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Design of a Pretreatment and Enzymatic Saccharification Scheme of Understory from Managed Pine Forest for a Biochemical-Refinery Platform: The Example of the Sweetgum Tree

Design of a Pretreatment and Enzymatic Saccharification Scheme of Understory from Managed Pine Forest for a Biochemical-Refinery Platform: The Example of the Sweetgum Tree

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Biological Engineering

By

Angèle Mezindjou Djioleu
University of Arkansas
Bachelor of Science in Biological Engineering, 2010

December 2012
University of Arkansas

ABSTRACT

   The possibility of using sweetgum from southern pine dominated forests as a biobased

refinery feedstock was investigated. First, a baseline hydrolysis scheme for sweetgum wood and

bark was designed. Sweetgum wood and bark were pretreated with 0.98% (v/v) sulfuric acid at

140°C for 30, 40, 50, 60 or 70 min and at 160°C for 30, 40, 50 or 60 min. The water insoluble

solid (WIS) fraction was hydrolyzed with a cellulase enzyme cocktail. Results showed that

maximum xylose and glucose yields from the wood were 82 and 86%, respectively. Similarly,

the respective maximum yields of xylose and glucose from the bark were 93 and 24%.

Concentrations of detected inhibitory compounds such as furfural, hydroxymethylfurfural

(HMF), formic acid and acetic acid ranged from 0.1 to 32.3 g/ 100 g of raw dry biomass. The

second part of this project investigated the effect of adding oak wood, sweetgum bark, or oak

bark, to sweetgum wood on xylose and glucose yields obtained from dilute acid pretreatment and

enzymatic hydrolysis. Carbohydrate recoveries for each species and mixed biomass samples

were obtained by using previously established hydrolysis protocols at 160°C for 20 min. Mixed

biomass samples were prepared to reflect real-life forestry harvesting scenario and consisted of

70% sweetgum wood plus 1) 30% sweetgum bark; 2) 30% oak bark; 3) 30% oak wood. 100%

sweetgum was the control. Results showed that oak wood yielded 35% of its theoretical xylose

content and sweetgum wood, 65%. Both woody species resulted in higher glucose and lower

formic acid recoveries than their respective bark material. Analysis of data with the Dunnett

Control's test in JMP 10.0 showed contamination of sweetgum wood did not have a significant

effect (P > 0.05) on hydrolysis except with sweetgum bark which exhibited a significantly higher

xylose concentration than the control. In conclusion, sweetgum wood was a good source of

carbohydrate for a biobased refinery, but the removal of bark might be necessary to achieve

desirable yields. It is important to note that all the above results were obtained with intensively washed pretreated biomass, which will not be realistic for a real-life sustainable biorefinery.

This thesis is approved for recommendation
to the graduate council.

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Dr. Danielle Julie Carrier

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Finally, my sincere gratitude to the SunGrant Initiative for financing this project

DEDICATION

      I would like to dedicate this thesis to my mother who always supported me in pursuing my education and taught me the value of hard work. I want to thank my fiancé, Sylvain Tientcheu, and my son, Brad Tientcheu, for all the love and patience that they demonstrated during all those years and for being at my side during this journey, especially through all hard times.

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1. INTRODUCTION

Southern pine forests produce nearly 60% of the softwood lumber in the U. S.; in Arkansas, nearly 75% of all produced timber is from pine-dominated forests (Arkansas Forestry Commission, 2008). However, hardwood competition in the pine forest understory is a major impediment to pine forest growth. Therefore, southern pine forests are intensively managed (Wear and Greis, 2002). Annually, more than $150 million are spent reducing or eliminating competition in southern pine forests, primarily through the use of herbicides (Siry, 2002).

The hardwood understory is composed of a mixture of sweetgum (*Liquidambar styraciflua* L.), oaks (*Quercus spp.*), elms (*Ulnus spp.*), maples (*Acer spp.*), dogwoods (*Cornus spp.*), and other miscellaneous hardwood species, which compete with pine for site resources. In Arkansas, the quantity of logging residue ranges from 1.71 to 2.03 million dry tons annually, and total forest based biomass resources are approximately 50 million dry tons annually (Gan and Smith, 2006; Jackson, 2007). Instead of being a nuisance, this hardwood understory growth could become an important source of biomass for lignocellulosic-based biorefineries, especially because sweetgum is a fast-growing hardwood. Capturing biomass from fuel-reduction thinning and understory harvests could raise forest based biomass production from 2.3 to 5 million dry tons annually in the state of Arkansas alone (Pelkki, 2007).

Although not yet deployed, lignocellulosic-based refineries present potential for the production of fuels and chemicals (Kamm and Kamm, 2004). In standard biorefineries, biomass is deconstructed into simple sugars that can be used to produce either biofuels or other biochemical products (Wyman, 1994). There are several steps involved in biomass deconstruction, of which pretreatment is the most important. Of the evaluated pretreatment techniques, dilute acid presents advantages such as low cost and ease of use (Sannigrahi *et al*., 2011).

The goal of this project was to demonstrate that sweetgum harvested from pine forest understory could be used as feedstock in a lignocellulosic-based refinery using dilute acid pretreatment.

2. LITERATURE REVIEW

2.1. Lignocellulosic Biomass

Lignocellulosic material is the most abundant form of organic carbon; approximately 170×10^9 tons of biomass are produced yearly by photosynthesis (Kamm and Kamm, 2004). Examples of such material include: forestry products, including hardwoods or softwoods; forestry waste, such as sawdust and forest debris; herbaceous energy crops, like switchgrass, alfalfa or miscanthus; agricultural residues, including corn stover, wheat straw or sugar cane bagasse; and municipal solid wastes.

As shown in Figure 1, there are three main components in lignocellulosic material structure: cellulose, hemicellulose and lignin. Cellulose is the major biomass component consisting, depending on the species, of about 35–50% of the structure. Cellulose is composed of approximately 10,000 D-glucopyranosyl units linked together with a 1-4-β bond in a highly crystalline structure. About 20-35% of biomass structure is made up of hemicellulose, which consists of xylose backbone polymers with arabinose, galactose, glucose, or mannose branches. In some species, such as hardwood, hemicellulose has acetyl group branching off the xylan backbone. Hemicellulose is linked to cellulose with hydrogen bonds, and this results in biomass structure strengthening. The third biomass component is lignin and accounts for 15-25% of most types of lignocellulosic material. Unlike cellulose and hemicellulose, lignin is a polyphenolic polymer and does not hydrolyze into reduced sugars. Lignin envelops both hemicellulose and cellulose and protects the biomass against pests and diseases (Ragauskas *et al*., 2006; Wyman, 1994).
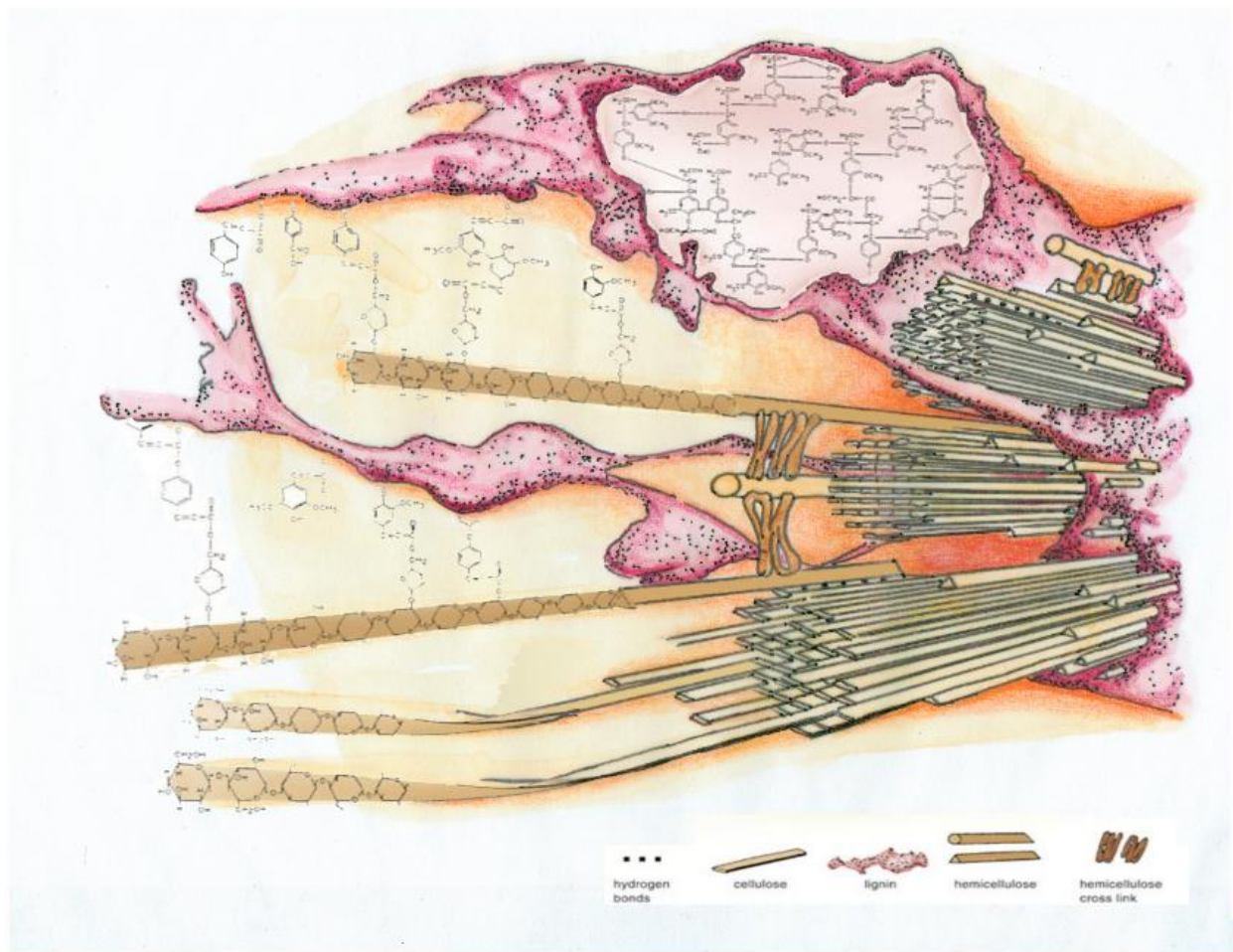
Figure 1. Structure of lignocellulosic biomass
Drawing from Zoe Smith

2.2. Biochemical-Based Refinery

The U.S. Department of Energy defined a biorefinery as a facility that converts feedstock into a spectrum of valuable products, based on the petrochemical refinery. Kamm and Kamm (2004) identified three biorefinery systems that are currently being investigated: 1) lignocellulose feedstock (LCF) biorefinery; 2) whole crop (WC) biorefinery; and 3) green biorefiney. LCF biorefinery converts lignocellulosic material into a variety of products. Among the three biorefinery systems, LCF has the greatest chances of being deployed because: 1) an abundance of lignocellulosic biomass; 2) the availability of energy crops and food crop residues; 3) cycling of carbon, reducing green house gas emissions; and 4) competition between petrochemical and future biobased products markets. WC biorefineries are based on the conversion of cereals such as rye, wheat, and maize. Although WC biorefineries strive to exploit all parts of the crop to be more profitable, they still remain major competitors to food industries. Finally, green biorefineries are different from LCF and WC biorefineries in the sense that their principal products are not fuels, but compounds extracted from the phytosynthetically active parts of the feedstock. Examples of compounds extracted from green biorefineries include amino acid, proteins, hormones, dyes, enzymes, and organic acids (Kamm and Kamm, 2004). An overview of a biorefinery is shown in Figure 2.

Feedstock (s):
- Food and feed grains
- Lignocellulosic biomass (wood, herbaceous, forestry and agricultural waste)
- Municipal solid waste (paper, cardboard)

Conversion processes:
- Biological (bacterial, enzyme)
- Chemical (acid hydrolysis)
- Thermochemical (combustion, pyrolysis gasification)
- Physical (milling)

Products:
- Fuels (ethanol butanol)
- Chemicals (furfural, organic acid)
- Materials (polymers)
- Value-added compounds (phytochemicals)

Figure 2. Overview of a biorefinery

A biochemical-based biorefinery is a type of LCF biorefinery that combines chemical and biological approaches in converting raw biomass. As shown in Figure 3, there are four unit operations in a biochemical-based biorefinery: a) pretreatment; b) enzymatic hydrolysis; c) fermentation; and d) product separation. The two first steps are the focus of this project because they are the limiting factors for large-scale establishment of biorefineries due to their inherent costs (Wyman, 1994).
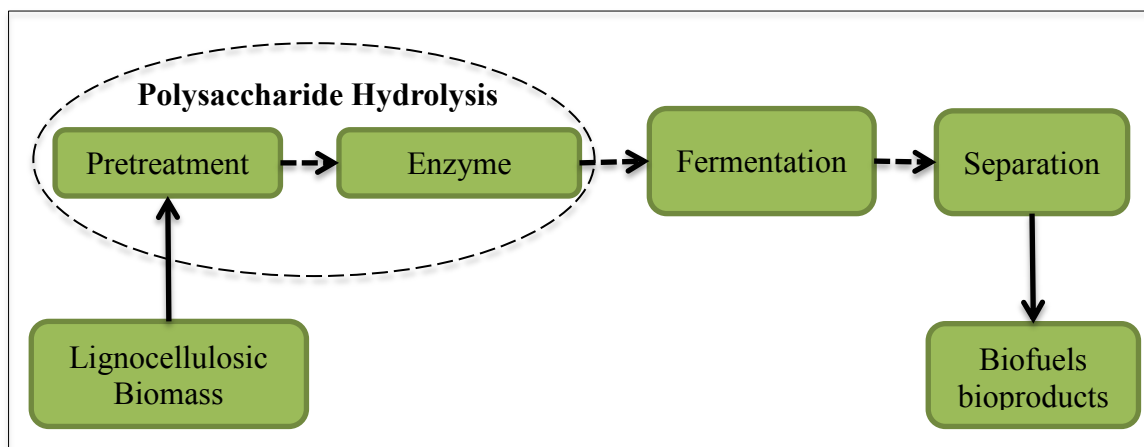
Figure 3. Schematic representation of a biochemical-based biorefinery

In biochemical-based biorefineries, pretreatment is the most important processing step because it is aimed at disrupting biomass structures to facilitate enzymatic hydrolysis. Without pretreatment, expensive enzyme cocktails cannot saccharify plant cell walls. Characteristics of effective pretreatments are: 1) opening of cellulose crystalline structure to facilitate hydrolysis; 2) prevention of sugar degradation, especially hemicellulose sugars; 3) limitation of the formation of lignin degradation compounds that can inhibit fermentation; and 4) environmental and cost friendly (Mosier *et al*., 2005).

Biochemical-based biorefineries use chemical pretreatments as opposed to physical handling. Depending on the nature of chemicals used or pH conditions, chemical pretreatments can either be acidic, alkaline or water-based. Examples of such pretreatment are: uncatalyzed steam explosion, liquid hot water, dilute acid, ammonia fiber explosion (AFEX), and lime. These different types of pretreatment have a variety of effects on the biomass. For instance, all of them improve cellulose accessibility. However, dilute acid mainly removes the hemicellulose and disrupts the lignin's structure, whereas AFEX has minor effects on hemicellulose but, removes and alters lignin (Mosier *et al*., 2005).

