An Abstract of the Thesis of

John J. Fenske for the degree of Master of Science in Food Science and Technology presented on June 17, 1994.

Title: Quantitative Polysaccharide Analysis of Lignocellulosic Biomass

Abstract approved: _______________________________

Michael H. Penner

Lignocellulosic biomass is a potential source of fermentable sugars such as glucose. Enzymatic hydrolysis of cellulose is a viable method of solubilizing the glucose from biomass, but the cellulose fraction of native lignocellulosic material is shielded from enzymatic attack by the lignin-hemicellulose matrix surrounding it. Pretreating lignocellulosic biomass with dilute sulfuric acid at high temperatures solubilizes hemicellulose, rendering the cellulose fraction more susceptible to enzymatic hydrolysis. Evaluation of dilute-acid, high-temperature pretreatments depends on polysaccharide analysis of the two fractions resulting from a pretreatment, prehydrolyzed solids(PHS) and prehydrolyzate liquid(PH). The polysaccharide analysis is based on a method described by the National Renewable Energy Laboratory and involves a two-stage sulfuric acid hydrolysis followed by HPLC quantification using ion-moderated partition chromatography and refractive index detection. The subject of this thesis is identifying and quantifying the sources of error associated with the polysaccharide analysis and the error associated with the evaluation of the effects of pretreatment on the polysaccharide fractions of switchgrass and poplar. This was addressed by conducting replicate polysaccharide analyses on single samples of native biomass, PHS, and PH. The variability associated with these measurements was compared to the variability associated with replicate analyses of identically pretreated biomass. It was found that the use of sugar standards to

correct for sugar destroyed during the analysis adds error and most likely overestimates the amount of sugar from biomass actually destroyed. It is evident that assuming a volume after neutralization of the hydrolyzed biomass sample is more reproducible than measuring the volume. When using a batch-type reactor and the temperature and acid parameters used in this study,140°C-180°C, 0.6-1.2 % sulfuric acid (w/w), it is evident that the major source of error in evaluating pretreatment conditions is the pretreatment itself, not the analysis.

Quantitative Polysaccharide Analysis of Lignocellulosic Biomass

by
John J. Fenske

A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Completed June 17, 1994
Commencement June, 1995

APPROVED:

Associate Professor of Food Science and Technology, in charge of major

---

Department Head, Food Science and Technology

Dean of Graduate School

Date thesis is presented: June 17,1994

Typed by John J. Fenske

Table of Contents

Introduction	1
Literature Review	4
Methods	14
Pretreatment Protocol	14
Analytical Protocol	15
Sugar Recovery after minimal exposure to acid	19
Glucose recovery after minimal exposure to acid with internal standard	19
Results and Discussion	21
Analytical sources of variability	21
Sensitivity of solids analysis	35
Sensitivity of prehydrolysate analysis	39
Reproducibility of pretreatment conditions	39
Results of pretreatment	43
Miscellaneous sources of error	53
Recommendations for future work	54
Bibliography	55

List of Figures

Figure	Title	Page
1	Random error associated with quantitative measurements of standard sugar solutions using HPLC	24
2	Weight percent xylan vs. original dry matter recovered in poplar PHS	48
3	Weight percent xylan vs. original dry matter recovered in switchgrass PHS	49
4	Weight percent xylan vs. percent of original dry matter recovered in prehydrolyzed switchgrass solids	50
5	Weight percent xylan vs. percent of original dry matter . recovered in prehydrolyzed poplar solids	51

List of Tables

Table	Title	Page
1	Percent recovery of sugars from mixtures after minimal exposure to 4% acid	28
2	Percent recovery of sugars after minimal exposure to 4% Acid	30
3	Percent Sugar Recovery After Two- and One-Stage Acid Acid Hydrolysis	32
4	Random error associated with quantitative analysis of the polysaccharide fraction of native switchgrass and poplar	36
5	Random error associated with quantitative analysis of the polysaccharide fraction of pretreated poplar solids(PHS) and poplar prehydrolyzate(PH)	38
6	Random error associated with replicate pretreatments	41
7	Compostion of Switchgrass PHS and PH	44
8	Compositions of Poplar PHS and PH	46

QUANTITATIVE POLYSACCHARIDE ANALYSIS OF LIGNOCELLULOSIC BIOMASS

Introduction

Biomass is the mass of materials generated by the growth of any living organism, including plants, animals, and microorganisms. Lignocellulosic biomass is plant material composed of three major components, lignin, cellulose, and hemicellulose. Wood, straw, corn cobs, yard waste, and even municipal solid waste are lignocellulosic biomass.

Biomass is the raw material of many products, including cattle feed, paper, structural materials such as fiberboard, useful chemicals like furfural, and fermentable sugars such as glucose. However, the glucose is not readily available, and its extraction requires a pretreatment step to make the biomass more susceptible to cellulytic attack and an enzymatic hydrolysis to cleave the cellulose to glucose. Pretreatment results in a solid fraction, called prehydrolyzed solids(PHS), and a liquid fraction, called prehydrolysate(PH).

Fermenting the cellulose present in lignocellulosic biomass to ethanol requires the solubilization of glucose. This can be done either enzymatically or through chemical means such as strong acid treatments. Cellulytic enzymes do not perform well on native biomass because of the interfering presence of a hemicellulose-lignin matrix(Grohmann *et al.*, 1984). One way of preparing the native lignocellulosic biomass for cellulytic attack is to prehydrolyze the feedstock with dilute acid at a high temperature(Knappert *et al.*, 1980; Grohmann et al., 1984; Grethlein et al., 1984). Pretreatment yields a solids

stream, or prehydrolyzed solids(PHS), and a liquid stream, or prehydrolyzate(PH).

Dilute acid and high temperature solubilize hemicellulose preferentially to cellulose. The solubilization of hemicellulose is thought to be the result of an acid-catalyzed hydrolysis of glycosidic bonds within the hemicellulose as well as the cleavage of lignin-hemicellulose ester bonds(Wayman, 1990).

PHS, which has a higher proportion of cellulose to hemicellulose, resulting from the high-temperature, dilute-acid pretreatment, is more susceptible than native cellulose to cellulytic enzymes, so polysaccharide composition can be used as an indicator of cellulase susceptibility after dilute-acid pretreatment. That is, hemicellulose removal correlates with cellulytic susceptibility (Knappert *et al.*, 1980). For this reason, it is necessary to have an accurate, reproducible method of analyzing PHS for saccharide content. If the saccharides in the PH are also of interest, the PH is analyzed for saccharide content.

Most polysaccharide analyses of lignocellulosic biomass are based on a method named for the Swedish chemist Peter Klason. The method involves a two-stage sulfuric acid treatment, the first being a strong sulfuric acid(72% w/v) treatment at near-ambient temperatures designed to solubilize the polysaccharides, the second is a high temperature hydrolysis carried in 4% acid. The method was actually designed to gravimetrically determine the lignin content of wood, but it serves quite well for polysaccharide determination. There are many variations on the Klason method, but most are merely temperature and acid concentration changes.(TAPPI, 1993; NREL, 1991; Kaar, 1991)

Quantitative methods for polysaccharide analysis differ mainly in the way the resulting monosaccharides are detected. The sugars may be converted to

their alditol acetates and separated via gas chromatography(Blakeney *et al.* 1983). They may also be measured without derivitization following separation by HPLC. The most common method of quantification in HPLC-based methods makes use of a refractive index detector. The HPLC separation of monosaccharides can be achieved by ion-moderated partition chromotography. The column packing is a cross-linked divinyl benzene polymer with a counter-ion, which may be lead, calcium, silver, or hydronium. Different counter-ions and degrees of cross-linking yield different sugar separations (Lee *et al.*, 1989). An alternative, HPLC-based method is to separate the sugars by anion exchange chromatography under highly alkaline conditions and detect them with a pulsed amperometric detector, which affords greater sensitivity. (Lee, 1990).

