.009
	furfural	0.970	0.007	0.128	1.271	0.005	0.088	0.262	0.004	0.055
Lignin Degradation Products	adipic acid	0.005	0.002	0.002	0.002	0.002	0.001	0.004	0.004	0.003
	3,4-dihydroxybenzoic acid	0.015	0.004	0.003	0.022	0.026	0.009	0.005	0.006	0.004
	3,5-dihydroxybenzoic acid	0.050	0.003	ND	0.005	0.003	0.000	0.013	0.002	0.001
	2,5-dihydroxybenzoic acid	0.019	0.045	0.003	0.004	0.013	0.002	0.004	0.016	0.010
	3,4-dihydroxybenzaldehyde	0.030	0.017	0.140	0.200	0.004	0.103	0.204	0.018	0.208
	salicylic acid	0.047	0.018	0.009	0.014	0.033	0.008	0.029	0.026	0.015
	4-hydroxybenzeldehyde	0.074	0.039	0.100	0.045	0.020	0.109	0.027	0.051	0.082
	vanillic acid	0.114	0.021	0.012	0.054	0.048	0.018	0.042	0.028	0.015
	homovanillic acid	0.030	0.002	0.020	0.027	0.002	0.013	0.012	0.004	0.038
	4-hydroxyacetophenone	0.007	0.001	0.004	0.003	0.000	0.002	0.002	0.002	0.005
	caffeic acid	0.007	0.010	0.011	0.017	0.150	0.032	0.002	0.005	0.007

	syringic acid	0.077	0.013	0.011	0.044	0.009	0.012	0.046	0.031	0.017
	vanillin	0.096	0.073	0.045	0.111	0.052	0.039	0.080	0.103	0.235
	4-hydroxybenzoic acid	0.009	0.007	0.002	0.002	0.001	0.001	0.002	0.005	0.002
	benzoic acid	0.012	0.051	0.046	0.003	0.054	0.048	0.020	0.067	0.062
	syringaldehyde	0.033	0.114	0.205	0.411	0.097	0.357	0.362	0.166	0.199
	4-hydroxycoumaric acid	0.168	0.104	0.141	0.353	0.076	0.163	0.323	0.141	0.128
	ferulic acid	0.035	0.086	0.144	0.068	0.118	0.093	0.132	0.095	0.101
	sinapic Acid	0.005	0.054	0.045	0.005	0.006	0.064	0.011	0.005	0.005
	3-hydroxy-4-methoxycinnamic acid	0.024	0.003	0.003	0.016	0.007	0.006	0.026	0.010	0.006
	4-hydroxycoumarin	0.000	ND	ND	0.000	ND	ND	0.000	ND	ND
	ortho-toluic acid	0.000	0.000	0.000	0.001	0.000	0.000	0.001	0.000	0.000
	para-toluic acid	0.013	0.002	0.002	0.013	0.003	0.003	0.005	0.003	0.002

APPPENDIX C

EVALUATION OF ALAMO-DERIVED EXPERIMENTAL LINE SWITCHGRASS FOR BIOCONVERSION TO ETHANOL

Introduction

There has been a recent interest in switchgrass as a possible biomass feedstock. A new switchgrass cultivar was developed in Georgia to create a more robust switchgrass for growth in the southeastern United States. ADEL (Alamo-derived Experimental Line) switchgrass (*Panicum virgatum* L.) resulted from crossing two lowland cultivars of switchgrass, Alamo and Klanow, and produced a 30% average annual yield increase over either parent (9). In a long-term field study to determine productivity on a hectare basis ADEL parent, Alamo switchgrass, produced between 16,000 and 17,500 kg/ha annually (six year average) (9). We evaluated ADEL switchgrass using pressurized batch hot water pretreatment for bioconversion to ethanol (10).

Materials and Methods

ADEL switchgrass was harvested as a second harvest after a 1yr of growth in December 2007. Pretreatment, fermentation, and analytical analyses were conducted as described in Chapter 4.

Results and Discussion

PBHW pretreated ADEL released 105.3 ± 7.5 mg sugar/g grass after enzymatic digestion, but ADEL switchgrass without PBHW pretreatment (untreated) did not liberate any additional sugars after 24 h (Table C.1). Ethanol and reducing sugar concentrations over the course of fermentation for untreated and PBHW pretreated ADEL are show in Figure C.1. When fermented, PBHW pretreated ADEL switchgrass produced 149.1 mg ethanol/g grass (46% of maximum theoretical yield) compared to 67.6 mg/g grass, only 20% of its maximum theoretical yield (Table C.1).

Table C.1 Enzymatic digestion, maximum ethanol concentrations, and percent theoretical yields for PBHW pretreated and untreated ADEL switchgrass. Values are reported on a dry matter basis. Experiments were conducted in triplicate.

Fermentation Substrate	-24h Enzymatic Digestion (mg sugar/g grass)	0h Enzymatic Digestion (mg sugar/g grass)	Percent Cellulose and Hemicellulose	Maximum Ethanol Concentration	Percent Theoretical Yield
ADEL (PBHW)	55.3 ± 4.0	105.3 ± 7.5	69.6	149.1 ± 8.9	46
ADEL (Untreated)	53.7 ± 5.7	52.4 ± 2.8	73.2	67.6 ± 8.6	20