There are advantages and disadvantages associated with each pretreatment technique. In general, water-based pretreatments have the advantage of not using expensive catalysts and the formation of fermentation-inhibitory compounds is limited. However, water-based processes produce xylose oligomers, which inhibit enzymatic hydrolysis of the pretreated biomass and require an extra hydrolysis step to break down the oligomers before fermentation. On the other hand, catalyzed pretreatment such as dilute acid, AFEX, and lime usually yield highly digestible biomass, but the cost associated with the catalyst used can make the process very expensive. Sulfuric acid is inexpensive, but its corrosiveness dictates that equipment used for dilute acid

pretreatment should be constructed from stainless steel, increasing capita costs. In addition, the cost to detoxify liquid hydrolysates, containing fermentation-inhibitory compounds produced during dilute acid pretreatment and the excessive amount of water used to wash the pretreated biomass prior to enzymatic hydrolysis, also increase its overall cost. Dilute acid pretreatments are further discussed below. The elevated price of ammonia and its recycling cost is the principal disadvantage of AFEX. Additionally, AFEX is not effective for forestry biomass such as hardwood and softwood. Finally, prolonged pretreatment periods are disadvantages associated with lime pretreatment. Low pressures and temperatures are used, but pretreatment takes several days instead of minutes or hours (Mosier *et al*., 2005).

In biochemical-based biorefineries, enzymatic hydrolysis is conducted with an enzyme cocktail containing high cellulase activity. Enzymatic hydrolysis can be carried out under two different scenarios: 1) performed separately from the fermentation step, named separate hydrolysis and fermentation (SHF); and, 2) enzymatic hydrolysis and fermentation conducted simultaneously, named simultaneous saccharification and fermentation (SSF). Of these two scenarios, SSF is preferred because enzymatic hydrolysis and fermentation are performed in the same vessel. Also, cellulose hydrolysis into glucose is maximized in SSF because fermentation of glucose, as it is produced, drives the cellulose hydrolysis reaction forward (Mosier *et al.*, 2005; Wyman, 1994).

Cellulase is a mixture of three enzyme activities (endoglucanase, exoglucanase, β-glucosidase), which is mainly produced today by genetically modified strains of the fungus *Trichoderma reesei.* Endoglucanase cleaves cellulose inside the chain; exoglucanase breaks off two units of glucoses at the end of the chain into cellobiose; and β-glucosidase breaks cellobiose units into two glucose molecules that can be fermented into ethanol or other biobased products.

Cellulose conversion efficiency improved with β-glucosidase and xylanase supplementations. Xylanase hydrolyzes xylan into xylose. Xylanase-supplemented cellulase increased glucose yields by 50% for AFEX-pretreated biomass; approximately 57% for lime-pretreated biomass; and 14% for dilute acid pretreatment. Therefore, commercial preparations, such as Accelerase® 1500 produced by Genencor, are cocktails that contain a cellulase and xylanase enzymes (Kumar and Wyman, 2009; Wyman, 1994).

2.3. Dilute Acid Pretreatment

Among the chemical pretreatment techniques that improve cellulose digestibility, dilute acid pretreatment has the most potential to be used in a large-scale setting (Sannigrahi *et al*., 2011). Although any strong acid can be used, sulfuric acid (H_2SO_4) has been the most popular mainly because of its affordable price. It is usually conducted at low temperatures ($< 140°C$) for a long time or at high temperatures ($> 160°C$) for a short time. Dilute acid pretreatments have increased cellulose digestibility for a wide range of feedstocks, ranging from hardwoods to grasses and agricultural residues (Mosier *et al*., 2005). Reasons for this success are mainly attributed to hemicellulose removal from the cell wall and disruption of the crystalline structure of cellulose. While low temperatures are not efficient at disrupting the crystalline structure of cellulose, high temperatures promote xylose degradation into inhibitory products and premature hydrolysis of cellulose.

In early studies on dilute acid pretreatment of lignocellulosic biomass, effectiveness of the pretreatment was mainly measured by cellulose digestibility of the ensuing biomass. Cellulose digestibility is defined as the amount of cellulose, in the pretreated biomass, converted to glucose during enzymatic hydrolysis. However, recent studies have shown that maximizing xylose recovery during dilute acid pretreatment has the potential to improve economical viability

of the process. Moreover, with the development of engineered microorganisms that can ferment 5-carbon sugars (pentoses), xylose is no longer considered a waste product in the sugar-to-ethanol conversion process. For example, genetically modified *Escherichia coli* could produce 0.92 g/L/h of ethanol from a broth containing 95 g/L of xylose. *Zymomonas mobilis* is another useful engineered microorganism capable of producing 0.32 g/L/h from fermentation medium supplemented with 60 g/L xylose concentration (Chung et al., 2005; Dien et al., 2003; Saha et al., 2005). Consequently, efficiency of dilute acid pretreatment is now measured from combining xylose and glucose yields after hydrolysis rather than solely glucose yields (Lloyd and Wyman, 2005).

One of the main disadvantages of dilute acid pretreatment is the formation of sugars and lignin degradation compounds, such as furfural, 5-hydroxymethylfurfural (HMF), formic acid, and acetic acid, that could inhibit enzymatic hydrolysis or sugar fermentation. Furfural results from the degradation of xylose; HMF from glucose; and both can further degrade into formic acid. The acetyl group released from the hemicellulose during pretreatment forms acetic acid (Palmqvist Hahn-Hagerdal, 2000). Study on ethanol production from xylose showed that presence of these compounds in the prehydrolysate inhibits xylose fermentation. Fermentation of xylose contained in a prehydrolysate of poplar and corn stover yielded 67% and 80%, respectively (Fenske *et al*., 1998). Both yields were significantly lower than the control of 90%. Cantarella *et al*. (2004) also showed that formic acid concentrations of 11.5 g/L could significantly inhibit cellulose saccharification, yielding glucose concentration of 10 g/L instead of 30 g/L obtained in the absence of formic acid. Therefore, reporting concentration of these inhibitory compounds in pretreatment hydrolysate would provide another angle to measure the efficiency of the pretreatment.

2.4. Hydrolysis of Sweetgum Wood and Bark

Sweetgum wood, as a potential feedstock for a biochemical-based biorefinery, was studied by Torget *et al*. (1990). Sweetgum wood was pretreated with dilute sulfuric acid (0.45-0.55 (v/v) H_2SO_4) at 140 and 160°C for times ranging from 0 to 60 min, in a stainless steel stirred reactor. Results showed that, at higher temperatures, hemicellulose hydrolyzed faster. Nighty eight percent of hemicellulose was hydrolyzed in less than 20 min at 160°C; similar yields were obtained by hydrolyzing at 140°C for times from 30 to 60 min. Enzymatic hydrolysis of dilute acid pretreated biomass showed improvement of cellulose digestibility; best results were with pretreatments at 160°C. Nighty to one hundred percent cellulose digestibility was observed for biomass pretreated at 160°C for 5 to 10 min, while 80 to 90% digestibility was obtained with biomass pretreated at 140°C for more than 30 min. Temperature did not influence lignin solubilization; 15 to 18% of lignin was removed with both temperatures.

In exploring the possibility of using whole trees in biorefineries, Torget *et al*. (1991) investigated the hydrolysis of sweetgum bark. Temperatures ranging from140 to 160 °C and acid concentrations of 0.50 to 0.65% v/v% were used to hydrolyze sweetgum bark. Hydrolysis of sweetgum bark was much more complex than that of sweetgum wood. All xylan and approximately 17% of Klason lignin were hydrolyzed with hot water prior to acid addition; up to 50% of sweetgum bark mass was loss after pretreatment. Although, all the hemicellulose was removed during pretreatment, enzymatic attack of pretreated sweetgum bark was not successful at releasing glucose at both temperatures investigated. Maximum cellulose digestibility of 25 % was observed. Concentrations of acetic acid and furfural in liquid hydrolysates after pretreatment were reported. The concentrations of acetic acid and furfural at 140°C and 160°C were 1.9 and

0.2 g/L, and 2.2 and 0.6 g/L, respectively, indicating that higher temperatures favored sugar degradation.

Martin *et al*. (2010) also investigated the hydrolysis of hemicellulose in sweetgum wood and bark. A 65°C water-extraction of shikimic acid prior to dilute H_2SO_4 (0.98% v/v) pretreatment at 130°C for 50 min increased xylose yield by 21 and 17% from sweetgum bark and wood, respectively. This work showed how extraction of value-added compounds could be integrated into a biorefinery prior to hydrolysis in order to increase the economical efficiency of the conversion of lignocellulosic biomass into fuels.

Spindler *et al*. (1991) investigated the simultaneous saccharification and fermentation (SSF) of dilute sulfuric acid (0.45% v/v) pretreated sweetgum wood at 140°C for 60 min. SSF was conducted at 37°C for 3 and 8 days with either *Saccharomyces cerevisiae* or a mixture of *S. cerevisiae* and *Brettanomyces clausenii*, in a fermentation broth containing cellulase supplemented with an excess of β-glucosidase. Results showed that with *S. cerevisiae*, 86% of theoretical ethanol yield was obtained after 3 days. On the other hand, mixtures of *S. cerevisiae* and *B. clausenii* only yielded 59% after 3 days and 84% after 8 days of theoretical ethanol yields, respectively. In general, pretreated sweetgum wood was a good feedstock for ethanol production.

2.5. Hydrolysis of Oak Wood and Bark

Knappert *et al*. (1980) investigated the effects of temperature and acid concentration on glucose yields from dilute acid pretreatment and enzymatic hydrolysis of oak wood. Oak wood was pretreated for 0.22 min with sulfuric acid concentrations raging from 0.4 to 1.2% (w/w) and at temperatures from 160 to 220°C. In general, oak wood responded positively to the acid pretreatment. Enzymatic hydrolysis of non-pretreated oak biomass resulted in 21.3% cellulose digestibility; pretreated material, 189 °C with either 0.6 or 1% w/w sulfuric acid, and

enzymatically hydrolyzed for 48 h displayed 100% cellulose digestibility. The increase in cellulose digestibility was attributed to nearly complete removal of hemicellulose, a reduction of cellulose degree of polymerization from 606 to 398, and disruption of cellulose crystalline structures. No scientific literature covering oak bark hydrolysis was located.

2.6. Hydrolysis of Mixed Biomass

Hydrolysis of mixed biomass *per se* has not received a lot of attention as compared to pure biomass. Jensen *et al*. (2008) investigated mixture effects on the kinetics of hemicellulose hydrolysis of mixed biomass during dilute acid pretreatment. Kinetic parameters for hemicellulose hydrolysis of aspen, balsam, basswood, red maple and switchgrass were established; xylose concentrations were predicted using the developed kinetic model. For all the tested biomass samples, the difference between predicted and experimental xylose concentrations were less than 0.5 g/L. Additionally, xylose concentrations in hydrolysates stemming from pretreated mixed biomass samples also could be predicted by combining the weighted kinetic parameter for each sample in the mixture. For example, experimental xylose yield of 6 g/L from hydrolysis of biomass mixtures, containing 50% balsam and 50% switchgrass, could be predicted by combining half of the kinetic parameters of balsam and switchgrass. There was no synergistic or antagonist effects on the xylose yield from biomass mixture.

2.7. Conclusion

In summary, sweetgum wood is a good source of sugars for the production of lignocellulosic ethanol. Dilute sulfuric acid pretreatment significantly improved its cellulose digestibility; and, fermentation of its released glucose can be converted to ethanol. However, optimum pretreatment conditions for sweetgum wood to maximize xylose yields, which will, in

turn, increase ethanol yields have not been evaluated. Moreover, the formation of degradation compounds has not been tracked, leading to a lack of understanding between generation of these compounds and pretreatment severity. Furthermore, all the studies reported on hydrolysis of sweetgum species were always conducted with 100% of pure debarked sweetgum wood. However, it is more likely that sweetgum will be harvested along with other understory species present in the understory of pine plantations. The contribution of sweetgum bark material to the carbohydrate material balance also needs to be ascertained. Investigating the possibility for biorefineries to handle, as a feedstock, whole sweetgum tree contaminated with other biomass will give a realistic picture of the conversion of sweetgum to ethanol.

3. OBJECTIVES

The goal of this project was to demonstrate that sweetgum wood harvested form southern-pine-dominated plantation understories could be used as feedstock in biochemical-based biorefineries. In addition, this work also investigated the possibility for biorefineries to handle whole sweetgum trees, including sweetgum wood and bark, or whole sweetgum trees mixed with oak wood and bark. Realization of this goal will be one step towards increasing the use of understory biomass, limiting the release of herbicides in the environment, and translating forestry logistics to biorefinery applications. Specific objectives were:

1) Investigate the effects of temperature and time during dilute acid pretreatment on xylose and glucose yield from dilute acid pretreatment and enzymatic hydrolysis of sweetgum wood and bark

2) Determine saccharification conditions for maximum xylose and glucose recovery for sweetgum wood and bark

3) Investigate the effect of adding sweetgum bark, oak wood or oak bark to sweetgum wood on glucose and xylose recovery from saccharification under optimum conditions

4. MATERIALS AND METHODS

4.1. Biomass Description

Bark and wood from sweetgum and oak were obtained from Dr. Matthew Pelkki and Dr. Philip Tappe, School of Forest Resources, University of Arkansas, Monticello, AR. The feedstock was in the form of 1 cm × 1 cm chips. The mature trees were harvested with a chainsaw from a pine plantation understory in Drew County, AR. All the branches were removed and only the stem was used. Bark was separated from the wood with a chain flail debarker. Samples of each individual species were milled to pass through a 20 mesh (0.84 mm) screen using a Wiley Mini Mill (Thomas Scientific, Swedesboro, NJ) and samples were dried in a 105°C oven until sample moisture was less than 5%, as determined using an MB45 Moisture Analyzer (Ohaus Corporation, Pine Brook, NJ).

4.2. Compositional Analysis of Natural Biomass

A 10-g sample dry biomass for sweetgum and oak wood and bark was submitted to a 24-h ethanol extraction according to the method described in the National Renewable Energy Laboratory (NREL) LAP/TP-510-42619 protocol (Sluiter *et al*., 2008a). Contents of structural carbohydrates and acid insoluble lignin (AIL) of ethanol-extracted biomass were determined following NREL LAP/TP-510-42618 protocol (Sluiter *et al*., 2008b). Composition of raw biomass is given in Table 1.