When authors report results of polysaccharide analysis of biomass, it is customary to give an estimate of the error associated with the measurement, usually in the form of a standard deviation or confidence interval. This value may reflect variability in the analytical method as well as in the substrate, and generally no attempt is made to describe the sources of variability. In reports of new analytical work, e.g. a new analytical technique, the substrates have generally been extracted to remove interfering compounds. Since the primary focus of these analyses was to analyze pretreated biomass, and an industrial-scale application of dilute acid pretreatment would not include an extraction step, it was decided to work solely with native, unextracted biomass. This thesis describes a method, based on the National Renewable Energy Laboratory's(NREL) suggested method for determining the saccharide content of lignocellulosic biomass(NREL, 1991), and the sources of variability associated with the analysis.

Literature Review

Biomass can be defined as the mass of materials generated by the growth of any living organism, including plants, animals, and microorganisms. The term biomass can also refer to agricultural byproducts such as corn cob and wheat straw. Lignocellulosic biomass is plant material composed of three major components, lignin, cellulose, and hemicellulose. It includes such stuff as wood, straw, corn cobs, yard waste, and even municipal solid waste. The relative amounts of each of the three components varies greatly according to biomass source (Wayman, 1993).

Biomass is the raw material of many products, including cattle feed, paper, structural materials such as fiberboard, useful chemicals like furfural, and fermentable sugars such as glucose. The glucose is not readily available, as will be explained shortly, and its extraction requires a pretreatment step to solubilize the hemicellulose, and an enzymatic hydrolysis to solubilize glucose from the cellulose. Pretreatment results in a solid fraction, called prehydrolyzed solids(PHS), and a liquid fraction, called prehydrolysate(PH). But first consider each of the three main components of lignocellulosic biomass.

Lignin is the primary aromatic component of lignocellulosic biomass. It acts as a kind of glue for plant material. It is part of the lignin/hemicellulose matrix which encrusts cellulose fibers. The lignin "molecule" as such is ill-defined in the sense that its structure is always different. Lignin appears to form via a free-radical, oxidative condensation reaction of three monomers, coumaryl, coniferyl, and sinapyl alcohols(Wayman, 1993). The resulting linkages between the monomers are complex and are primarily ether bonds. These precursors are made in an offshoot of the shikimic acid pathway, the same one used by plants

to synthesize phenylalanine and tyrosine. Commercially, lignin is primarily used for its physical properties, such as a binding agent in dyes, animal feed, concrete and charcoal. Much of the lignin produced in the paper industry is burned to fuel boilers for in-house energy production. Yet, the building blocks of lignin make it a source of compounds one might not expect to derive from lignocellulosic biomass, such as vanillin. From vanillin, L-dopa, a drug used in the treatment of Parkinson's disease can be made(Sjöström , 1993).

The quantification of lignin is a two-stage acid procedure named for the Swedish chemist Peter Klason, who suggested in 1897 that lignin's chemical structure was similar to coniferyl acid. The first step is the removal of materials not of interest, which is accomplished with successive extractions with hot water, hot water/ethanol mixture, and finally hot ethanol. The materials removed are called extractives and include compounds such as waxes, polysaccharide gums, resins, phenolics and free sugars. They are removed because their presence in biomass is quite variable, and could consequently make the lignin measurement quite variable , even when comparing members of the same species (TAPPI method 264, 1993).

After extraction, the biomass is dried at 105°C to constant weight, a portion is weighed, and mixed with 72%(w/w) sulfuric acid. The strong acid disrupts the hydrogen bonds between the polysaccharides, making them soluble. The acid also begins to catalyze the hydrolysis of the polysaccharide, yielding free sugars. The strong acid is then diluted to 4%(w/w) and the resulting mixture is boiled with refluxing for four hours to complete the hydrolysis of the polysaccharides. The liquid is filtered through a tared, sintered glass crucible, dried and weighed. The dark brown material in the crucible is Klason lignin, or acid-insoluble lignin, and is reported on a dry weight basis(TAPPI method 222).

During the two-stage acid hydrolysis for lignin quantification, some of the lignin is solubilized. This material is called acid-soluble lignin, and its quantification has been the topic of some debate. Kaar *et al.* showed that acid-soluble lignin is not chemically distinct from acid-insoluble lignin, but merely lignin which is solubilized by the harsh acid and heat treatments of the Klason lignin procedure (Kaar, 1991). It is measured by reading an absorbance in the UV range(205-280nm), and using an extinction coefficient of approximately $110gL^{-1}g^{-1}$, which is based on experiments with model compounds. However, there are sugar degradation products in the solution, hydroxymethyl furfural and furfural, which also absorb at the longer UV wavelengths, so that 205nm is now the accepted wavelength(TAPPI method 223) .

Cellulose is an unbranched polymer of β 1-4 linked glucose. In nature, the degree of polymerization has been measured to be between 8000 and 15,000 (Sjöström, 1993). In trees, the glucose used to make cellulose is synthesized in the leaves as a product of photosynthesis, then transported to the site of polymerization, the secondary cell wall of the cambial layer. Through hydrogen bonding, the chains align to form microfibrils, which in turn align to form tightly-packed structures called fibrils, which in turn make up fibers. The fibrils may be envisioned as a handful of uncooked spaghetti, with the individual noodles representing microfibrils. The cellulose fibers provide plant materials with their mechanical strength. Although cellulose is chemically homogeneous, physically it is heterogeneous. There are crystalline and amorphous regions of the microfibril. Crystalline regions are where the glucose chains align, while the amorphous regions contain non-aligned chains. It is thought that the crystalline regions are more recalcitrant to enzymatic hydrolysis than the amorphous portions (Sjöström , 1993).

Because of the tight hydrogen bonding between cellulose chains, cellulose is a relatively inert polymer. It is insoluble in water and common solvents such as ethanol and chloroform. It is soluble in strong acids, such as 72% H_2SO_4, 85% H_3PO_4, and 41% HCl, and also in alkaline copper solutions such as copper-ammonium hydroxide complex. The copper solutions are particularly useful in measuring the degree of polymerization of cellulose, since the acidic solutions not only solubilize, but also hydrolyze the cellulose chains. In contrast to acidic solutions, KOH and NaOH will swell but not dissolve cellulose(Sjöström , 1993).

Cellulose represents the major source of fermentable sugars in most woods, while in grasses such as switchgrass, the hemicellulose fraction can be nearly as large as the cellulose. Cellulose can be saccharified to cellobiose, a β 1-4 glucose dimer, by the action of exo-glucanases, and the cellobiose can be cleaved to two glucose molecules by cellobiohydrolase. The resulting glucose can then be fermented to ethanol by yeast. However, in native plant material, the cellulose is not readily hydrolyzed. It is embedded in a hemicellulose/lignin matrix which hinders access of the cellulase enzymes.

The term hemicellulose refers to a group of polysaccharides which are formed in the early stages of plant cell wall development. It is distributed throughout the cell wall in wood, but is most concentrated in the primary wall. The major hemicellulose components have a xylan backbone. Branching from the xylan units are other sugars, including galactose, glucose, arabinose and mannose. Each biomass sample has its own hemicellulose profile and quantity with varying degrees of acetylation.

Hardwood hemicellulose is a β 1,4 linked xylopyranose polymer with a degree of polymerization of about 200. About one in ten xylose units has a single, terminal side chain which consists of 4-O-methylglucuronic acid linked

directly to carbon 2 of xylose. About 7 of every 10 residues has an *O*-acetyl group attached at carbon 2 or 3 or both. Hardwoods usually contain about 3-5% glucomannans, with a glucose:mannose ratio of approximately 1:2. The glucomannan is an unbranched β 1,4 linked polymer of glucose and mannose units (Coughlan, 1992).

The xylans in grasses have the same backbone as the wood xylan discussed above, but grass xylans are more highly branched, contain large amounts of arabinose units, and uronic acids make up a smaller proportion of the total xylan weight. The arabinose units are probably linked to carbon 2 of xylose.

In lignocellulosic biomass, some of the hemicellulose is thought to be esterified to lignin. For example, arabinoxylan isolated from barley straw was found to have *p*-coumaric acid, one of the monomeric components of lignin, esterified to 1 of every 31 arabinose residues, and another lignin monomeric component, ferulic acid, was esterified to every 15 arabinose residues (Coughlan, 1992).