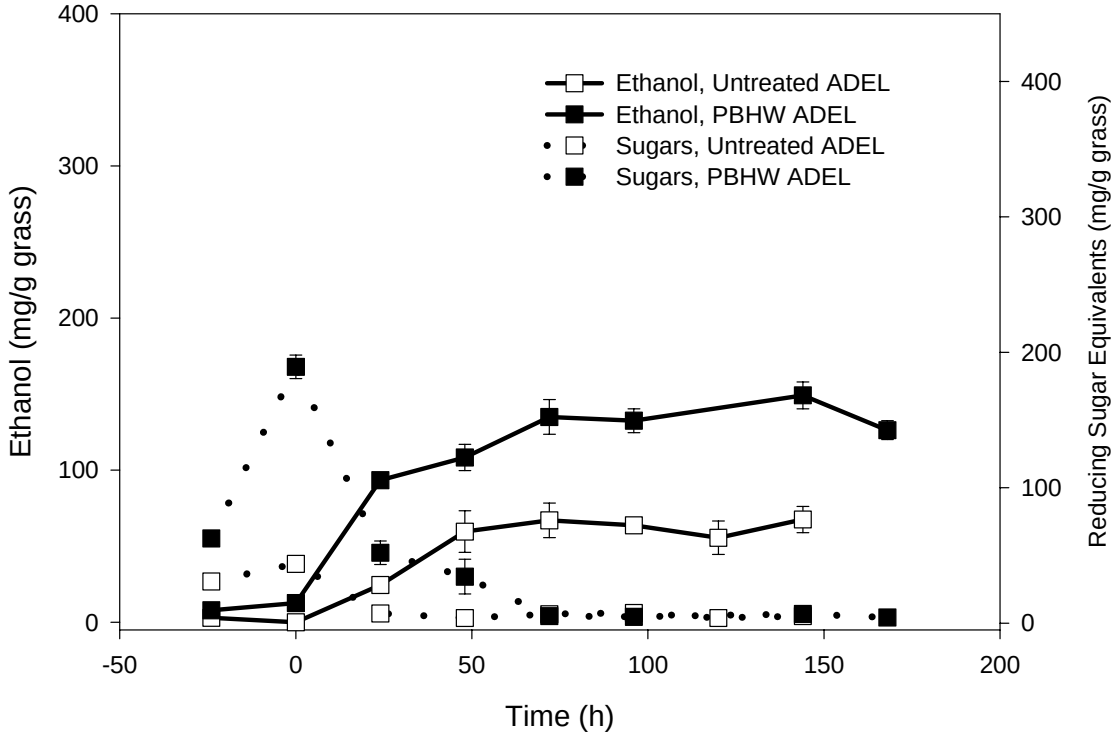

Figure C.1 Average ethanol production and reducing sugar concentrations during fermentation of untreated and PBHW-pretreated ADEL switchgrass. PBHW- pretreatment was carried out at 5% w/v solids in the reactor at 230°C for two minutes. The -24 hour time point corresponds to the beginning of the 24 hour enzymatic digestion. Bacterial inoculation occurred at time 0 hour. Reducing sugars are removed at approximately the same rate ethanol is produced. Fermentations were performed in triplicate.

NDF, ADF, ADL, and IVDMD analyses were conducted on PBHW pretreated and untreated ADEL was also conducted (Table C.2). Based on these analyses, PBHW pretreated ADEL resulted in no solubilization of cellulose, but 26% hemicellulose solubilization. When this was enzymatically digested, cellulose dissolution increased by 26% but there was no further hemicellulose solubilization. Enzyme digested untreated ADEL solubilized 15% of the cellulose and 11% of the hemicellulose. When dissolution of total fermentable material was evaluated, untreated ADEL had 26% solubilized and PBHW pretreated had 52%.

Table C.2 NDF, ADF, ADL, and IVDMD in untreated and PBHW pretreated and enzyme digested ADEL solids. Samples were pooled prior to analysis (n=3). Enzyme digested solids were evaluated after 24h of enzymatic digestion.

Fermentation Substrate	NDF	ADF	ADL	IVDMD
Untreated ADEL	78.3	46.1	5.1	33.0
PBHW ADEL	76.1	52.1	6.6	38.7
Enzyme Digested Untreated ADEL	68.6	40.0	5.1	33.3
Enzyme Digested PBHW ADEL	64.6	39.2	5.7	39.1

When compared to Tifton 85 bermudagrass and Merkeron napiergrass (Chapter 4), ADEL performed considerably worse whether it was pretreated or left untreated. This was due presumably to the reduced sugar release from enzymatic digestion since the measured potential inhibitor levels in the ADEL treatments are comparable to those of T85 and Merkeron. Although ADEL had the highest level of cellulose and hemicellulose in untreated grass compared to the other two cultivars, all of the ADEL treatments had much lower IVDMD values than the other two grasses (Table 4.2 and Table C.2). The increased maturity of the switchgrass in this study corresponds to a higher stem to leaf ratio compared to the other grasses, resulting in lower IVDMD values. Inhibitor levels were comparable for T85, Merkeron, and ADEL, so no

definitive conclusion can be drawn as to whether there are inhibitors interfering with enzymatic hydrolysis or fermentation. Also, there may be other inhibitory compounds affecting the bioconversion process that were not evaluated in this study. Regardless of the inhibitors investigated here it is evident that innate digestibility of grasses, measured using traditional methods, correlates with commercial enzyme digestion and fermentation to ethanol.

ADEL switchgrass was harvested after a year of growth, which is much older than most grasses harvested for animal feed or energy feedstocks. Advantages to allowing such a long growth period include increased biomass yields at harvest and the replacement of nutrients and minerals to the soil that are normally stripped during more traditional harvesting practices, but at the cost of digestibility (personal communication with W. F. Anderson). For fermentation processes, digestibility of grass is a critical factor in the efficiency of the conversion process. This necessitates a balance between optimizing biomass yield with digestibility. Based on these data, younger grasses are a better choice as feedstocks for fermentation to ethanol.