Table 1: Compositional analysis of natural biomass (% dry weigh)

Biomass	Xylan	Glucan	[1]AIL	Extractives	Ash
[2]SG Bark	8.56 ± 0.76	21.20 ± 0.89	31.57 ± 0.28	15.95	5.76 ± 1.00
SG Wood	15.04 ± 2.92	45.00 ± 1.27	20.37 ± 3.00	2.31	0.27 ± 0.1
Oak Wood	14.41 ± 2.05	48.06 ± 0.1	16.05 ± 0.78	3.26	0.06 ± 0.1
Oak Bark	11.29 ± 1.48	25.35 ± 2.93	25.02 ± 1.27	11.47	5.02 ± 0.8

Data are means of 3 replications ± 1 standard error

1: Acid insoluble lignin

2: Sweetgum

4.3. Dilute Acid Pretreatment

A 1-g sample of dry biomass was soaked in 10 mL of 0.98% (v/v) sulfuric acid (H_2SO_4) in 50-mL centrifuge tubes for 12 h. The mixture was placed in a 32-mL stainless steel pretreatment tube (14.22 mm inner diameter, 5.59 mm wall thickness, 200 mm length) with an additional 10 mL of H_2SO_4. Pretreatment tubes containing raw biomass and acid were heated in a fluidized sand bath (Techne Incorporated, Burlington, NJ) at the desired temperature and for the desired time. Illustration of the pretreatment set-up is shown in Figure 4. After pretreatment, the tubes were immediately submerged into cold tap water for 1 min; slurry contents were poured into 15-mL centrifuge tubes for separation into a liquid fraction (prehydrolysate) and a solid fraction (pretreated biomass). The pretreated biomass was washed by stirring in 30 mL of Millipore filtered water on a stir plate, set at 300 rpm for 30 min. The water-insoluble-solid (WIS) fraction was separated from the wash water by vacuum filtration through a Büchner funnel containing Whatman No. 1 filter paper from VWR Scientific Products (West Chester, PA). The WIS fraction was stored at 4°C for a maximum of 3 days until used for enzymatic hydrolysis. The prehydrolysate and wash water were recovered and stored for a maximum of 3 days at 4°C before xylose, glucose, and degradation compounds determination.

Dry wood
chips

Willey miller
(biomass grinding to
20 mesh)

Reactor tubes
containing ground
biomass with 0.98%
(v/v) H_2SO_4 at 5%
(w/w) slurry loading

Tube in sand bath
(heating
equipment)

Liquid hydrolysate
after pretreatment

Washed pretreated
biomass
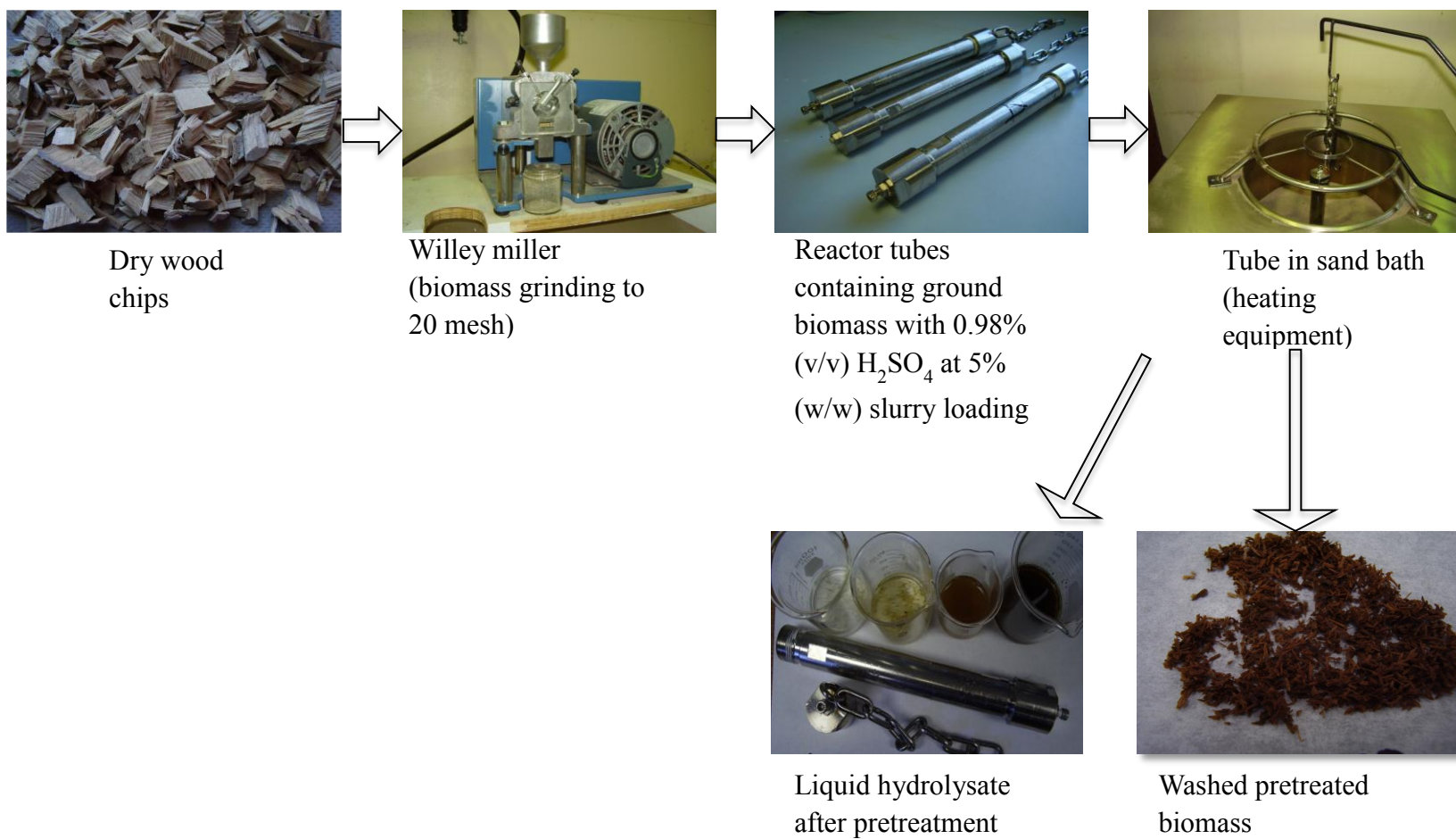
Figure 4. Schematic representation of dilute acid pretreatment set up

4.4. Enzymatic Hydrolysis

A commercially available enzyme cocktail, Accellerase®1500, donated by Genencor (Danisco US Inc., Rochester, NY) was used to hydrolyze the WIS fraction. The enzyme cocktail had an endoglucanase activity of 2200 to 2800 CMC U/g and a β-glucosidase activity of 525 - 775 pNPG U/g (provided by the manufacturer). The WIS fraction was mixed in a 50-mL amber bottle with 5 mL of sodium citrate buffer (pH = 4.8), 0.5 mL of enzyme and 4.5 mL of Millipore filtered water. The Amber bottles were placed in a shaking water bath (Thermo Electron Corporation, Winchester, VA) at 55°C and 100 rpm for 24 h. The enzymatic hydrolysis set-up is illustrated in Figure 5. The resulting slurries were poured into a 15-mL centrifuge tube, submerged in boiling water to stop the reaction, and centrifuged at $3000 \times g$ for 2 min. The volume of the supernatant (enzymatic hydrolysate) was measured and the liquid was stored at 4°C for a maximum of 3 days until it was analyzed for sugar content; the pellet was discarded.

Washed pretreated biomass

Enzyme cocktail and citrate buffer

Water bath at 55°C with Amber
bottles containing pretreated biomass
and enzyme mixture

Figure 5. Schematic representation of enzymatic hydrolysis set up

4.5. Analytical Method

Sugars and degradation compounds in liquid hydrolysates were analyzed based on NREL

LAP/TP-510-42623 protocol (Sluiter *et al.*, 2008c).

4.5.1. Sugar Analysis

Five-mL aliquots of prehydrolysate, wash water and the enzymatic hydrolysate were

neutralized with calcium carbonate (Fisher Scientific, Fair Lawn, NJ) and filtered through a 0.2-

µm filter for xylose and glucose analyses with a Waters 2695 Separations module (Milford, MA)

equipped with Shodex precolumn (SP-G, 8 µm, 6×50 mm) and Shodex column (SP0810, 8 µm

$\times 300$ mm). Millipore filtered water (0.2 mL/min) was the mobile phase and the column was

heated to 85°C with an external heater. Sugars were detected with a Waters 2414 Refractive

Index Detector (Milford, MA). Examples of sugar chromatograms are shown in Figure 6. Sugars

concentration in liquid hydrolysates were determined based on calibration curves shown in

Figure 7, which were established using sugar standards, D-(+) glucose from Alfa-Aesar (Ward

Hill, MA) and D-(+) xylose from Sigma-Aldrich Inc (St. Louis, MO).
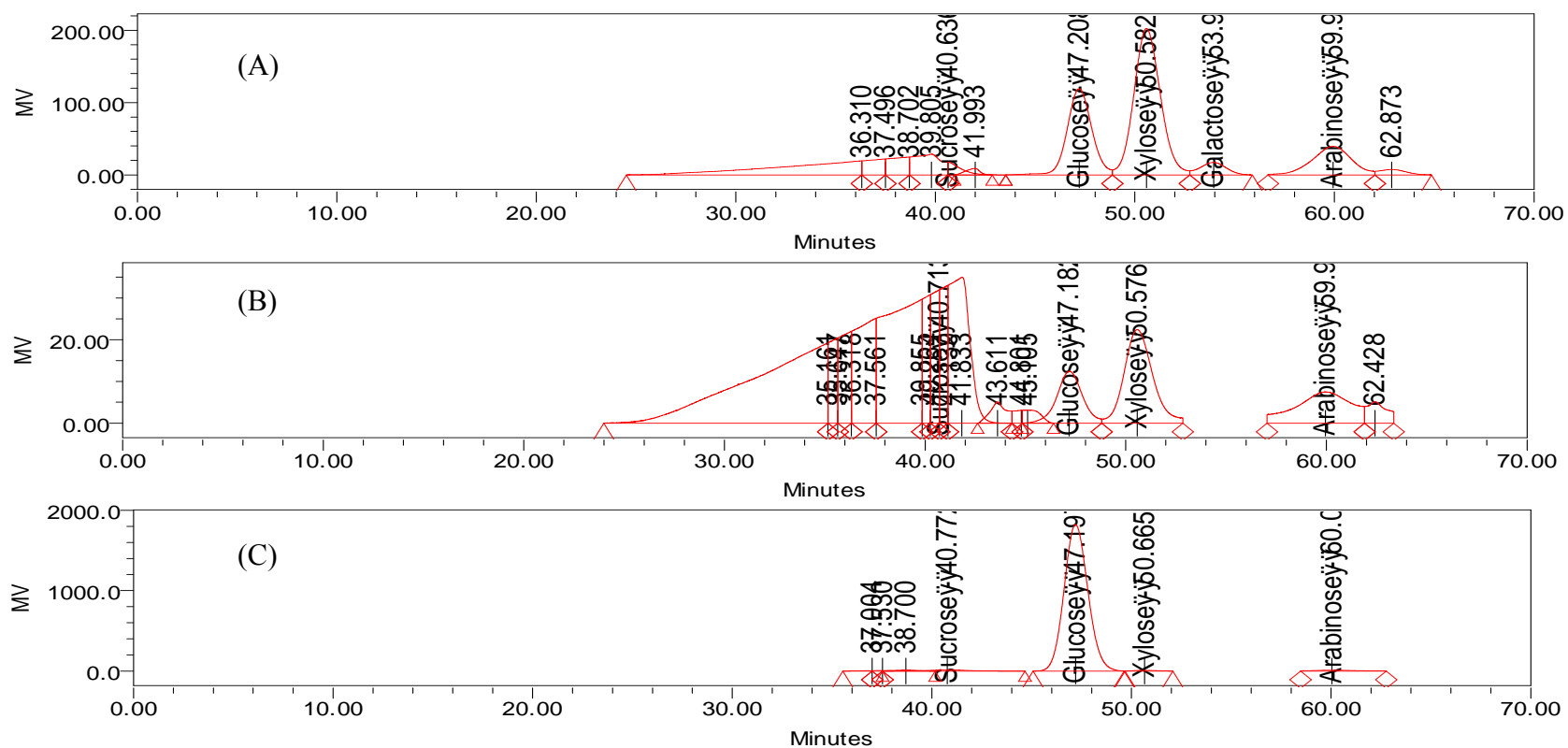
Figure 6. Chromatograms of sugar in liquid hydrolysates from dilute acid pretreatment and enzymatic hydrolysis of sweetgum wood. Retention times of glucose, xylose and arabinose were 47.2, 50.6, 60.0 min, respectively. (A), prehydrolysate; (B), wash water; and, (C), enzymatic hydrolysis hydrolysate.
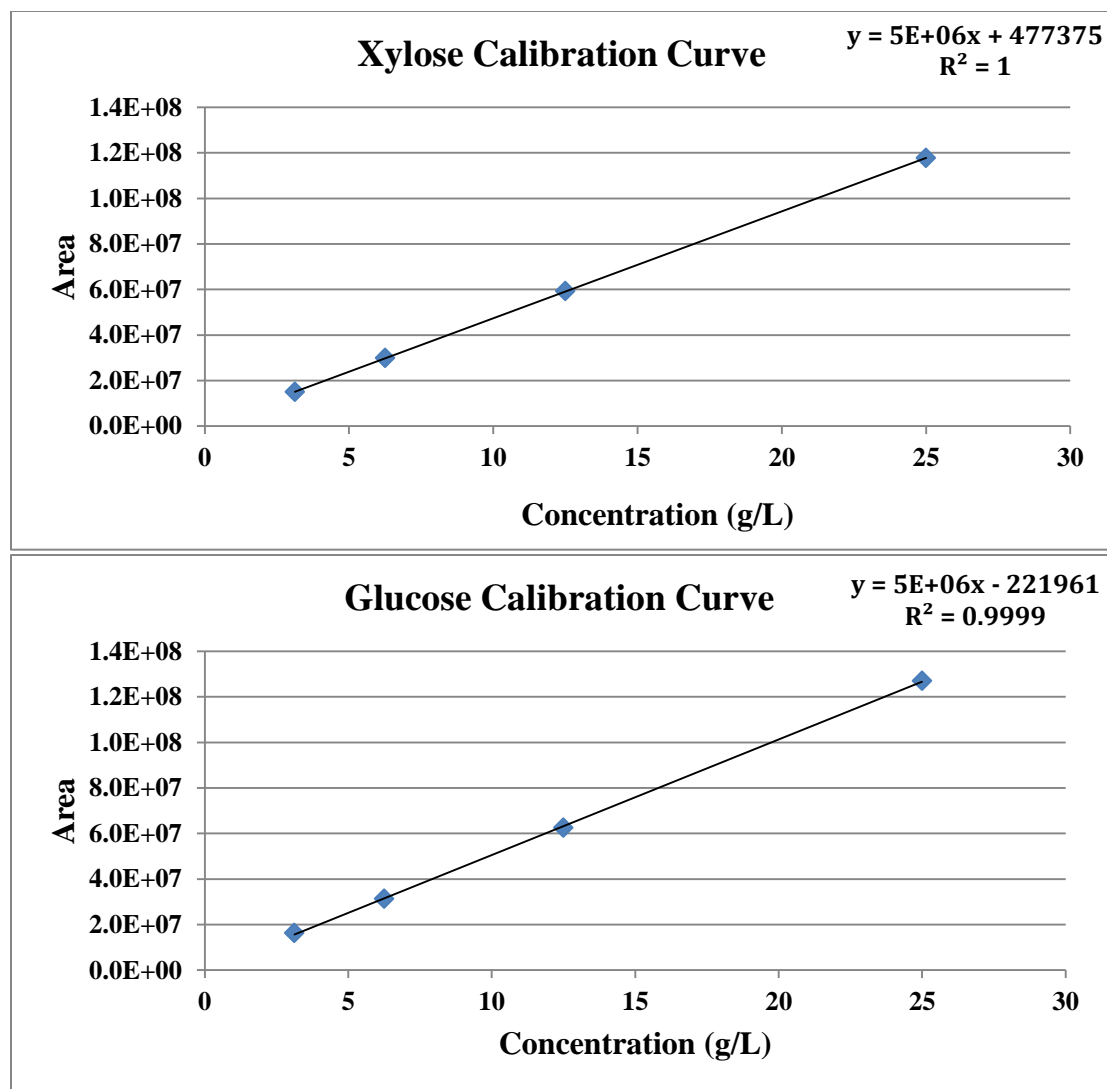
Figure 7. Xylose and glucose calibration curves for a Waters 2695 Separations module equipped with a Shodex precolumn (SP-G, 8 μm, 6 × 50 mm), Shodex column (SP0810, 8 μm × 300 mm) and a Waters 2414 Refractive Index Detector. Millipore filtered water flowing at 0.2 mL/min was the mobile phase