Hemicellulose is readily solubilized by a number of different methods, including autohydrolysis, which is the heating of biomass to high temperatures in the presence of steam. High temperatures not only catalyze the hydrolysis of hemicellulose, but also release acetic acid side chains from hemicellulose, thus decreasing the pH and further increasing hemicellulose solubilization. Other methods of solubilizing hemicellulose include base-catalyzed hydrolysis (Fox, 1989), ammonia freeze-explosion(Mes-Hartree, 1988), and dilute-acid pretreatment at high temperatures (Knappert, 1980). The solubilization is the result of cleavage of the glycosidic bonds in the polysaccharide chain as well as ester bonds from side chains to lignin(Coughlan, 1992).

The quantification of polysaccharides in biomass has special significance when devising protocols for the conversion of biomass to ethanol. First, there is

the necessity of evaluating different feedstocks. Different species of trees have different absolute and relative amounts of cellulose and hemicellulose, and this information is useful. For example, aspen wood was found to contain 45% glucan and 15% xylan(w/w), while white ash has 40% glucan and 18% xylan (Kaar, 1991) In contrast, switchgrass contains 31% glucan and 22% xylan(Himmel, 1985). This data, along with relative abundance data and growth rates, helps to choose from the many potential feedstocks for a biomass to ethanol operation.

Another reason polysaccharide analysis of biomass is vital when evaluating a pretreatment protocol is that the removal of hemicellulose from biomass is associated with a significant increase in susceptibility to cellulytic hydrolysis(Grethlein *et al.* 1980,1984;Knappert et al., 1980; Grohmann *et al.*, 1985, 1986; Torget *et al.* 1988). Any pretreatment of biomass removing hemicellulose results in PHS in which the cellulose fraction is much more susceptible to cellulytic attack. Thus, quantifying the removal of hemicellulose after pretreatment gives a valid indicator of the susceptibility of the remaining solids to cellulase hydrolysis.

If fermentation of the hemicellulose sugars which are solubilized in the pretreatment is desired, quantification of the sugars in the liquid resulting from the pretreatment, or prehydrolysate(PH), is helpful. Dilute-acid pretreatment, as well as autohydrolysis, result in the degradation of solubilized pentoses, especially xylose, to furfural. Furfural formation results in net losses of fermentable sugars, and is suspected to inhibit glycolysis(Banerjee *et al.*, 1981) and ethanol production by *Saccharomyces cerevisiae* (Boyer *et al.* , 1992).

The analysis of biomass for polysaccharides, both hemicellulose and cellulose is generally accomplished using a method very similar to that used to quantify Klason lignin. The objective is to solubilize, then hydrolyze the

polysaccharides. This is done by using strong sulfuric acid at near-ambient temperatures, followed by a weaker acid treatment(around 4%) at elevated temperatures. The elevated temperature treatment may be either four hours of boiling with reflux, or one hour at 121°C. Once the biomass has been hydrolyzed to a mixture of what is essentially Klason lignin and monosaccharides, the monosaccharides are separated and quantified.

There are a few variations on this method, and most involve different methods of separation and detection.

The Technical Association of the Pulp and Paper Industry(TAPPI) publishes a wood, pulp, and paper analytical methods handbook (TAPPI, 1993). In the method "*Carbohydrate composition of extractive-free wood and wood pulp by gas-liquid chromotography*", the biomass is dissolved in 72% sulfuric acid and kept in a 30°C water bath for one hour, then diluted to 4% acid, and autoclaved for one hour. The solution is then cooled, internal standard and a pH indicator are added, the solution is neutralized with barium hydroxide, precipitating barium sulfate . The solution is then centrifuged and the supernatant collected for reduction and acetylation.

The reduction involves adding excess borohydride and heating in a 60°C water bath for one hour. The excess borohydride is destroyed by addition of glacial acetic acid. Borate, which forms from the borohydride, complexes with the alditols and thus interferes with resolution. In order to remove the borate, the liquid is washed with maethanol and evaporated to dryness three times. The acetylation step that follows has another one hour incubation step, followed by three methylene chloride extractions and another concentrating step. The solutions are then injected onto a GLC column, which is calibrated with sugar standards which have been carried through the entire analytical procedure.

This method has some disadvantages for a laboratory interested in analyzing large numbers of pretreaed biomass samples. First, the numerous concentration and extraction steps prove time-consuming. Also, it involves the use of organic solvents such as methylene chloride as well as bad-smelling chemicals such as acetic anhydride. Possible advantages include separation of some alditol-acetate derivatives of monosaccharides such as rhamnose and fucose as well as better overall resolution of individual sugars.

Blakeney et al.(Blakeney *et al.*, 1983) also published a method for determining monosaccharide content of a hydrolyzed plant cell walls using alditol-acetate derivatives and capillary GLC. This method is performed in one test tube with no transfers or evaporations. The hydrolysis of the plant material was essentially the same as described in the TAPPI analysis. The reduction was again accomplished with sodium borohydride, but by using a catalyst in the acetylation step, interference from the borate was removed, so that the methanol extraction and evaporation steps were not necessary. The average run time for an injection was only 16 minutes, and this allowed elution of all the sugar derivatives of interest in a biomass analysis, glucose, xylose, galactose, arabinose, and mannose. This method would appear to be more feasible when processing large numbers of samples.

Again, a two-stage sulfuric acid hydrolysis to yield monosaccharides is consistently used by most authors. Differences exist in the method of detection, neutralization and whether or not an internal standard is included. In the method supplied to our laboratory by the National Renewable Energy Laboratory(NREL, 1991), the entire sample is neutralized, filtered and the volume of filtrate recorded. This sample is then injected on an HPLC column(Bio-rad Aminex 87-P) which separates the sugars of interest via ion-moderated partition chromotography. The solid phase consists of small beads

of cross-linked divinyl benzene, to which is bonded an amine phase. The solid phase has a net negative charge, which is countered with lead ions. The lead counter-ions interact with cis hydroxyl groups on the sugars, thus affording separation. The mobile phase is distilled, deionized water. The sugars are detected with a refractive index detector, so gradient elution is not possible. When the manufacturer- recommended guard column is used, a sloping baseline is obtained. The baseline is caused by ions present after the neutralization of sulfuric acid and it coelutes with the sugars, making an internal standard choice difficult. This sloping baseline can be eliminated with the use of a de-ashing guard column, as will be discussed in this thesis.

Another published method(Kaar *et al.*, 1991) uses the two-stage acid hydrolysis to obtain a solution of monosaccharides from biomass. The solution is then filtered, and the solids are weighed and reported as lignin(even though the hydrolysis conditions are not identical to the TAPPI lignin method), and the resulting solution is diluted in a volumetric flask. This solution then becomes the stock for monosaccharide as well as other analyses, such as uronic acid and furfural. For monosaccharide analysis, an internal standard is added. The pH is adjusted to 5.3 with $Ba(OH)_2$, and the supernatant is collected. The solution is passed through an ion-exchange resin, collected, evaporated to a smaller volume, and anlayzed by HPLC. The column is the same as described in the NREL method column and manufacturer-recommended guard column was used. The advantage of this method over the NREL method is that volume mesurements after neutralization become unnecessary. The use of an internal standard also corrects for injection size variation. There is one other advantage in using an internal standard which has to do with volume changes during neutralization. This will be discussed in the Results and Discussion method of this thesis.

An alkaline solution of monosaccharides can also be separated using anion-exchange chromostography and subsequently detected with a pulsed amperometric detector(PAD)(Lee *et al.*, 1989). Most sugars will ionize at pH 12-13, and thus can be separated by ion-exchange chromotography. Detection is then usually achieved with a PAD, which has greater sensitivity than a refractive index(RI) detector. However, anion exchange chromatography with PAD does have some practical disadvatages to ion-moderated partition with RI. The anion exchange column must be recharged quite frequently, and the detector is more sensitive to "dirty" samples, such as those obtained from hydrolysis of lignocellulosic biomass. Also, the highly alkaline mobile phase(pH 12-13) is hard on HPLC pumps and fittings, and thus cannot be left in the system overnight. In comparison, the distilled water mobile phase used with ion-moderated partition may be left running almost indefinitely with little or no harm coming to the costly HPLC equipment(Pers. comm., Waters technical services)

Methods

Pretreatment Protocol

Two biomass preparations, switchgrass and a hybrid poplar, were supplied by NREL. It had been air dried and milled to pass a 40 mesh screen. The "fines", any material which passes through a 120 mesh screen, were also omitted. This is to prevent problems with charring during pretreatment.