4.5.2. Degradation Compounds Analysis

Aliquots of the prehydrolysate and wash water were filtered through a 0.2-μm filter and analyzed for degradation compounds with a Waters 2695 Separations module equipped with a Bio-Rad Aminex HPX-87H Ion Exclusion 7.8 mm × 30 mm column, heated to 55°C. The mobile phase was 0.005 M H_2SO_4 flowing at 0.6 mL/min. Compounds were detected with a UV index using the Waters 2996 Photodiode Array detector. Furfural and HMF were detected at 280 nm; whereas, formic acid and acetic acid were detected at 210 nm. Chromatograms of degradation compounds are shown in Figure 8. Concentrations of compounds in liquid hydrolysates were determined with calibration curves shown in Figure 9, which were established with reference standards purchased from VWR (Scientific Products).
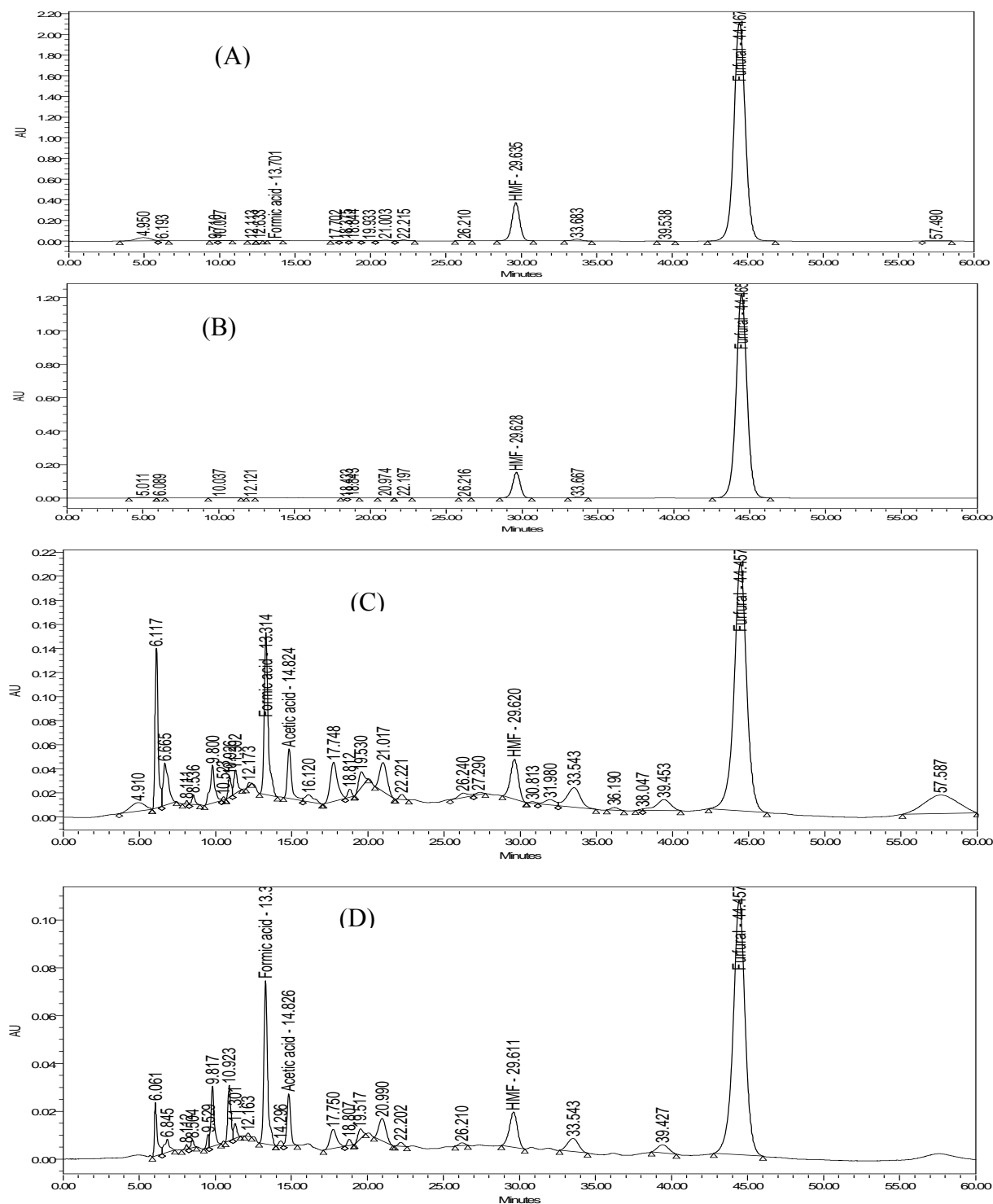
Figure 8. Chromatograms of degradation compounds in liquid hydrolysate from dilute acid pretreatment of sweetgum wood. Furfural and HMF, retention times of 44.5 and 29.6 min detected at 280 nm, respectively, in (A) dilute acid prehydrolysate and (B) wash water. Formic acid and acetic acid, retention times of 13.3 and 14.8 min detected at 210 nm, respectively, in (C) dilute acid prehydrolysate and (D) wash water
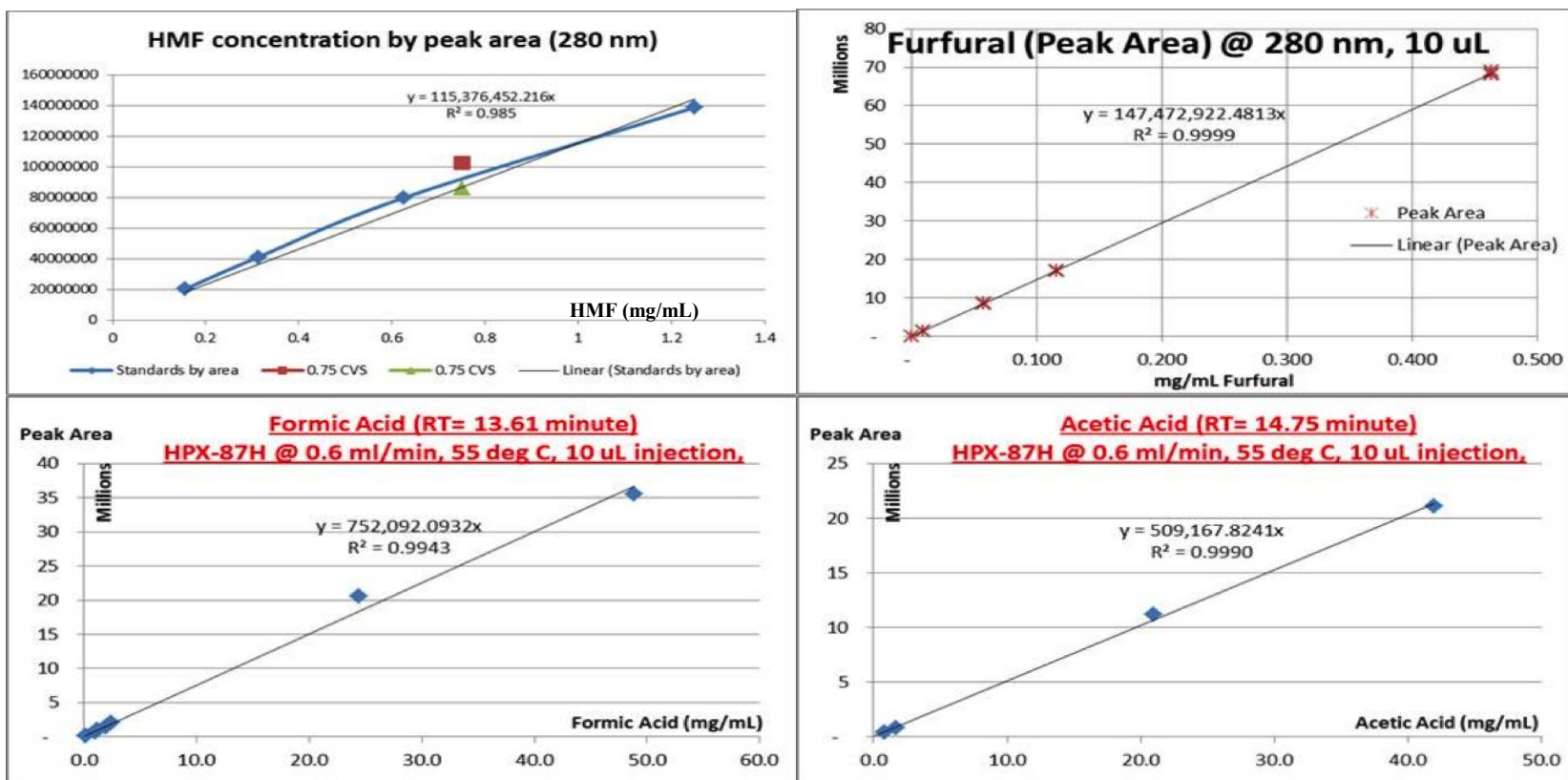
Figure 9. Calibration curves of HMF, furfural, formic acid, acetic acid for a Waters 2695 separations module equipped with a Bio-Rad Aminex HPX-87H Ion Exclusion 7.8 mm × 30 mm column and a Waters 2996 Photodiode Array detector. The mobile phase was 0.005 M H_2SO_4 flowing at 0.6 mL/min

4.6. Hydrolysis of Pure Sweetgum Wood and Bark

Sweetgum wood and bark were pretreated using the protocol described above at 140°C for 30, 40, 50, 60 or 70 min and at 160°C for 30, 40, 50 or 60 min. A completely randomized design (CRD) was used because of a limited number of available reactors. All pretreated biomass samples were subjected to enzymatic hydrolysis following the protocol described above. All pretreatment experiments were performed in triplicate.

4.7. Hydrolysis of Contaminated Sweetgum Wood

Pure sweetgum wood, sweetgum bark, oak wood, and oak bark were initially pretreated at 160°C for 20 min following the protocol described above. Because it was suspected that the conditions in the sand bath could change between operations, all runs were blocked by replication in order to minimize variations in results due to equipment failure. Mixed samples were also pretreated at 160°C for 20 min using a randomized block design (RBD). Mixed biomass samples were prepared as follow: 1) 70% sweetgum wood and 30% oak wood; 2) 70% sweetgum wood and 30% oak bark; and 3) 70% sweetgum wood and 30% sweetgum bark. One hundred percent of sweetgum wood was the control. All pretreated biomass samples were subjected to enzymatic hydrolysis following the protocol described above. All experiments were performed in triplicate.

4.8. Statistical Analysis

Xylose and glucose yields from mixed samples were run through an analysis of variance (ANOVA) procedure in JMP 9.0 (SAS Institute, Cary, NC) to identify any significant effect due to contamination. Means of each treatment levels were compared to the control (sweetgum wood) with the Dunnett's control test in JMP 9.0 (SAS Institute, Cary, NC). Significance was established for $P < 0.05$.

5. RESULTS AND DISCUSSION

5.1. Hydrolysis of Pure Sweetgum Wood

In order to design a hydrolysis scheme for sweetgum wood, the effects of the pretreatment time and temperature on glucose and xylose yields obtained from dilute acid pretreatment and enzymatic hydrolysis of sweetgum wood were investigated. Pretreatment was conducted at 140°C for 30, 40, 50, 60, and 70 min, and at 160°C for 30, 40, 50, and 60 min.

5.1.1. Dilute Acid Pretreatment

Figure 10 presents the yields of xylose and glucose recovered in sweetgum wood prehydrolysates and wash waters using various pretreatment times at 140°C and 160°C. Although the two liquid streams were analyzed separately, their carbohydrate contents were combined to calculate xylose and glucose yields as percentages of the theoretical amount in the dried biomass.

Xylose was the primary sugar recovered in the prehydrolysates and wash waters, indicating high hydrolysis of the hemicellulosic fraction of wood during pretreatment. At 140°C (as shown in Figure 10A), xylose yield modestly increased with pretreatment time up to a maximum value of 79% after 60 min. Conversely, at 160°C (Figure 10B) hemicellulose hydrolysis released its maximum, 71%, within 40 min of pretreatment, at which time xylose yields decreased.

Glucose also was detected in prehydrolysates and wash waters. Pretreatment time did not affect glucose recovery at 140°C, with less than 5% of the glucose recovered. However, at 160°C glucose yields increased with pretreatment time. Pretreatment at lower temperatures is ideal in achieving a high xylose recovery. More elevated temperatures, especially for prolonged periods of time, will result in considerable loss of xylose and premature hydrolysis of the cellulosic

fraction, which can result in glucose degradation. These findings are in agreement with studies performed on other feedstock with dilute acid pretreatment (Cara *et al*., 2008; Lloyd and Wyman, 2005; Torget *et al*., 1990).
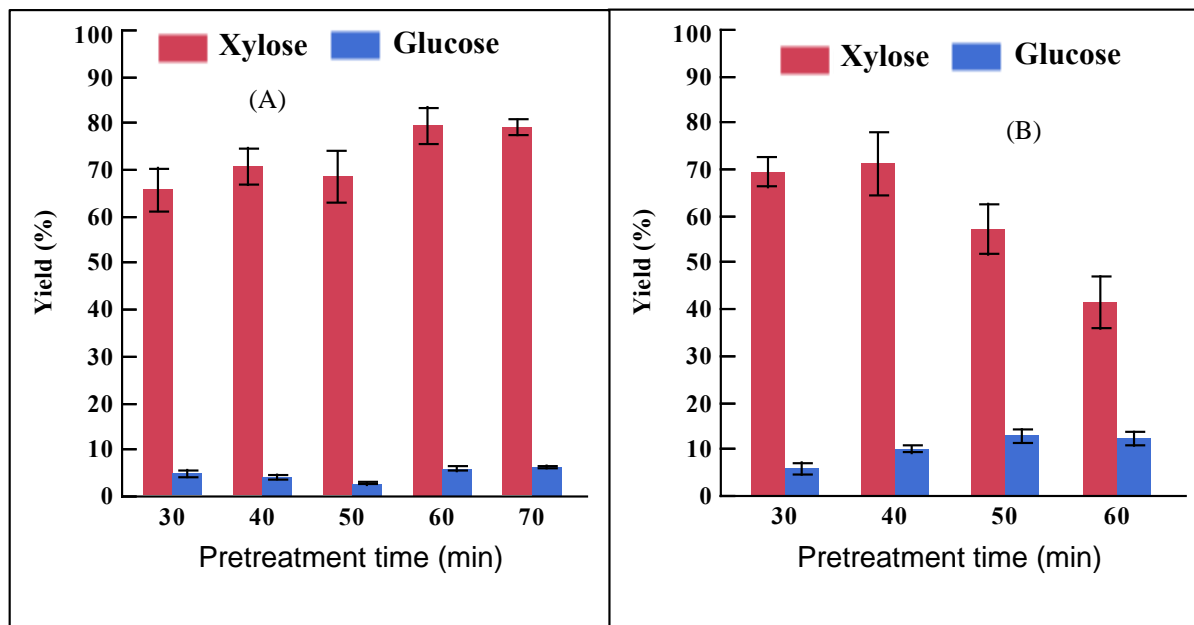
Figure 10. Prehydrolysate of sweetgum wood: xylose and glucose yields. Pretreatment occurred at (A): 140°C and (B): 160°C with 0.98% (v/v) H_2SO_4. The yields represent the amount of xylose and glucose recovered as a percentage of the theoretical amount in the raw biomass. Error bars standard error of 3 replications

An inherent and undesirable property of dilute acid pretreatment is the production of sugar and lignin degradation compounds which are inhibitory to enzymatic hydrolysis and detrimental to microorganisms used in sugar fermentation (Palmqvist and Hahn-Hagerdal, 2000). Furfural, HMF, formic acid and acetic acid were detected in prehydrolysates and wash waters from wood pretreatment (Table 2). Furfural and HMF result from xylose and glucose degradation, respectively, and both can further degrade into formic acid; acetic acid is released from acetyl groups of hemicellulose polymers (Palmqvist and Hahn-Hagerdal, 2000).

Concentrations (g/100 g of dried biomass) of degradation products increased with time and severity of pretreatment (Table 2). Sugar degradation was less severe at 140°C than at 160°C; the increase in degradation compounds, especially furfural and formic acid, at 160°C coincided with a decrease in xylose recovery. Even though there was a slight degradation of xylose at 140°C, xylose recovery did not decline with pretreatment time because, at lower temperature, the rate of xylan hydrolysis is higher than its degradation rate (Lloyd and Wyman, 2005).

Table 2

Degradation compounds (g/100g of natural biomass) produced from 0.98% (v/v) sulfuric acid pretreatment of sweetgum wood

Pretreatment conditions		Prehydrolysate				Wash water [a]			
Temperature (˚C)	Time (min)	Acetic Acid	Furfural	Formic Acid	HMF	Acetic Acid	Furfural	Formic Acid	HMF
140	30	4.6 ± 0.9	0.1 ± 0.0	1.8 ± 0.3	0.0 ± 0.0	2.5 ± 0.2	0.2 ± 0.1	0.8 ± 0.2	0.0 ± 0.0
140	40	2.9 ± 0.5	0.2 ± 0.3	1.8 ± 0.6	0.0 ± 0.0	2.7 ± 0.3	0.4 ± 0.2	0.9 ± 0.3	0.0 ± 0.0
140	50	2.9 ± 0.2	0.2 ± 0.0	1.8 ± 0.2	0.0 ± 0.0	3.1 ± 0.3	0.1 ± 0.0	0.9 ± 0.1	0.0 ± 0.0
140	60	3.6 ± 0.3	0.3 ± 0.6	3.4 ± 1.2	0.0 ± 0.0	2.5 ± 0.5	0.5 ± 0.3	1.5 ± 0.8	0.0 ± 0.0
140	70	3.7 ± 0.1	0.3 ± 0.0	3.6 ± 0.2	0.0 ± 0.0	2.7 ± 0.3	0.2 ± 0.0	3.0 ± 0.3	0.0 ± 0.0
160	30	4.8 ± 2.0	0.7 ± 0.2	8.1 ± 2.5	0.1 ± 0.0	3.2 ± 0.4	0.6 ± 0.1	5.4 ± 1.6	0.0 ± 0.0
160	40	4.3 ± 0.1	1.2 ± 0.1	10.4 ± 0.8	0.1 ± 0.0	2.7 ± 0.1	0.7 ± 0.1	4.8 ± 0.2	0.0 ± 0.0
160	50	5.0 ± 0.4	1.3 ± 0.1	10.6 ± 0.8	0.2 ± 0.1	3.2 ± 0.2	1.0 ± 0.1	5.6 ± 0.2	0.1 ± 0.0
160	60	3.7 ± 0.7	1.6 ± 0.4	6.6 ± 1.3	0.2 ± 0.1	3.7 ± 0.6	1.8 ± 0.4	5.5 ± 1.5	0.2 ± 0.1

Data are means ± standard error of three replications

[a] Water used for washing pretreated biomass

5.1.2. Enzymatic Hydrolysis

The effects of pretreatment time on xylose and glucose yields from enzymatic hydrolysis of sweetgum wood pretreated at 140°C and 160°C are depicted in Figure 11. Xylose and glucose yields were calculated as percentages of the theoretical amount in the dried biomass and should be differentiated from cellulose digestibility reported by Torget *et al.* (1990). As expected, most of the glucose was solubilized during enzymatic hydrolysis for both pretreatment temperatures; however, biomass pretreated at 140°C (Figure 11A) was less responsive to enzymatic attack than the one pretreated at 160°C (Figure 11B), shown here by a higher glucose recovery at 160°C than at 140°C. Although most of xylose present in sweetgum wood was solubilized during pretreatment at 140°C, complete removal of the hemicellulose during pretreatment did not translate to higher digestibility. It is possible that performing enzymatic hydrolysis for more than 24 h could improve glucose yields; however, our results showed that glucose yields increased only 10% after 48 h of enzymatic hydrolysis. Moreover, 24 h was the time recommended by the enzyme manufacturer for maximum activity of the enzyme.

Seventy four percent of glucose was recovered in enzymatic hydrolysates of biomass pretreated at 160°C (Figure 11B) and better digestibility of pretreated sweetgum wood was observed with increasing pretreatment times. Obtaining more digestible material from pretreatment conducted at harsher conditions has previously been reported (Foston and Ragauskas, 2010); hydrolysis of the amorphous section of the cellulose, observed in this work, resulted in higher glucose concentrations during prolonged pretreatment at 160°C. Kabel et al. (2007) attributed the relationship between high temperature and cellulose degradability to the disruption of lignin structures during pretreatment; however, lignin structures in natural and pretreated sweetgum wood were not analyzed in our work.
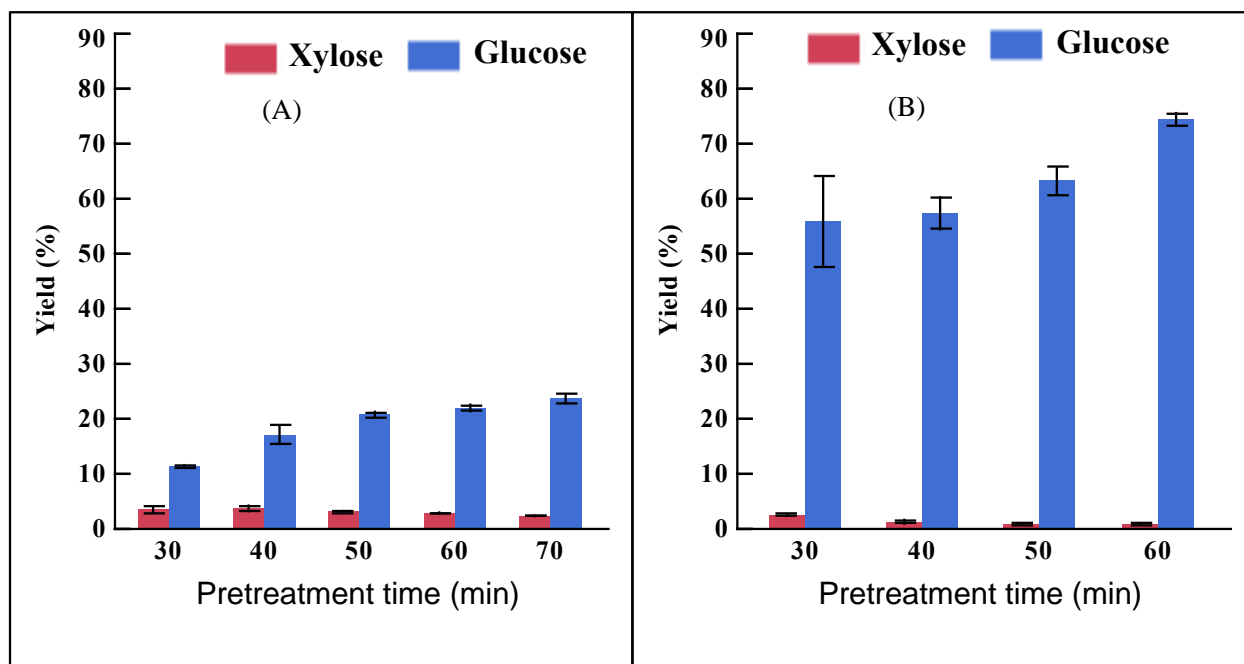
Figure 11: Enzymatic hydrolysate of pretreated sweetgum wood: xylose and glucose yields. Pretreatment occurred at (A): 140°C and (B): 160°C with 0.98% (v/v) H_2SO_4. The yields represent the amount of xylose and glucose recovered as a percentage of the theoretical amount in the raw biomass. Error bars are standard error of 3 replications.

5.1.3. Overall Yields

The dilemma between maximizing xylose recovery during pretreatment and producing highly digestible cellulosic material occurred because the conditions for maximum xylose recovery did not correspond to conditions for maximum glucose recovery. Similar results have been observed (Lloyd and Wyman, 2005). One solution to this problem could be to maximize total fermentable sugars yields (TFS) (TFS = xylose + glucose) from pretreatment and enzymatic hydrolysis of the biomass, as reported by Lloyd and Wyman (2005). Yields of xylose, glucose and TFS expressed as percentages of theoretical amounts present in dried wood (sugar yields) or as amount of sugars (g) produced from 100 g of dried biomass (raw biomass yields) are depicted in Table 3. In general, at 140°C xylose, glucose, and TFS yields modestly increased with pretreatment time. Up to 47% of TFS were recovered after 70 min of pretreatment; these pretreatment conditions yielded maximum xylose recovery of 82%. Any sugar cocktail (xylose + glucose) obtained at 140°C contained low amount of glucose and, for a fermentation process; this is not the ideal sugar stream. Pretreatment at 160°C yielded a maximum TFS of 72% after 60 min of pretreatment; these pretreatment conditions also gave maximum glucose recovery of 86%. At 160°C, an increase in pretreatment time did not have an effect on TFS yields; however, the sugar stream obtained at times before 40 min had a higher percentage of xylose than streams obtained after 40 min, which had a higher percentage of glucose. This occurred because the xylose concentration in the sugar stream decreased while the glucose concentration increased with pretreatment time.

Table 3

Sugars produced from 0.98% (v/v) sulfuric acid pretreatment and enzymatic hydrolysis of sweetgum wood

Pretreatment conditions		Sugar yields[a]			Raw biomass yields[b]		
Temperature (˚C)	Time (min)	Xylose	Glucose	TFS[c]	Xylose	Glucose	TFS[c]
140	30	68.9 ± 5.1	13.4 ± 3.3	31.5 ± 3.3	15.6 ± 1.1	6.3 ± 2.1	21.9 ± 2.3
140	40	74.1 ± 4.4	20.8 ± 2.1	38.2 ± 2.7	16.8 ± 1.0	9.8 ± 1.0	26.5 ± 1.9
140	50	71.3 ± 5.4	23.1 ± 0.7	38.8 ± 2.2	16.1 ± 1.2	10.8 ± 0.3	27.0 ± 1.5
140	60	82.1 ± 3.9	27.4 ± 1.0	45.2 ± 1.8	19.9 ± 1.4	15.8 ± 3.2	35.7 ± 4.2
140	70	82.0 ± 1.8	30.4 ± 1.2	47.2 ± 0.7	18.6 ± 0.4	14.3 ± 0.5	32.8 ± 0.5
160	30	71.4 ± 3.2	55.0 ± 3.2	64.6 ± 5.0	16.2 ± 0.7	28.7 ± 3.8	44.9 ± 3.5
160	40	72.1 ± 7.0	66.8 ± 2.3	68.5 ± 0.8	16.3 ± 1.6	31.3 ± 1.1	47.7 ± 0.6
160	50	54.0 ± 8.1	74.8 ± 2.3	68.1 ± 1.1	13.1 ± 1.2	35.1 ± 1.1	47.3 ± 0.8
160	60	41.9 ± 5.6	86.2 ± 0.6	71.8 ± 2.2	9.5 ± 1.3	40.4 ± 0.3	49.9 ± 1.5

Data are means ± standard error of three replications

[a] Percentage of theoretical yield.

[b] Yields in g/100 g of natural material.

[c] Total fermentable sugars

5.2. Hydrolysis of Pure Sweetgum Bark

The possibility to use sweetgum bark as feedstock for a biorefinery was also assessed. Tree bark usually is not considered an ideal candidate, mainly because it is not a substantial source of carbohydrate when compared to tree wood. The sweetgum bark used for this study contained on a dry basis 21.2% glucan and 8.56% xylan (Table 1). In addition, bark biomass contains extractives than can potentially interfere with enzymatic hydrolysis or fermentation. However, using the whole tree would simplify supply chain processing and increase the amount of carbohydrate available per tree harvested. Even though bark should be integrated in the biomass conversion process, wood will dictate the process parameters; therefore, sweetgum bark in this study was submitted to the same pretreatment and enzymatic hydrolysis conditions as for sweetgum wood.