The biomass was pretreated at 10% solids (w/w) in a 0.6L Parr batch-type, stirred reactor equipped with a self-built acid-injection device and a temperature controller, which controlled both the heating mantle and the cooling device. Cooling was accomplished with a solenoid valve which controlled water flow through the cooling coil. In a typical pretreatment, biomass(30g weighed to the nearest 0.1 mg) and approximately 240 g of water(weighed to the nearest 0.01g) were added to a Pyrex™ reactor liner. The liner was then placed in the reactor, sealed and the agitator was started. The reactor was then brought to the desired temperature. It took approximately 20 minutes to reach 180°C. After the desired temperature was attained, approximately 20g of acid solution(weighed to the nearest 0.01 g) was injected in to the reactor. The injection took approximately three seconds. The reaction was terminated by opening the cooling water valve and removing the reactor from the heating mantle. This termination protocol dropped the temperature from 180°C to 70°C in about two minutes.

The pretreated biomass was filtered in a tared, two-piece plastic Buechner funnel and the solids were washed with approximately 1300 mL distilled water. The solids, or prehydrolyzed solids(PHS), were dried by placing the

upper portion of the funnel in a 45°C oven for 60 hours, and then analyzed for polysaccharides. The filtrate, or prehydrolysate(PH), was stored at 4°C until analyzed for polysaccharide.

Analytical protocol

Procedure for PHS and unpretreated biomass

Native biomass samples were dried at 45°C overnight, and weighed for analysis. Pretreated samples, already dried at 45°C, were removed from the Buechner funnel and placed in a mortar and ground. Moisture determinations were made on the 45°C-dried material to correct for moisture content.

To determine the moisture content of the 45°C-dried material, approximately 1.0 g of sample was weighed to the nearest 0.1 mg into a pre-dried and weighed aluminum dish. After drying overnight at 105°C and cooling in a dessicator, the sample was again weighed and the percent solids calculated in order to correct for moisture content of the 45°C-dried biomass.

$$\textit{(Eq. 1)} \quad \%\ \text{solids} = 100 \times \frac{\text{SW after } 105^{\circ}\text{C}}{\text{SW before } 105^{\circ}\text{C}}$$

where SW is sample weight.

For the polysaccharide analysis, approximately 0.3 g of 45°C-dried material was weighed to the nearest 0.1 mg, transferred to a test tube, and 3.0 mL of 72% (w/w) sulfuric acid(Sigma™) was added. The sample was mixed with a stir rod until completely wetted and placed in a 30°C water bath. The samples were incubated in a 30°C water bath for two hours and stirred every half hour to assure complete mixing. After two hours, the samples were removed from the

water bath and placed in an ice bath. The samples were transferred to 250 mL Pyrex screw-cap bottles using 84 mL of distilled water per sample, resulting in a 4%(w/w) acid solution. The screw cap was tightened on each sample and the samples were autoclaved for 65 minutes at 121°C, including 2-3 minute autoclave warm-up time. After autoclaving, the samples were cooled under cold, running tap water, transferred to 100 mL volumetric flasks and diluted to the mark with distilled water. These steps were repeated with an equal weight of a mixture of 45°C-dried sugar standards. The results obtained from these sugar correction samples were used to estimate sugar decomposition caused by the harsh acid and temperature conditions of the analysis.

Approximately 20 mL of the hydrolyzed biomass was poured into 50 mL beaker and then neutralized with calcium carbonate to pH 6, or slightly higher, by addition of approximately 2 grams of calcium carbonate. Calcium sulfate precipitate formed. The pH was checked with pH paper, although once foaming has ceased upon the addition of new calcium carbonate, the solution was found to be neutral or slightly alkaline. Using a syringe, about one mL of supernatant was drawn off for HPLC analysis and pushed through a 0.45 μm filter into an autoinjector vial. A waiting period of approximately 45 minutes was required following neutralization prior to HPLC sample preparation to prevent a precipitate from forming in the autosampler vials.

A series of sugar solutions in distilled water were used for HPLC calibration. The calibration sugar standards, the acid-treated sugar standards(sugar correction samples), and the hydrolyzed biomass samples were analyzed by HPLC. The all-Waters HPLC system included 501 pumps, a 401 refractive index detector, a 717 plus autosampler, and Baseline 810 integration software. The column was a Bio-rad Aminex HPX-87P, the flow rate

was 0.6 mL/min. of Milli-Q double distilled water, and the column was controlled at 85°C.

Calculations for solids

A calibration curve for each of the sugars based on area of peaks was created. The curve was forced through the origin. That is, zero concentration was assumed to give zero response. The effect of forcing the curve through the origin on the calibration curve slope was less than 1 part in 100. From the curves, the concentration of sugars present in each biomass sample and sugar correction sample was calculated. A volume of 100 mL, the volume of the volumetric flask to which the hydrolyzed biomass was added before neutralization, was assumed.

The amount of each sugar recovered from the sugar correction samples after two-stage acid hydrolysis was determined to give an estimate of sugar loss from biomass samples caused by the analysis itself.

$$(Eq.\ 2) \qquad SRF = \frac{MC,\ g/L}{AC,\ g/L}$$

where SRF is the sugar recovery factor(<1.0), MC is the measured concentration of sugar correction samples and AC is the added, or known, concentration of the samples used to determine the sugar correction factor.

The sugar recovery values were used to adjust sugar concentrations of biomass as follows:

$$(Eq.\ 3) \qquad ASC = \frac{SC_{biomass},\ g/L}{SRF}$$

Where ASC is the adjusted sugar concentration of the hydrolyzed biomass sample and SC is the measured sugar concentration of the hydrolyzed biomass sample.

The weight percent polysaccharide of each sugar in the biomass sample, on a 105°C dry weight basis was then calculated with the equation

(Eq.4) $$\text{weight percent polysaccharide} = 100 \times \frac{\text{ASC, g/L} \times 0.1\ \text{L} \times \text{MWF}}{\text{SW,g} \times (\%\ \text{solids}\ /100)}$$

Where the 0.1L represents the volume of the volumetric flask, SW is sample weight and MWF is the correction factor accounting for the addition of a water molecule to each sugar as it is hydrolyzed from the polymer and is

(Eq. 5) $$\text{MWF=MW sugar - MW } H_2O\text{/MW sugar}$$

where MW is molecular weight. The percentage of any given polysaccharide remaining in the PHS after a pretreatment was calculated as

(Eq. 6) $$\%\ \text{Orig. polysacc. in PHS} = 100 \times \frac{\text{wt.\% glycan} \times \text{dry weight PHS}}{\text{original weight of glycan in reactor}}$$

Procedure for prehydrolysate saccharide analysis

75 mL of PH was transferred to 250 mL Pyrex™ bottles and 4.179 g of 72% sulfuric acid was added to make a 4% acid solution, the solution was then autoclaved as in the solids analysis. The sugar correction samples were added to 4% sulfuric acid solutions and autoclaved with the PH samples.

Calculations for prehydrolysate

As for the solids calculations, a sugar recovery value was calculated and used to adjust the sugar concentrations of the prehydrolysate. The amount of polysaccharide in the PH as a percent of the biomass added to the reactor was calculated with the equation

$$(Eq.\ 7)\ \%\ \text{orig. polysac. in PH} = 100 \times \frac{\text{ASC, g/L} \times \text{vol. of PH, L} \times \text{MWF}}{\text{original weight of glycan in reactor}}$$

Sugar Recovery After Minimal Exposure to Acid

A standard sugar mixture was added to 87 mL 4% acid solution, immediately neutralized with calcium carbonate, filtered, and the volume of filtrate was used to calculate the sugar recovery(treatment A, measured volume). In the assumed volume, no filtration treatment, B, sugar mixture was added to 87 mL 4% acid, diluted to 100 mL in a volumetric flask, neutralized, and supernatant was collected and prepped for HPLC injection. A volume of 100 mL was assumed to calculate sugar recovery. In treatment C, assumed volume with filtration, sugar mixture was added to 87 mL 4% acid, diluted to 100 mL in a volumetric flask, neutralized, and filtered *without* washing. A volume of 100 mL was assumed for calculating sugar recovery.