5.2.1 Dilute Acid Pretreatment

The effects of pretreatment time on xylose and glucose yields from prehydrolysates and wash waters of bark pretreated at 140°C and 160°C are shown in Figure 12. Sugar recovery from sweetgum bark pretreatment did not follow the same trend as for sweetgum wood pretreatment. Xylose loss occurred faster at 140°C (Figure 12A) than at 160°C (Figure 12B). More xylose was recovered at 160°C than at 140°C. These results were in contrast to results obtained for sweetgum wood because harsher pretreatment conditions of the wood yielded lower xylose recovery. The significant difference between the response of the bark and the wood to pretreatment could be attributed to the significant difference between their respective compositions.
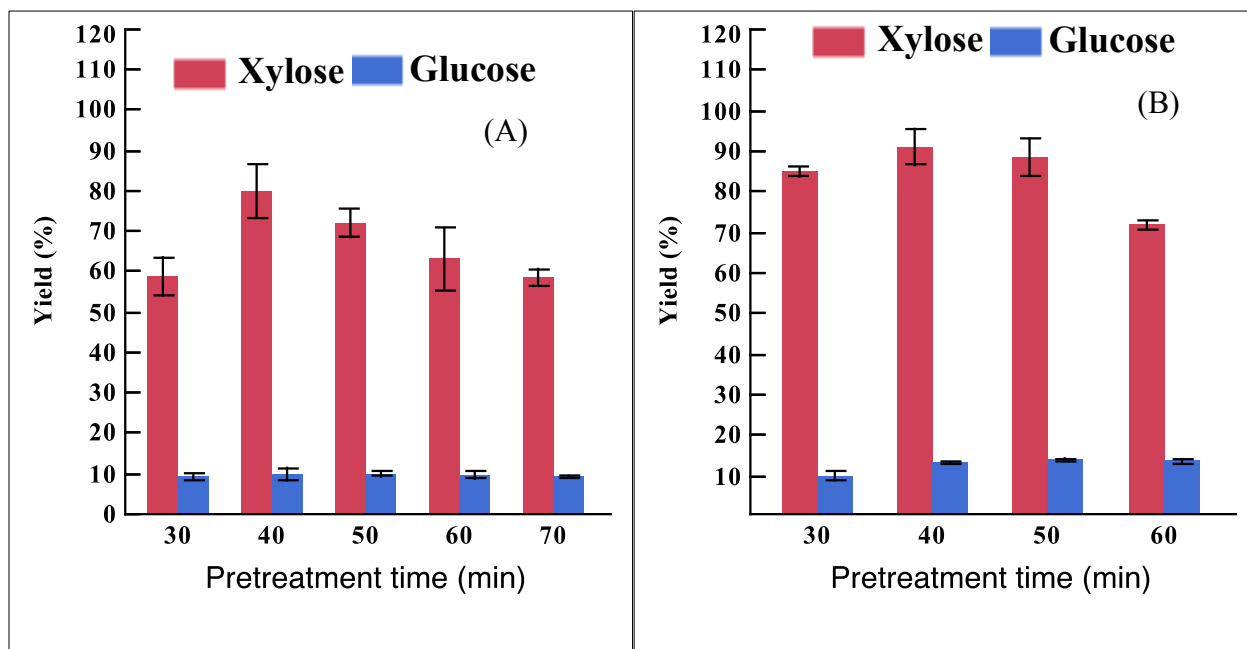
Figure 12. Prehydrolysate of sweetgum bark: xylose and glucose yields. Pretreatment occurred at (A): 140°C and (B): 160°C with 0.98% (v/v) H_2SO_4. The yields represent the amount of xylose and glucose recovered as a percentage of the theoretical amount in the raw biomass. Error bars are standard error of 3 replications

Furfural, HMF, formic acid and acetic acid were present in the prehydrolysate and wash water from the bark pretreatment (Table 4). Concentrations of these by-products in pretreatment liquid streams were lower at 140°C than at 160°C. It was expected that concentration of furfural and formic acid would be higher at 140°C than at 160°C given that a higher loss of xylose occurred at 140°C. Concentrations of formic acid in the bark prehydrolysate and wash water, especially at 160°C, were over 11 g per 100 g of natural biomass. When combining formic acid recovery in the prehydrolysate and wash water obtained from pretreatment at 160°C for 40 min, formic acid yield was 43% of the dried biomass. Thus, it is more likely that for sweetgum bark, reactions other than sugar degradation could be responsible for xylose loss and formation of formic acid during pretreatment. High extractive content of bark could be the origin of such elevated amount of formic acid in the prehydrolysate. The presence of those inhibitory compounds at such elevated concentrations in the pretreatment liquid streams could be one reason why bark is not an ideal candidate as a feedstock for a biorefinery.

Table 4
Degradation compounds (g/100g of natural biomass) produced from 0.98% (v/v) sulfuric acid pretreatment of sweetgum bark

Pretreatment conditions		Prehydrolysate				Wash water[a]			
Temperature (˚C)	Time (min)	Acetic Acid	Furfural	Formic Acid	HMF	Acetic Acid	Furfural	Formic Acid	HMF
140	30	1.4 ± 0.2	0.0 ± 0.0	16.1 ± 0.4	0.0 ± 0.0	1.6 ± 0.1	0.0 ± 0.0	4.8 ± 1.1	0.0 ± 0.0
140	40	3.0 ± 1.2	0.0 ± 0.0	19.7 ± 2.2	0.0 ± 0.0	1.5 ± 0.1	0.0 ± 0.0	3.9 ± 1.5	0.0 ± 0.0
140	50	1.6 ± 0.4	0.1 ± 0.0	14.5 ± 3.9	0.0 ± 0.0	2.0 ± 0.3	0.1 ± 0.0	7.6 ± 3.4	0.0 ± 0.0
140	60	1.5 ± 0.2	0.1 ± 0.0	12.5 ± 2.1	0.0 ± 0.0	2.0 ± 0.4	0.1 ± 0.0	7.7 ± 3.0	0.0 ± 0.0
140	70	1.1 ± 0.0	0.1 ± 0.0	9.0 ± 0.2	0.0 ± 0.0	2.6 ± 0.1	0.2 ± 0.0	11.9 ± 1.1	0.0 ± 0.0
160	30	3.9 ± 1.2	0.1 ± 0.1	21.6 ± 2.9	0.1 ± 0.0	2.6 ± 0.3	0.2 ± 0.0	14.7 ± 3.3	0.0 ± 0.0
160	40	7.9 ± 2.8	0.4 ± 0.0	32.3 ± 6.4	0.1 ± 0.0	2.0 ± 0.4	0.2 ± 0.0	11.2 ± 4.2	0.0 ± 0.0
160	50	6.3 ± 1.7	0.5 ± 0.0	26.9 ± 1.3	0.1 ± 0.0	2.2 ± 0.3	0.3 ± 0.0	10.8 ± 1.7	0.0 ± 0.0
160	60	2.6 ± 0.5	0.6 ± 0.2	16.7 ± 4.8	0.1 ± 0.0	2.5 ± 0.5	0.5 ± 0.1	10.8 ± 5.3	0.1 ± 0.0

Data are means ± standard error of three replications
[a] Water used for washing pretreated biomass

5.2.2. Enzymatic Hydrolysis

The enzymatic hydrolysis of sweetgum bark (Figure 13) was not as successful as for the hydrolysis of sweetgum wood. A maximum of 11% of glucose was recovered in bark enzymatic hydrolysate compared to 74% for wood. An increase in pretreatment time or temperature did not improve glucose yields.

This resistance to enzymatic attack after pretreatment has been reported to be inherent to sweetgum bark (Torget *et al*., 1991). Although hemicellulose was completely removed, only 22% of cellulose digestibility was observed for sweetgum bark pretreated at 160°C for up to 30 min. Torget *et al*. (1991) attributed sweetgum bark's resistance to enzymatic attack to its complex nature and to condensation of lignin in hot acid. Extractives, such as shikimic acid, reported by Martin *et al*. (2010) and high ash content could also contribute to sweetgum bark's recalcitrance. Moreover, Cantarella *et al*. (2004) showed that formic acid concentrations of 11.5 mg/mL inhibited the cellulose enzymatic cocktail; therefore, formic acid detected in bark prehydrolysates of our study could contribute to the recalcitrance observed in the bark. Insufficient washing of the pretreated pellet could exacerbate this recalcitrance. A better understanding of sweetgum bark structure and composition needs to be established to design optimum processing conditions to maximize saccharification of this feedstock system.
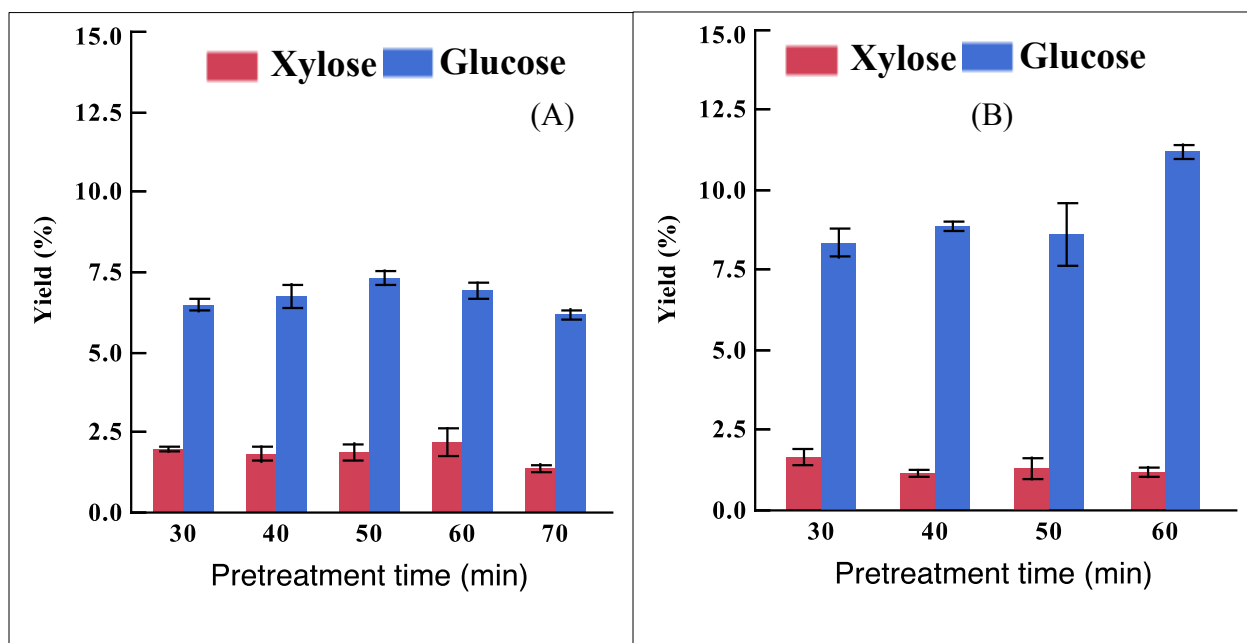
Figure 13. Enzymatic hydrolysate of pretreated sweetgum bark: xylose and glucose yields. Pretreatment occurred at (A): 140°C and (B): 160°C with 0.98% (v/v) H_2SO_4. The yields represent the amount of xylose and glucose recovered as a percentage of the theoretical amount in the raw biomass. Error bars are standard error of 3 replications.

Table 5 presents the sugar recoveries from pretreatment and enzymatic hydrolysis of sweetgum bark. Maximizing xylose or glucose yields in bark was possible because the recovery for both sugars occurred at 160°C. Moreover at 160°C, pretreatment time did not affect TFS or glucose yields; therefore, maximization of xylose recovery was the only factor affecting pretreatment conditions of sweetgum bark.

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Table 5
Sugars produced from 0.98% (v/v) sulfuric acid pretreatment and enzymatic hydrolysis of sweetgum bark

Pretreatment conditions		Sugar yields [a]			Raw biomass yields [b]		
Temperature (˚C)	Time (min)	Xylose	Glucose	TFS [c]	Xylose	Glucose	TFS [c]
140	30	60.0 ± 4.3	15.9 ± 1.2	29.5 ± 2.2	5.3 ± 0.4	3.2 ± 0.2	8.5 ± 0.6
140	40	81.4 ± 6.4	16.9 ± 1.4	36.7 ± 2.9	7.2 ± 0.6	3.4 ± 0.3	10.6 ± 0.8
140	50	73.6 ± 3.3	16.9 ± 0.5	34.3 ± 1.3	6.5 ± 0.3	3.4 ± 0.1	9.9 ± 0.4
140	60	65.3 ± 7.2	16.9 ± 1.3	31.8 ± 2.1	5.8 ± 0.6	3.4 ± 0.3	9.2 ± 0.6
140	70	60.2 ± 2.0	13.9 ± 0.3	28.1 ± 0.5	5.3 ± 0.2	2.8 ± 0.1	8.1 ± 0.1
160	30	88.2 ± 3.4	17.8 ± 1.0	40.3 ± 1.2	7.8 ± 0.3	3.6 ± 0.2	11.6 ± 0.4
160	40	93.5 ± 6.5	21.4 ± 1.0	44.5 ± 1.9	8.3 ± 0.6	4.3 ± 0.2	12.9 ± 0.6
160	50	91.8 ± 7.3	22.4 ± 1.4	44.9 ± 2.5	8.1 ± 0.6	4.4 ± 0.3	13.0 ± 0.7
160	60	72.7 ± 1.0	24.5 ± 0.7	39.3 ± 0.8	6.4 ± 0.1	4.9 ± 0.1	11.3 ± 0.2

Data are means ± standard error of three replications
[a] Percentage of theoretical yield
[b] Yields in g/100 g of natural biomass.
[c] Total fermentable sugars

5.3. Hydrolysis of Contaminated Sweetgum Wood

Previous hydrolysis study of pure sweetgum wood and bark showed that optimum hydrolysis condition of sweetgum wood, using our equipment and protocol, was 160°C for 60 min. The latter conditions yielded maximum TFS of 72%. Unfortunately, the sand bath, instrument used to conduct pretreatments, malfunctioned, and when repaired produced different fluidization conditions. With the repaired set-up, higher sugar degradation was observed when pretreating at 160°C for 60 min. Adjusting the pretreating conditions to 160°C, for 20 min limited sugar degradation and yielded 60% of TFS.

5.3.1. Hydrolysis of Pure Biomass

Table 1 summarizes the composition of sweetgum wood and bark, as well as oak wood and oak bark. Oak and sweetgum wood had similar composition; both barks were also alike in terms of composition. Both wood contained 45% glucan; bark contained only up to 21% glucan. More lignin, extractives, and ash were present in both barks as compared to both woods. Although bark biomass did not represent a considerable source of sugars, the effect of combining bark and woody biomass during pretreatment needed to be determined because of the potential to simplification of biomass handling process prior to hydrolysis.

Hydrolysis of pure sweetgum wood, sweetgum bark, oak wood, and oak bark was first investigated to determine the effect of pretreatment and enzymatic hydrolysis conditions on their respective xylose and glucose yields. Figure 14 presents xylose and glucose recoveries (left panel) and degradation compound productions (right panel) of all the biomass samples during pretreatment. Xylose and glucose are expressed as percentage of their respective theoretical yields (recovered/amount present in un-pretreated biomass); yields for acetic acid, furfural, formic acid, and HMF are expressed as g compound per 100 g natural biomass. Based on xylose

yields, pretreatment conditions were more suitable for both barks than for woods. Oak bark yielded the highest xylose, 80%, whereas oak wood yielded the lowest, 35%. Low xylose concentrations and elevated amounts of furfural determined in oak wood prehydrolysate showed that pretreatment conditions were particularly severe for oak wood. Xylose from sweetgum wood was 30% higher than from oak wood and this difference indicated that optimum conditions to maximize xylose yield for sweetgum wood might not be the best for oak wood. Furthermore, difference in xylose yields between bark and wood biomass ascertained the fact that pretreatment-induced hemicellulose hydrolysis was specific to the species on one hand, and to the plant part on the other hand.