As a control, sugars were added to 87 mL of distilled water, and transferred to a 100 mL volumetric flask. All samples and standards were injected three times in order and three separate calibration curves were made.

Glucose recovery after minimal exposure to acid with an internal standard

Glucose was added to 87mL 4% sulfuric acid, diluted to 100 mL in a volumetric flask, and with a volumetric pipette, 25mL was mixed with 5mL arabinose solution, also measured with a volumetric pipette. This solution was then neutralized with calcium carbonate and prepped for HPLC injection. A standard curve was created by mixing 25mL of various glucose standard solutions with 5mL of internal standard solution.

Results and Discussion

Analytical Sources of Variability

Instrumental sources of variability

In determining the effects of different pretreatment conditions on the polysaccharide composition of biomass, there will be variability. This error will have two primary sources, the pretreatment conditions and the analysis itself. It becomes necessary to know whether the differences between xylan solubilization between two sets of pretreatment conditions are the result of the conditions or simply an artifact of random error associated with the analysis. That is, when can one be certain that the differences observed are real? This question can be answered by determining the sensitivity of the method. One estimate of sensitivity is to triple the standard deviation associated with a measurement. One can then be fairly sure that any measurement outside this range will not be the result of random error. This value is similar to a confidence interval.

Variability and sensitivity are closely related, i.e. the more variable a method, the less sensitive it is. The variability, or error, associated with the analysis is the primary concern of thesis and can be considered to have two sources, instrumental variability and non-instrumental variability. Variability will be considered in terms of coefficient of variation, variance, and standard deviation.

Quantification of polysaccharides in my HPLC-based method depends on separation, detection, and peak integration of sugar solutions. The instrument

consists of an autoinjector, column, detector, and integration software. Each of these contributes to variability in peak area, and it is possible to estimate each contribution individually to get an estimate of the instrument-related error of the analysis. It is also possible to estimate the HPLC-derived error experimentally by injecting standard sugar solutions repeatedly.

The refractive index detector is capable of measuring changes in refractive index of 0.5×10^{-7} refractive index units(RIU)(Waters, 1993). According to (Weston, 1958), a change of 1%(w/w) of glucose in solution will yield an RIU change of 0.00143. Assuming a linear relationship between refractive index and concentration, this means that the smallest change in concentration that the detector cell alone is capable of measuring would be 0.004 mg/mL. Assuming a 300 mg sample that has been hydrolyzed to 100 mL, the smallest detectable change in glucose concentration, 0.004 mg/mL, represents 0.13% glucan(w/w) of a biomass sample. This is an estimate of the detector's capability, and does not include variability introduced by the injector or integrator.

Detector variability can be broken down to long-term and short term variability. Short term variability is call detector noise, and this is dealt with by degassing of the mobile phase and other measures. It is generally the same from sample to sample, unless something has gone drastically wrong, such as an air bubble in detector cell. Long-term drift should also not play much of a role, since the detector is autozeroed at the beginning of each 45 minute run. As a check for leng-term drift and sample stability, the calibration curve samples were usually injected at the beginning and end of a series of samples. The calibration samples injected 18 hours apart gave peak areas similar to those obtained by injecting samples immediately following each other.

The precision of the autoinjector is listed as 0.5% CV. According to this value, repeated injections of the same solution should have a volume CV, and

hence peak area CV, of approximately 0.5%. If the peak area CV is greater than 0.5%, then it may be attributed to sample, column, or detector variability. Sample variability is not an issue, since aqueous sugar soutions should be uniform.

If the column were dirty, it could influence the results by binding some sugars or affecting the sugars in some unforeseen fashion. However the peaks showed good Gaussian distribution with no evidence of peak tailing.

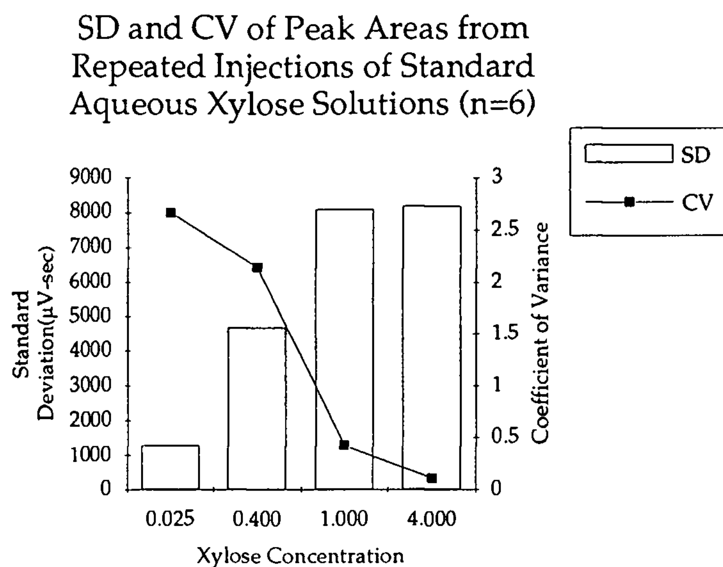
Another factor which could introduce variability is integration. Although the integration is performed by a software program, chromatograms have to be 'proofread' to ensure the integration makes sense. Although care is taken when start and endpoints are moved, they do not align perfectly.

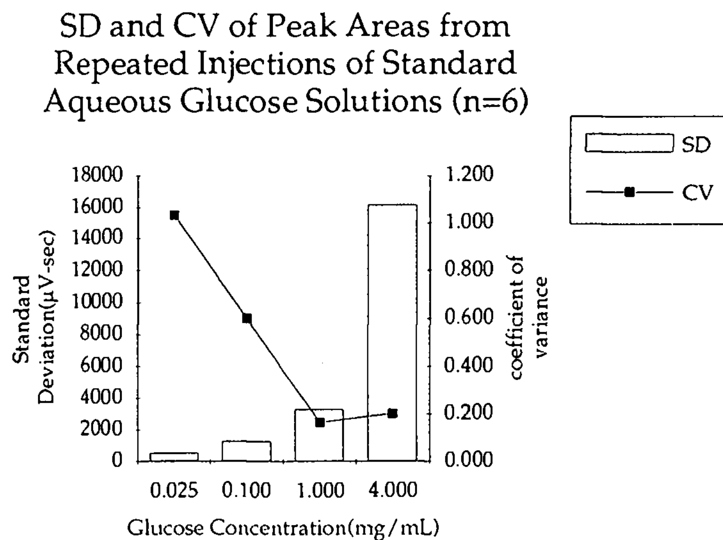
An estimate of the entire system's variability can be obtained by injecting standard sugar solutions repeatedly. Figure 1 shows a plot of standard deviation(SD) and CV of peak areas obtained by injecting four mixtures of glucose and xylose six times in a single day. Each solution was injected once in turn, and then the whole set was injected five more times. The coefficient of variance(CV) ranged from 1.0% to 0.2% for glucose and from 2.7% to 0.1% for xylose. The CV is higher at lower concentrations. This trend indicates that the standard deviation(SD) does not increase proportionally with increasing concentrations/peak areas, and thus that analysis of more concentrated solutions is more reproducible. However, the SD of the more dilute solutions is smaller, so that the minimum detectable change(MDC) in concentration will be smaller for dilute solutions.

The variability of the standard sugar analysis has implications for the sensitivity of the overall method. The glucose concentrations measured for solids analyses ranged from 1.0 mg/mL for native switch grass to 1.6 mg/mL for pretreated poplar. Peak area standard deviations from analysis of standard

Figure1

Random Error Associated with Quantitative Measurements of Standard Sugar Solutions Using HPLC [1]





[1] Four solutons of glucose and xylose were each injected once in turn, then the whole set was injected five more times.
Peak areas were determined by integration software.

glucose solutions at 1.0 mg/mL was 3500 μV-sec. Peak areas are converted to concentrations via a calibration curve. A typical calibration curve for glucose has the form and values

(Eq. 8) gluc. conc.(mg/mL) $= 5.02 \times 10^{-7} (\mu V\text{-sec})^{-1} \times$ peak area (μV-sec)

Multiplying the 1.0 mg/mL standard glucose solution peak area SD of 3500μV-sec by three and substituting that value into Eq. 8, the minimum detectable change(MDC) in concentration of the instrument when reading glucose concentrations of approximately 1.0 mg/mL is 0.005 mg/mL. With a volume of 100 mL, and a sample weight of approximately 300 mg, 0.005mg/mL translates to a minimum detectable difference of 0.2% glucan(w/w) of a biomass sample. This means that the instrument is limited to detecting differences of approximately 0.2% glucan(w/w), without any other laboratory error such as sample weighing, transfer, or moisture determinations.