Production of furfural and HMF were slightly lower in bark samples than in wood. However, both oak bark and sweetgum bark yielded higher formic acid contents than corresponding wood samples. Sweetgum bark yielded almost twice the amount of formic acid than that of oak bark, indicating that species affected its concentration. Results presented in the previous study of sweetgum bark pretreatment showed that sugar degradation was not the sole mechanism responsible for elevated formic acid concentrations detected in corresponding prehydrolysate. These results suggested that some other components such as extractives, may play a role in the production of high formic acid yields. Formic acid is known as a potent enzymatic hydrolysis and fermentation inhibitor (Panagiotou and Olsson, 2007; Palmqvist and Hahn-Hagerdal, 2000); because of this fact, it may be prudent to omit bark biomasses from pretreatment operations
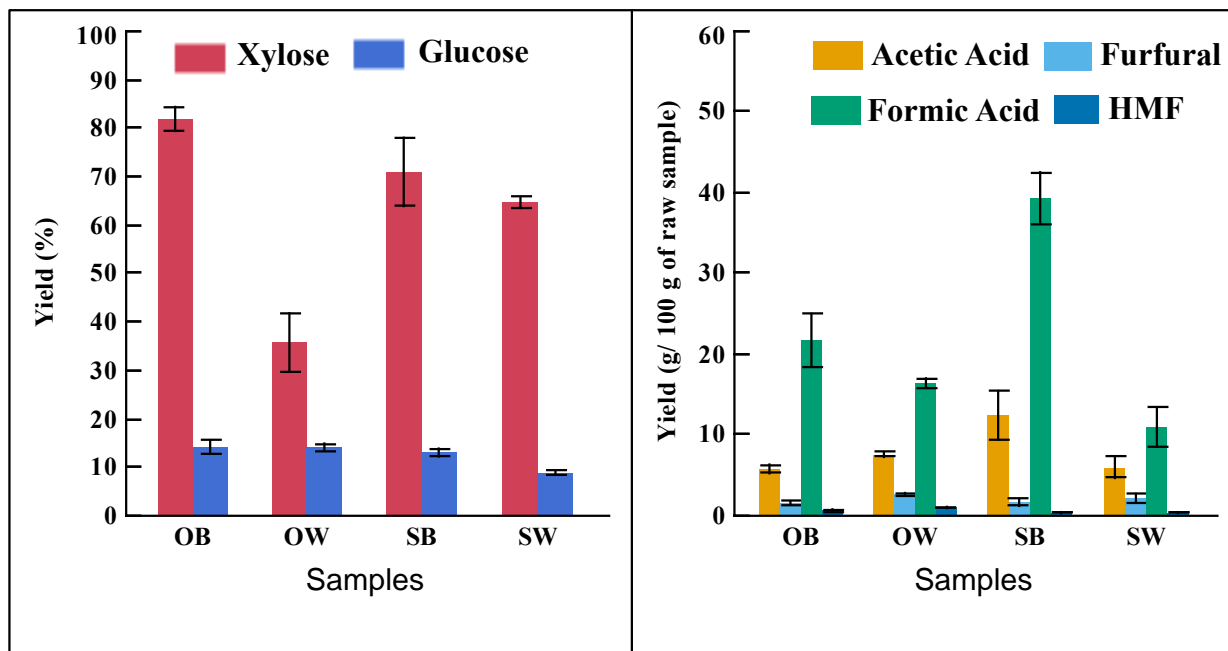
Figure 14. Recovery compounds from dilute acid pretreatment of lignocellulosic biomass samples. Pretreatment was done with 0.98%(v/v) H_2SO_4 at 160°C for 20 min. Samples are oak bark (OB), oak wood (OW), sweetgum bark (SB), and sweetgum wood (SW). Xylose and glucose yields are percentage of their respective theoretical amount in the raw sample. Error bars are standard error of 3 replications

Figure 15 presents oak bark, oak wood, sweetgum bark and sweetgum wood sugar yields obtained after subjecting pretreated samples to enzymatic hydrolysis. As for Figure 14, yields were expressed as percentages of theoretical yields (recovered/amount present in natural biomass). Only glucose was detected in all enzymatic hydrolysates, indicating complete hydrolysis of hemicellulose during pretreatment. Pretreated oak bark and sweetgum bark samples yielded only 10% glucose, suggesting that bark biomass was resistant to enzymatic hydrolysis. Data presented in the above study of sweetgum bark saccharification already highlighted its recalcitrance to enzyme. Lack of literature on oak bark hydrolysis limited our understanding of its low glucose yield. However, it is possible that similar mechanisms responsible for sweetgum bark's negative response to enzymatic hydrolysis also impeded oak bark's saccharification; Figure 16 shows that only 20% of cellulose in pretreated bark was actually converted to glucose.

Approximately 48% of glucose was recovered in both woody samples, as shown in Figure 15. For sweetgum wood, glucose yields, obtained by enzymatic hydrolysis, were significantly lower than previously determined values, of 74%. Cellulose digestibility studies of pretreated biomass, as shown in Figure 16, demonstrated that only 70% of the cellulose present in pretreated sweetgum wood was converted to glucose. As for oak wood, 92% of cellulose digestibility and 48% of glucose yield suggested that a major proportion of its cellulose was degraded during pretreatment.
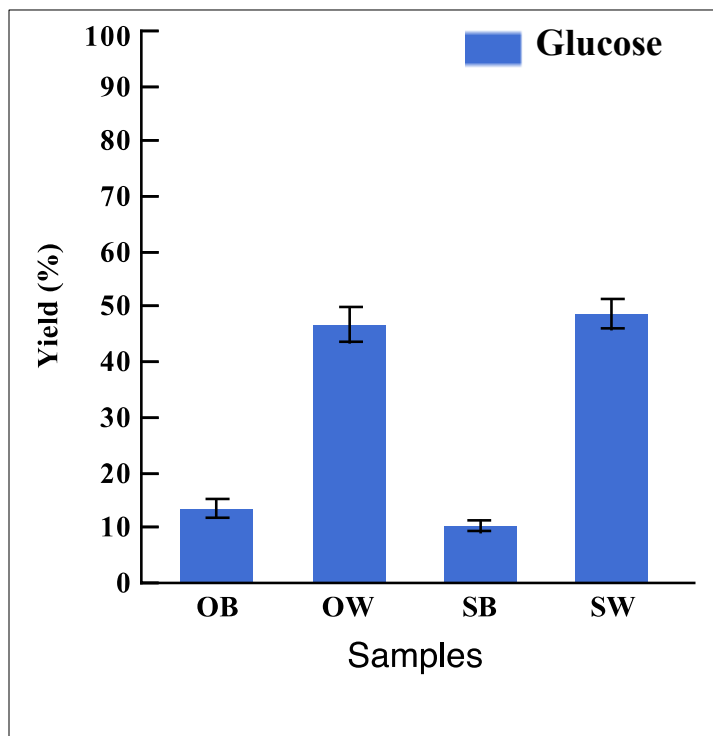
Figure 15: Sugar recovery from enzymatic hydrolysis of pretreated lignocellulosic biomass samples with 0.98%(v/v) H_2SO_4 at 160°C for 20 min. Samples are oak bark (OB), oak wood (OW), sweetgum bark (SB), and sweetgum wood (SW). Glucose yield is a percentage of its theoretical amount in the raw sample. Error bars are standard error of 3 replications.
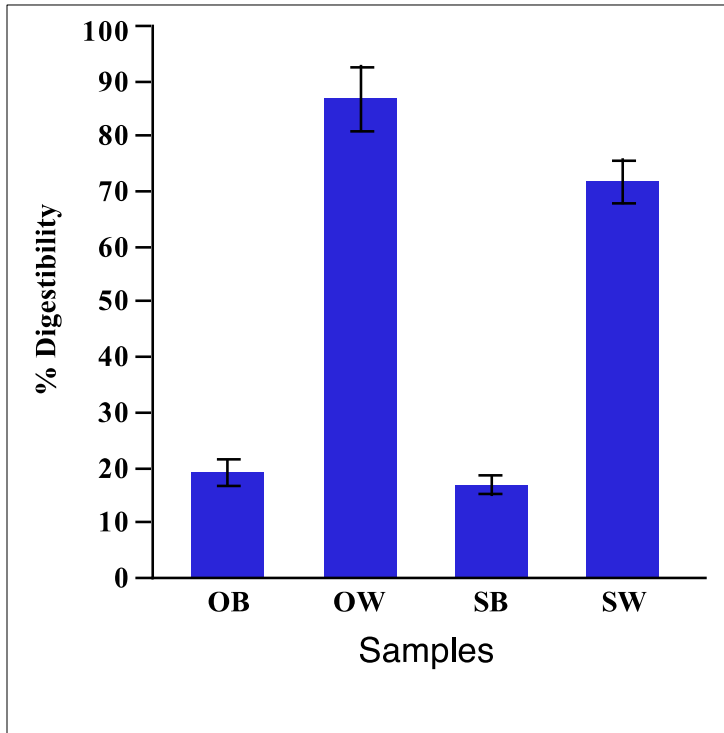
Figure 16. Cellulose digestibility of dilute sulfuric acid pretreated biomass. Acid concentration was 0.98% v/v, pretreatment temperature = 160°C and time = 20 min.

Combined xylose, glucose, and TFS yields from dilute acid pretreatment and enzymatic

hydrolysis of bark samples are illustrated in Figure 17. In summary, both barks yielded higher

xylose concentrations than their respective woods, while both woods yielded higher glucose and

TFS amounts.
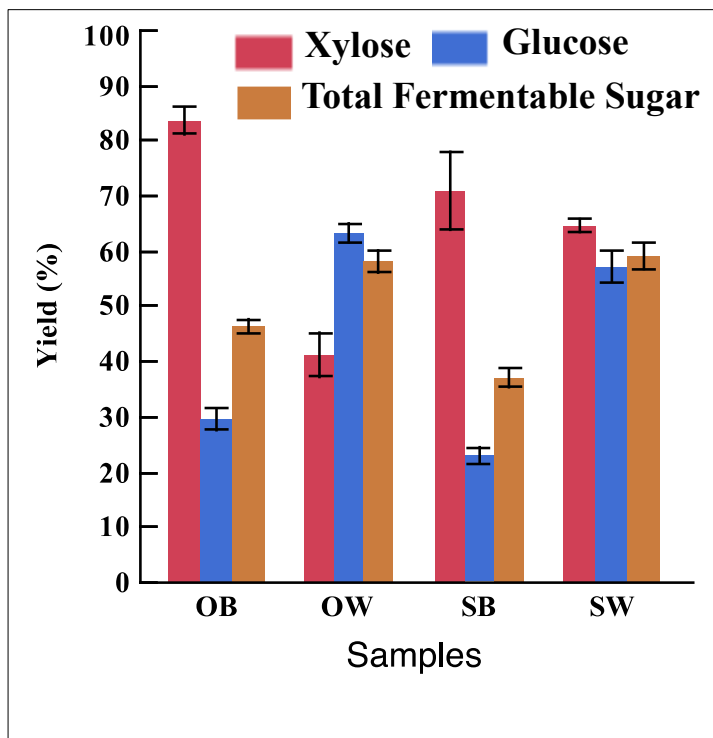
Figure 17. Sugar recovery from dilute acid
pretreatment and enzymatic hydrolysis of
lignocellulosic biomass samples. Pretreatment was
done with 0.98%(v/v) H_2SO_4 at 160°C for 20 min.

Samples are oak bark (OB), oak wood (OW),
sweetgum bark (SB), and sweetgum wood (SW). Sugar
yields are percentage of their theoretical amount in the
raw samples. Error bars are standard error of 3
replications.

5.3.2. Hydrolysis of Mixed Biomass

In this this work, the mixed biomass samples were prepared in the following manner: 1) 70% sweetgum wood mixed with 30% oak bark 2) 70% sweetgum wood mixed with 30% oak wood; 3) 70% sweetgum wood mixed with 30% sweetgum bark. The control was 100% sweetgum wood. Sugar yields from all three mixed samples were compared to the ones from 100% sweetgum wood in order to determine whether or not the addition of oak bark, oak wood or sweetgum bark would affect ensuing sugar recovery from sweetgum wood. To calculate the sugar yields from the mixed biomass samples, two scenarios were considered. In scenario A, it was assumed that all sugar recovered in the hydrolysates were solely stemming from sugar hydrolysis present in the sweetgum wood fraction of the mixed biomass. For example, under scenario A, xylose yield was calculated as follow:

$$\frac{Xylose\ mass\ recovered}{\%\ Xylose\ in\ sweetgum\ wood * 0.7 * mixture\ mass} * 100$$

With scenario A, oak bark, oak wood, and sweetgum bark were considered as non-significant source of sugars. This scenario could overestimate sugar yield because the net amount of sugar recovered in the hydrolysates would actually result from the hydrolysis of the sugar in the sweetgum wood fraction, but also from the other fration in the mixture. Scenario B took into consideration the possibility of having the other fractions also contribute to net sugar recovery; an example of xylose yield calculated under scenario B is given by:

$$\frac{Xylose\ mass\ recovered}{(\%\ xylose\ in\ sweetgum\ wood * 0.7 + \%\ xylose\ in\ oak\ bark * 0.3) * mixture\ mass} * 100$$

Data presented in Figure 18 presents xylose and glucose yields, from pretreatment, calculated according to scenario A (Figure 18A) and scenario B (Figure 18B), respectively. Data bars with a star (*) represent samples significantly different (P < 0.05) than the control. Results in Figure 18A showed that addition of oak bark and sweetgum bark significantly increased