In comparison, the xylose concentrations measured in biomass analyses ranged from 0.02 mg/mL for pretreated biomass to 1.0 and 1.3 mg/mL for unpretreated switchgrass and poplar samples respectively. The standard deviation of peak areas at 0.025 mg/mL is approximately 2100 μV-sec, while for 1.0 mg/mL, it is 8000 μV-sec. A typical calibration curve for xylose has the form and values

(Eq. 9) xylose conc.(mg/mL) $= 5.3 \times 10^{-7} (\mu V\text{-sec})^{-1} \times$ peak area (μV-sec)

Using the same argument as for glucose and the values from Figure 1 and Equation 9, three peak area standard deviations for a standard xylose solution of 0.025 mg/mL correspond to a xylose concentration of 0.003 mg/mL, or a xylan content of 0.1% by weight. At low xylose levels, as are found in harshly pretreated biomass, the sensitivity is greater, and differences of ~0.1%

xylan(w/w) are detectable and real. By contrast, mildly pretreated biomass has a higher xylose content, which translates into peak area standard deviations of approximately 8000 μV-sec. For higher xylose concentrations, as are found in native or mildly pretreated samples, three peak area standard deviations correspond to 0.4 % xylan(w/w) of a solids sample. This implies that for a xylose content in the higher ranges(14-20% w/w), differences of approximately 0.4% are real and reliably detected.

The sensitivity of 0.1% to 0.45% polysaccharide(w/w) represent the capabilities of the entire HPLC system, including autoinjector, column and detector. The MDC imply that differences in weight percent xylan and glucan of greater than 0.4% are measurable at higher concentrations and differences of 0.1% xylan are measurable at lower concentrations.

Neutralization and volume estimation of hydrolyzed biomass samples

The method of polysaccharide analysis described here is a modification of the method proscribed by the National Renewable Energy Laboratory(NREL). The modification is in the step after neutralization of the hydrolyzed biomass sample. NREL recommends neutralizing the entire 87mL volume of 4% acid solution, filtering the solution with a minimum of wash water, and measuring the volume of filtrate(NREL, 1991). It was not possible to wash the precipitate quantitatively, because the minor component sugars soon became too dilute for detection by refractive index. In the assumed volume method described here, the 87mL are diluted to 100 mL in a volumetric flask, an aliquot is neutralized, filtered for HPLC injection, and a volume of 100 mL is assumed.

In work involving multiple replications of the measured volume method proscribed by NREL using the measured volume method, the percentage of sugar recovered varied by as much as 15% for triplicate measurements in a given trial. From day to day, the values varied by more. For example, recoveries ranged from 65% to 99% for glucose and 70 to 90% of the xylose. This introduces a lot of variability into calculations of xylan and glucan present after dilute-acid pretreatment. It seemed possible that the day-to-day sugar recovery values might be different due to autoclave temperature fluctuations or reactions running longer or shorter than others. However, that did not explain same-day triplicate measurements which might show 75%, 85% and 90% glucose recovery.

It was proposed that the major source of variability in sugar recovery was the recovery of liquid from the filtration. This volume varied much more than the actual sugar concentrations, which implied that the washing was not quantitative and recovery values were dependent on the volume recovered, rather than on actual differences in sugar degradation. An experiment was designed to test how much sugar is lost in the filtration step and is described in the methods section under Sugar Recovery After Minimal Exposure to Acid.

The results are shown in Table 1. The low recoveries attained when the filtrate volume measured are due to water retention by the large filter cake. This is not a problem as long as the filter cake retains a reproducible amount of water. However, if the filter cake retains more sugar solution for some samples than others, for example if the vacuum source fluctuated, the volume of filtrate would change, thus changing the sugar recovery factor.

When the volume was assumed to be 100 mL after addition of calcium carbonate, with and without filtering, glucose was recovered at levels slightly higher than the control. This indicates that there is little or no entrapment or

Table 1

Percent Recovery of Sugars from Mixtures
After Minimal Exposure to 4% Acid[2]

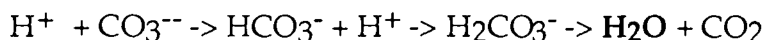
	Treatment A *measured volume filtered*		Treatment B *assumed volume no filtration*		Treatment C *assumed volume filtered*		Blanks	
	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose	Glucose	Xylose
Mean	88.0	89.2	101.9	100.1	102.2	100.2	99.6	99.6
SD	1.0	1.1	1.0	1.0	1.1	1.1	0.5	0.8
CV	1.2	1.2	1.0	1.0	1.1	1.1	0.5	0.8

[2]The means, SD, and CV reported are the result of three samples for each treatment condition

adsorption of sugars in the calcium sulfate precipitate which forms when calcium carbonate is added. Also, the smaller standard deviation of the assumed volume measurements implies that it is more reproducible that the measured volume method. Another factor to consider is that a volumetric flask is more precise than a graduated cylinder.

The results in the Table 1 show that the assumed volume method sugar recovery values are actually slightly higher than they should be. In order to eliminate possible interference from the other sugars in the mixture, another experiment was conducted using glucose and xylose separately. These experiment were conducted exactly as those discussed above, except that individual sugars were used instead of sugar mixtures. The results are shown in Table 2.

Both glucose and xylose are recovered in higher concentrations after being subjected to 4% acid for about 2 minutes and then neutralized. It seems likely that effective or actual volume changes are occurring, perhaps through precipitation of calcium sulfate or exclusion of the sugars from water by the calcium sulfate crystals. The amount of water generated by the neutralization of the acid would be



$H^+ + CO_3^{--} \rightarrow HCO_3^- + H^+ \rightarrow H_2CO_3^- \rightarrow \mathbf{H_2O} + CO_2$

so 1 mole of sulfuric acid yields one mole of water,

87 mL x (0.04 g acid/g≈mL) = 3.48 g H_2SO_4

3.48 g H_2SO_4 x 1 mole/100g = 0.0348 mole H_2O = 0.626 g H_2O.

0.63 g of H_2O in 100 mL represents a 0.6% volume increase, yet the concentrations of sugars after neutralization went up, indicating an effective volume decrease. The volume decrease appeared to be approximately 4-5%. It could be that when the sugars are dissolved in 87 mL of 4% acid and then diluted to 100 mL, the sugar is in 100 mL of freely accessible liquid. When the

Table 2

Percent Recovery of Sugars after Minimal Exposure to 4% Acid[3]

	No acid exposure		*Minimal acid exposure*		
	Glucose	Xylose	Glucose	Xylose	Glucose, using internal standard
Mean	98.9	100.6	104.5	105.0	99.4
SD	0.5	0.5	0.4	0.5	0.7
CV	0.5	0.5	0.4	0.5	0.7

[3]The means, SD, and CV reported are based on three samples for each treatment

acid is neutralized, the sulfate ion which previously made up a substantial portion of that 100 mL volume becomes a part of the solid calcium sulfate and is no longer available to solubilize the sugars. That is, the removal of sulfate ions from solution effects a decrease in volume and increase in concentration for the sugars.

Another experiment was designed to see if the high sugar recoveries after minimal exposure to 4% acid could be accounted for by using an internal standard. The experiment is described in the Methods section and the results are also shown in Table 2. The results indicate that an internal standard can eliminate the high recoveries, and hence, the high recoveries are due to a volume change.

Sugar Recovery Factors

A compilation of sugar recovery values after exposure to the two-stage(PHS analysis) and one-stage(PH analysis) acid hydrolysis is shown in Table 3. It is divided into a compilation of ten separate days' values, and into the values obtained on one day. Because of the larger variability, i.e. a higher CV, in xylose recoveries after the two-stage(PHS) hydrolysis compared to the one-stage(PH) hydrolysis(Table 3), it was proposed that a major source of variability in saccharide determinations of lignocellulosic biomass is the sugar correction factor, and specifically, the first stage of the two-stage hydrolysis.