xylose yield from sweetgum wood. Pure sweetgum wood yielded 52% xylose of theoretical available xylose; the addition of oak bark and sweetgum bark resulted in 80 and 90% xylose recoveries, respectively. In Figure 18B the addition of sweetgum bark enabled the highest xylose recovery, 72%, which was significantly different than that of 52% obtained for the control made up solely of sweetgum wood. These results indicated that sweetgum bark possibly affected hemicellulose hydrolysis of the mixture during pretreatment by preventing xylose degradation. As shown in Figure 19, furfural yields were 2.07 and 1.44 g per 100 g of dried sample for 100% sweetgum wood and combination of sweetgum wood and bark, respectively. These results may be useful; they suggests that debarking the tree prior to hydrolysis operations may not be necessary. However, a caveat must be placed. In all the work performed in this thesis, the pretreated biomass was rinsed with at least thirty times volumes of water prior to enzymatic hydrolysis. In the interest of water usage minimization, the rinsing step may not be possible at the deployment scale; in that case, the use of bark would not be recommended.
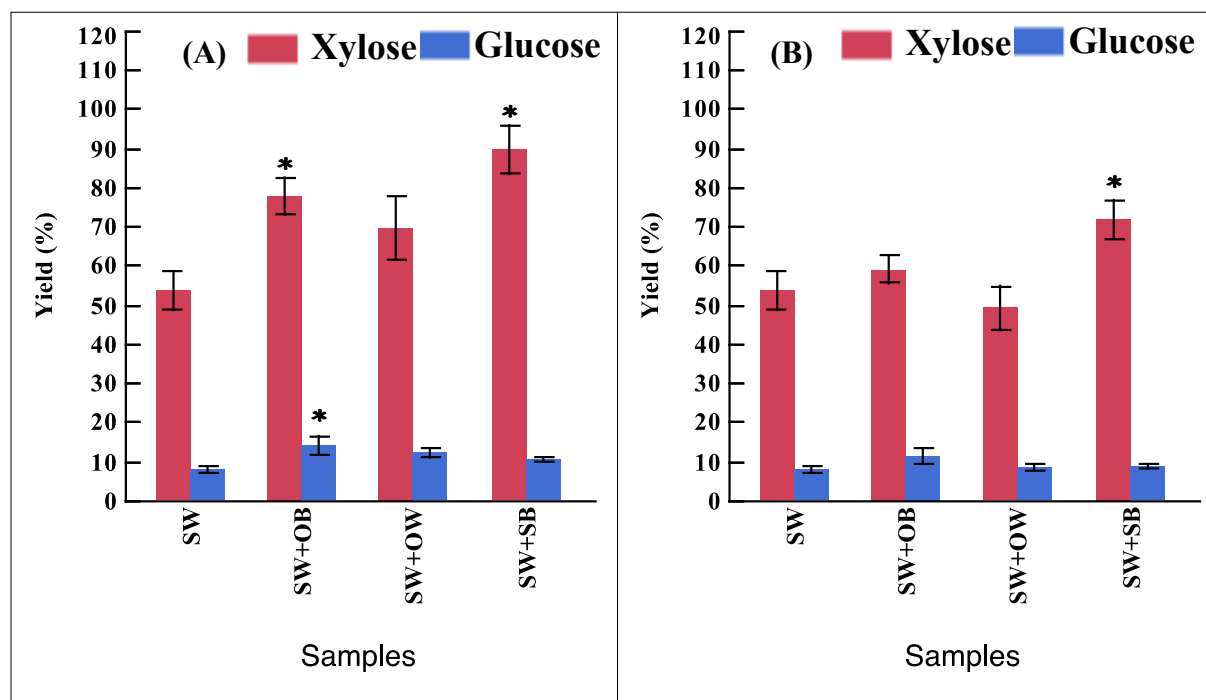
Figure 18: Sugar recovery from dilute acid pretreatment of lignocellulosic biomass samples. Pretreatment was done with 0.98%(v/v) H_2SO_4 at 160°C for 20 min. Samples consist of 100% sweetgum wood (SW) or 70% SW + 30% contaminant. Contaminants include oak bark (OB), oak wood (OW), or sweetgum bark (SB). Xylose and glucose yields are percentage of their respective theoretical amount in (A): sweetgum wood fraction and (B): entire sample. Error bars are standard error of 3 replications. *Samples are significantly different from the control (SW).
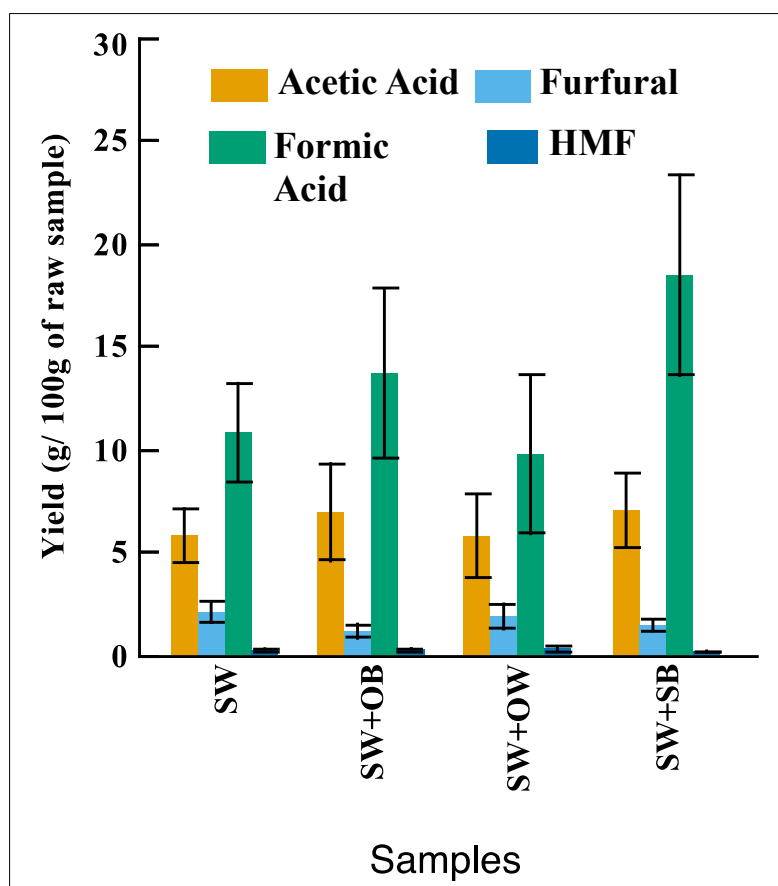
Figure 19. Degradation products from dilute acid
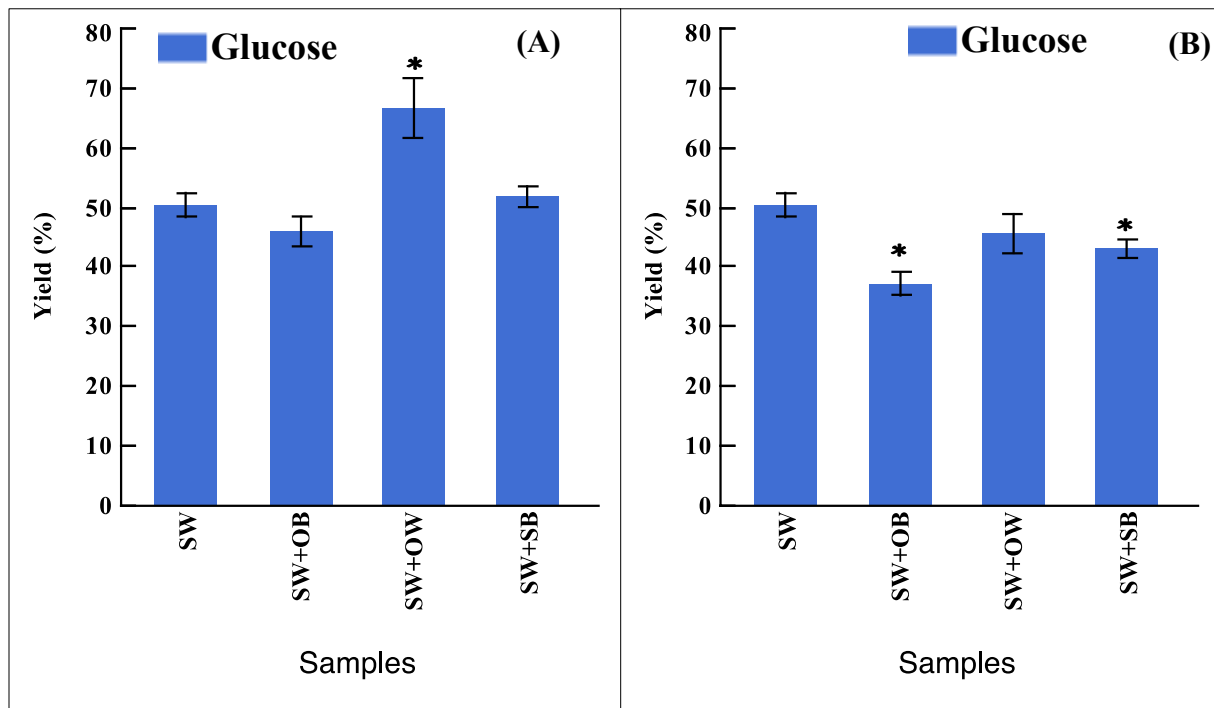pretreatment of lignocellulosic biomass samples.
Pretreatment was done with 0.98%(v/v) H_2SO_4 at 160°C
for 20 min. Samples consist of 100% sweetgum wood
(SW) or 70% SW + 30% contaminant. Contaminants
include oak bark (OB), oak wood (OW), or sweetgum
bark (SB). Error bars are standard error of 3 replications.

Oak wood did not significantly influence xylose yields (P > 0.05). Although oak is a good source of xylose, as shown in Table 1, most of its five-carbon sugar was degraded during pretreatment using the tested conditions, 160°C for 20 min; therefore the addition of oak wood did not significantly increase xylose yields. Figure 20A shows that the combination of sweetgum and oak woods afforded the highest glucose yields, 68%, during enzymatic hydrolysis. The difference between glucose yield from sweetgum-oak-wood combination and the control indicated that oak wood significantly increased glucose concentrations in enzymatic hydrolysate (Figure 20A). Conversely, addition of oak bark or sweetgum bark did not increase glucose yields stemming from enzymatic hydrolysis. Interestingly, the protective mechanisms that prevent cellulose hydrolysis of bark did not inhibit cellulose hydrolysis from sweetgum wood mixed with sweetgum or oak bark (Figure 20A).

Figure 20. Sugar recovery from enzymatic hydrolysis of pretreated lignocellulosic biomass samples with 0.98%(v/v) H_2SO_4 at 160°C for 20 min. Samples consist of 100% sweetgum wood (SW) or 70% SW + 30% contaminant. Contaminants include oak bark (OB), oak wood (OW), or sweetgum bark (SB). Glucose yield is a percentage of its theoretical amount in (A): sweetgum wood fraction and (B): entire sample. Error bars are standard error of 3 replications. *Samples are significantly different from the control (SW).

Another approach in analyzing the presented data was to predict sugar yields from mixtures, based on mixture fractions. Predicted sugar yields were calculated by adding weighted yields for pure sweetgum wood with weighted yields for pure oak wood, oak bark, or sweetgum bark. Experimental yields were obtained by adding mixture yields, calculated with scenario B, from pretreatment and enzymatic hydrolysis.

Comparison between predicted and experimental yield is shown in Figure 21. In general, the absolute value of the difference between the predicted and experimental yields was less than 11% of the experimental yield. Experimental xylose yield from sweetgum wood-bark combination was about 20% significantly higher than predicted value; this difference was basically due to the fact that sweetgum bark prevented the xylose degradation of sweetgum wood. The synergistic effect of sweetgum bark could not be captured with the predicting model. The trend observed in Figure 21 showed that, for the most part, studying the hydrolysis of pure biomass species present in a mixture could be sufficient to determine the amount of sugar that would be recovered from the hydrolysis of the mixture. Jensen *et al.* (2008) reached similar conclusion with the hydrolysis of hemicellulose from softwood, hardwood, and switchgrass mixtures during dilute acid pretreatment. The results presented by Jensen *et al.* (2008) did not extend to enzymatic hydrolysis nor did they include any bark biomass.
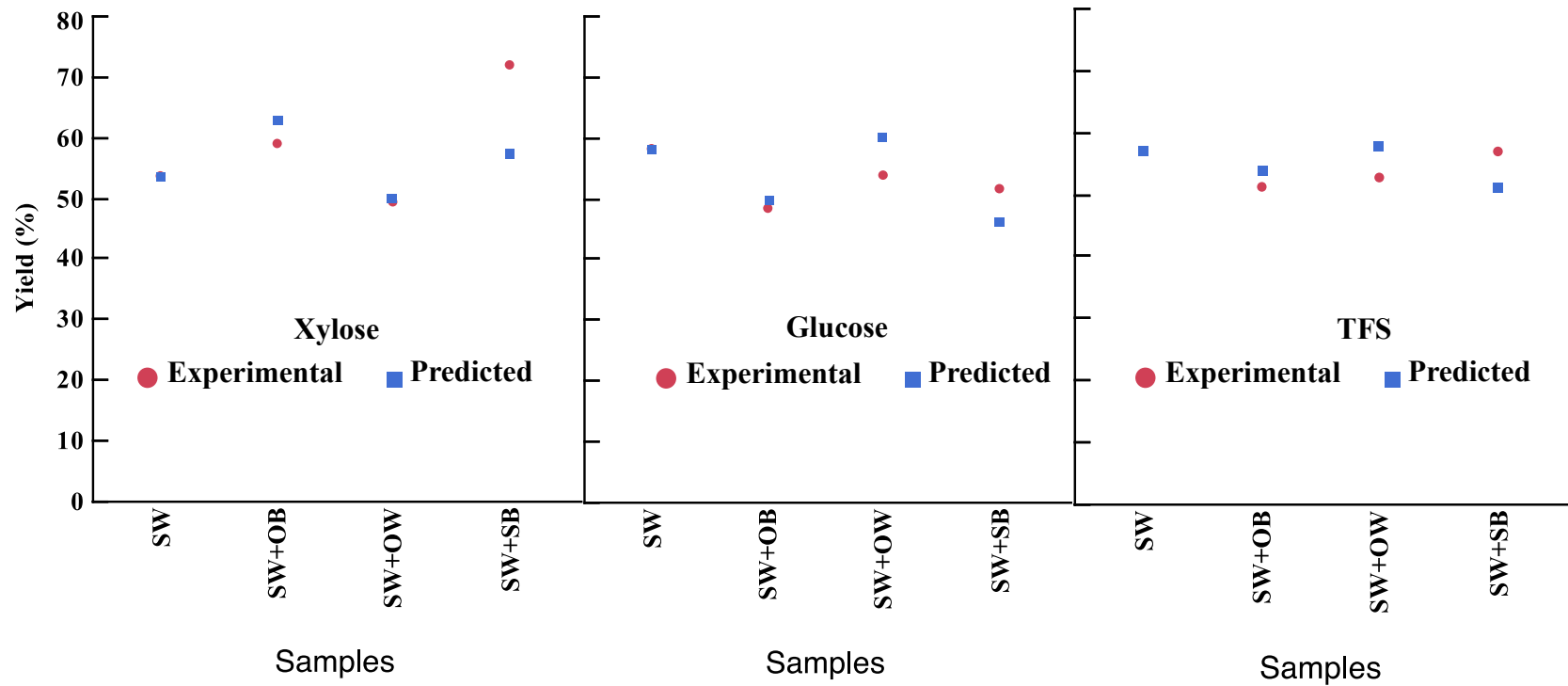
Figure 21. Comparing experimental and predicted sugar recovery data from dilute acid pretreated and enzymatic hydrolysis of lignocellulosic biomass samples. Pretreatment was done with 0.98%(v/v) H_2SO_4 at 160°C for 20 min. Samples consist of 100% sweetgum wood (SW) or 70% SW + 30% contaminant. Contaminants include oak bark (OB), oak wood (OW), or sweetgum bark (SB). Error bars are standard error of tree replications.

6. CONCLUSION

The possibility to use sweetgum wood from southern pine-forests as a feedstock in a biochemical-based biorefinery was investigated. High xylose and glucose yields were obtained from hydrolysis of 100% sweetgum wood. However, it was not possible to optimize pretreatment conditions to attain simultaneously maximum xylose and glucose yields. Therefore, maximizing total fermentable sugars with higher glucose content was a better approach to design an optimum hydrolysis scheme of 100% sweetgum wood for ethanol production. The best pretreatment conditions were 160°C for 60 min with 0.98% (v/v) sulfuric acid.

Hydrolysis of sweetgum wood contaminated with sweetgum bark, oak wood, and oak bark was also investigated. This work was actually the first to investigate the hydrolysis of oak bark into fermentable sugars. Contamination of sweetgum wood did not suppress its hydrolysis; a tendency of sweetgum bark to prevent xylose degradation during pretreatment was also observed; and it was possible to predict sugar yield from contaminated biomass by studying the hydrolysis of each biomass in the mixture. However, the excessive amount of formic acid produced by both bark during pretreatment could prevent the utilization of bark biomass because it would require intensive washing of pretreated biomass and detoxification of prehydrolysate before saccharification and fermentation in order to remove the formic acid. In sum, sweetgum wood from pine understory could be a good feedstock for a biorefinery however removal of the bark could be necessary to avoid additional unit operations.

Future work should investigate the contamination effects on the fermentation of released sugars from sweetgum wood hydrolysis. Some effort could also be done to determine the contamination effects on the amount of water needed to wash the pretreated biomass before saccharification.

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