The correction factor is more variable from day to day than it is within a single day. This is not a problem when comparing measurements made on the same day, but when data from many days are compared, the chance for error increases. This is especially true when comparing PHS polysaccharide content,

Table 3

Percent Sugar Recovery After Two- and One-Stage Acid Hydrolysis[4]

Collation of ten days	*Two-stage acid hydrolysis*		*One-stage acid hydrolysis*	
	glucose	xylose	glucose	xylose
Mean	98.6	88.5	98.3	90.7
SD	2.0	3.3	1.1	0.8
number of samples	20	21	16	16
CV	2.0	3.7	1.1	0.9
One Day				
Mean	99.0	89.1	100.5	93.8
SD	0.3	0.7	0.3	0.7
number of samples	3	3	3	3
CV	0.3	0.8	0.3	0.7

[4] Collation of ten-day data and one day data are percent sugar recovery values obtained after subjecting sugar standards to the acid hydrolysis conditions used for prehydrolyzed solids(two-stage) and prehydrolyzate(one-stage) polysaccharide analysis

because the correction factor was found to be more variable for PHS analyses than it was for PH analyses.

Also, for PHS analyses, the sugar correction factor represents an overestimate of the actual sugar loss from biomass solids. It is an overestimation because glucan will be degraded more slowly that free glucose, because the glucose in glucan spends less time as a vulnerable monomer than does the monomeric glucose used to calculate a correction factor. Comparing PHS from pretreatment conditions analyzed on the same day is not likely to be a problem, however, if analyzed on different days with different sugar correction factors, the results could be biased by a sugar correction factor.

In an experiment to quantify this overestimation, monomeric glucose and Avicel™, a microcrystalline cellulose, were subjected to the two-stage acid hydrolysis and the sugars yielded were quantified by HPLC. Avicel was approximately 97.5% (w/w)glucan, which made direct calculation of the glucose recovery difficult. In order to estimate whether or not more of the glucose from the monosaccharide sample was degraded, two peaks which are thought to be degradation products of glucose, hydroxymethyl furfural and levoglucosan(Gey, 1991) were integrated. These peaks were not present in the untreated glucose calibration standards. The ratio of the combined areas of the degradation product peaks to the glucose peak was compared. For Avicel, the average degradation peak area was 1.37% (SD=0.015) of the glucose peak area, while for monomeric glucose, the value was 1.45%(SD=0.018).

This indicates that, under the two-stage acid hydrolysis conditions, the monomeric glucose sample undergoes more degradation reactions than the glucose in the polymeric sample. When one considers a biomass sample, where the sugars are not only polymeric, but also encrusted in a lignin-hemicellulose matrix, it seems likely that the overestimation of sugar degradation is even

greater. Again, this is not a problem for comparing pretreatment effects as long as the correction factors from day to day are similar, but if they change a great deal and are indeed an overestimate, the bias introduce could be considerable.

The inclusion of a sugar correction factor, which has the same inherent variability as the analysis itself, introduces more variability into the final polysaccharide determination. In a solids analysis involving a two-stage acid hydrolysis , approximately 98% of the glucose and 88% of the xylose from the correction factor sample is recovered(Table 3), while approximately 99% of the glucose and 91% of the xylose is recovered from a one-stage prehydrolysate analysis. The CV of a sugar correction factor performed in triplicate on a single day is the same as that of sugar standard solutions, as can be seen by comparing CV values in Table 3 for the one day measurements and the CV values of the blanks in Table 1, both of which range between 0.3 and 0.7%, which indicates day to day variablility, not same day variability.

From the coefficients of variation in Table 3, it can been seen that the sugar recovery values from the ten-day data are more variable for the two-stage treatment than for the one-stage treatment. The CV of the ten-day data for xylose recovery after the one-stage treatment is 0.9%, while that of the two-stage treatment is 3.7%. A similar trend was seen for the recovery of glucose, where for the ten-day data, after a one-stage treatment the CV of recovery was 1.1% and after a two-stage treatment, the CV was 2.1%. Some of the variability might be due to the delay in diluting the 72% acid to 4% acid, the effects of which can be minimized by placing samples in an ice bath before transferring.

In order to reduce the day-to-day variability in sugar recovery values, it appears important to minimize the time spent transferring sample from the test tube to the autoclave bottles and to keep the samples on ice before transferring. It seems likely that the sugar correction value is an overestimate of actual

biomass sugar degradation, since during the first stage of the hydrolysis, not all of the sugars are in the monomeric form, and thus not all are subject to acid degradation. This means that days on which the sugar recovery was low will give higher sugar yields of biomass, especially for solids analysis, where the sugar correction factor is an overestimate of actual sugar losses from biomass.

Sensitivity of Solids Analysis

The MDC values calculated in the previous section represent the capability of the HPLC system under ideal conditions, i.e. the analysis of standard sugar solutions. In contrast, Table 4 shows data obtained from native poplar and switchgrass analysis performed in triplicate on two separate days. Each value represents a separate weighing and two-stage acid hydrolysis followed by a single injection of the resulting liquid. The values reported are the weight percent of the sugar polymers, calculated by converting the peak areas to sugar concentrations, adjusting the concentrations by a correction factor, and converting the concentration to a weight percent of the original dry matter.

The standard deviations of glucan content measurements of raw poplar and switchgrass were 0.3% (w/w) and the CV was 0.8% . The results for xylan were a little more variable, with an SD ranging from 0.1% to 0.5% (w/w) and a CV of 0.6% to 2.35% for switchgrass and poplar respectively. The average of these SD values is 0.3%, so that three standard deviations are approximately equal to 1.0%(w/w), meaning that differences of about 1% in the weight percent of glucan and xylan of biomass are significant and measurable using the methods described here. This includes weighing, moisture analysis, volumetric glassware use and two quantitative transfer steps. This minimum detectable

Table 4

Random error associated with the quantitative analysis of the polysaccharide fraction of native switchgrass and poplar [5]

	Poplar	Switchgrass
% Glucan(w/w)		
Mean	38.96	31.82
SD	0.26	0.32
CV	0.66	0.99
% Xylan(w/w)		
Mean	14.53	20.24
SD	0.09	0.48
CV	0.61	2.35

[5] Error estimates are based on six analyses performed independently on two days(n=6)

change of 1.0% glucan content is approximately twice as large as that calculated from the peak area SD of pure sugar standards, and five times greater than the estimated capability of the detector cell alone(Sensitivity of HPLC section).

The 1.0% minimum detectable change in weight percent polysacharide is based on measuring relatively high sugar concentrations from biomass, in fact the highest in the study, since they were determined using native biomass. Remember that the peak area standard deviations in Figure 1 were smaller at lower concentrations, and that smaller standard deviations mean greater precision. This relationship would fall apart as one approached the detection limit of the detector, since the signal to noise ratio would become too low, However, as will be shown in the next paragraphs, measuring small amounts of xylan in PHS can have greater sensitivity than measuring large amounts.

The data in Table 5 show the results of triplicate analysis of duplicate runs for two sets of pretreatment conditions for poplar. The conditions were 180 °C, 0.6 minutes, 0.9% acid(runs A and B) and 180 °C, 0.56 minutes, 1.0% acid(runs C and D). These treatments are considered harsh and solubilized over 90% of the xylan present in the native feedstock. The standard deviations for weight percent glucan content of the pretreated solids ranged from 0.1% to 0.5%. These standard deviations are very close to those obtained from analysis of native poplar(Table 4) and indicate that the error associated with the analysis of PHS is similar to that associated with the analysis of native feedstock.

Three standard deviations of the glucan measurement for PHS corresponds to a maximum of 1.5% weight percent glucan. There was very little xylan in the PHS of all four of these runs, and the standard deviations of weight percent xylan ranged from 0.01 to 0.03% for the triplicate measurements of individual runs. Three standard deviations for xylan content represent 0.1 %(w/w) xylan. This is significantly less than the value obtained for native biomass(1.0%, Table

Table 5
Random error associated with the quantitative analysis of the polysaccharide fraction of pretreated poplar solids(PHS) and poplar prehydrolysate(PH)[6]

	Prehydrolysate				*Prehydrolyzed Solids*			
	Glucose(mg/mL)		Xylose(mg/mL)		Wt.% glucan		Wt.% Xylan	
0.6 min., 180°C, 0.9% acid								
	run A	run B	run A	run B	run A	run B	run A	run B
Mean	0.346	0.389	2.018	2.124	50.7	51.7	1.7	1.4
SD	0.001	0.003	0.007	0.017	0.3	0.2	0.01	0.01
CV	0.33	0.68	0.37	0.82	0.5	0.4	0.8	0.8
Wt. Sugar in PH ± 3SD(mg)	479-489	538-560	2794-2856	2920-3068				
0.56 min., 180°C, 1.0% acid								
	run C	run D	run C	run D	run C	run D	run C	run D
Mean	0.390	0.377	2.131	2.062	52.0	51.8	1.4	1.4
SD	0.001	0.003	0.006	0.011	0.5	0.1	0.02	0.03
CV	0.29	0.81	0.28	0.52	0.9	0.2	1.4	2.5
Wt. Sugar in PH ± 3SD(mg)	550-559	530-556	3001-3052	2923-3015				

[6] Error estimates are based on triplicate analyses of two duplicated sets of pretreatment conditions
Four runs in total are represented, and a set of error estimates was calculated for each

4), which indicates that when smaller amounts of xylan are present(e.g. pretreated biomass), the minimum detectable change is smaller than when larger(e.g. native biomass).

Sensitivity of Prehydrolysate Analysis

The data from triplicate analyses on the PH from two sets of pretreatment conditions performed on poplar in duplicate are shown in Table 5. The standard deviation for glucose in the prehydrolysate ranges from 0.001 to 0.003 mg/mL for any individual run. Three SD represent a approximately 12 mg of glucan, or 0.1% of the original glucan added to the reaction vessel for both poplar and switchgrass.

A typical xylose concentration of a PH sample was 3.0 mg/mL. From Figure 1 and Equation 9, three SD of a standard xylose solution of this concentration gives a value of 0.012 mg/mL. In contrast, the xylose concentration values in Table 3 are for a PH sample hydrolyzed in triplicate. It is not surprising that three times the xylose concentration SD for a given a PH sample in Table 5 anges up to 0.06 mg/mL. With a prehydrolysate volume of 1400 mL, this represents 74 mg of xylan, or 1.8 % of the original xylan in a 30g poplar sample added to the vessel, or, 1.3% of the xylan in a 30g sample of switchgrass.

From these reproducibility studies, the minimum detectable difference between prehydrolysate samples is approximately 2% (w/w) of the total xylan and about 0.1% (w/w) of the total glucan added to the reaction vessel.

Reproducibility of pretreatment conditions

The calculated minimum detectable changes(MDC) in polysaccharide content of PHS and PH represent an estimate of the sensitivity of the analytical procedure. It was determined that differences in 1.0%(w/w) glucan in the PHS were real. The weight percent xylan MDC for PHS is harder to define. It is lower(0.1%) at low xylan content, and higher at high xylan content(0.5%). In PHS analyses, the MDC is approximately 2.0% of the total xylan and 0.1% of the total glucan added to the reaction vessel.

The next task is to estimate how much variability is associated with the pretreatment process itself. An estimate of this variability may be obtained by repeating a set of pretreatment conditions and analyzing the resulting PH and PHS. If the polysaccharide contents of the PH and PHS from replicate pretreatment runs are found to vary much more than replicate analyses of a single PHS and PH sample, the pretreatment itself introduces variability. Using switchgrass, one set of pretreatment conditions, 160°C, 0.9% acid, 3 minutes was performed three times, and the resulting PHS and PH were analyzed in duplicate, and the mean values were used to calculate the polysaccharide content mean, SD, and CV values for this set of pretreatment conditions(n=3). It should be noted that these conditions are harsh enough to solubilize 90% of the xylan originally present in the native switchgrass, and thus small variations in pretreatment conditions are not as likely to be detected as under milder pretreatment conditions. A summary of the results is shown in Table 6.

The standard deviation for weight percent glucan in the solids from the triplicate runs is about the same as for triplicate measurements of the material from a single run, which indicates no detectable variability is introduced by the pretreatment. In contrast, the SD for weight percent xylan of the three PHS samples was 0.2%, ten times greater than the 0.02% value for replicate analyses of the same PHS(Table 5). This indicates that the hydrolysis of xylan from the

Table 6
Random error associated with replicate pretreatments[7]

	Native Switchgrass	*Prehydrolyzed Solids*						*Prehydrolysate*		
Component	Weight %	Weight %			% Recovery of Component			% Recovery of Component		
		mean	SD	CV	mean	SD	CV	mean	SD	CV
dry matter	100.0				56.0	0.4	0.6			
glucan	31.8	50.4	0.5	0.9	88.5	1.2	1.3	17.0	1.1	6.5
xylan	20.3	3.4	0.2	6.0	9.5	0.6	5.8	84.9	3.0	3.6
galactan	0.0	0.0			0.0					
arabinan	3.5	0.5	0.1	24.7	7.7	2.3	29.8	107.4	2.1	2.0
mannan	0.4	0			0.0			35.9	1.0	2.9
ash	7.1	5.4	0.2	2.8	43.0	1.2	2.8			
klason lignin	19.5	31.6	0.4	1.1	91.0	1.0	1.1			
acid soluble lignin	3.7	1.6	0.1	3.7	23.8	0.9	3.6			

[7]One set of pretreatment conditions(160°C, 0.9% acid,3 minutes)was performed three times and the resulting prehydrolyzed solids and prehydrolyzate were analyzed in duplicate.

biomass was variable. The total variance in the weight percent xylan of the PHS from the three replicate pretreatments can be thought of as

$$(Eq10)\quad \sigma^2_{total} = \sigma^2_{pretreatment} + SEM^2_{analysis}$$

Using the mean of duplicate analyses of the PHS and PH reduces the error associated with analysis, and the square of the standard error of the mean is used instead of the square of the SD to calculate the error associated with the analysis. Calculating $SEM^2_{analysis}$(0.0005) from the SD values in Table 5 and the σ^2_{total}(0.04) from the SD values in Table 6, and substituting into Equation 10, yields a $\sigma^2_{pretreatment}$ equal to 0.039, or nearly equal to σ^2_{total}. This indicates that 99% of the variability associated with evaluating the effect of pretreatment conditions on xylan content of the PHS is due to the pretreatment, not the analysis.

Calculating the $\sigma^2_{pretreatment}$ using other parameters, such as weight percent glucan of the PHS and percent of original xylan recovered in the PH, reveals that the 80% of the variance is due to $\sigma^2_{pretreatment}$. The reduced influence of pretreatment on the total variance can be explained by xylan's greater susceptibility to acid hydolysis. The hydrolysis of xylan is expected to be more variable than that of glucan, since glucan is less susceptible to pretreatment than xylan, which is the very reason the pretreatment is used, i.e. to solubilize hemicellulose preferentially to cellulose. It should again be noted that the conditions used to calculate the $\sigma^2_{pretreatment}$ were harsh, and thus more reproducible than would be expected for comparatively mild pretreatment conditions. If conditions were chosen where less of the xylan were solubilized, it is likely that the variation would be greater. In fact, duplicate runs were performed on switchgrass under milder conditions(1 min., 160°C, 1.2% acid) and the percent of xylan recovered in the PHS was found to be 12.1%

and 23.5% on these two duplicate runs(Table 7). For poplar, three sets of milder conditions were performed in duplicate(1 min., 160°C, and 0.6,0.9 and 1.2% acid), and the duplicates ranged from 10% to 25% in the percent of xylan recovered in the PHS and PH(Table 8).

It seems that the larger source of error is the pretreatment itself and that the focus of continued work with this reactor design should be aimed at establishing its reproducibility over a wider range of preteratment conditions.

Results of pretreatment

The xylan content of prehydrolyzed solids and prehydrolysate are shown in Tables 7 and 8, and graphed in Figures 2 and 3. Both graphs show many of the pretreatment conditions yield solids which lie within the 1%(w/w) sensitivity limit, calculated from the SD of repeated solids analysis. However, remember that at lower xylan content(reproducibility of PHS analysis section, Table 5), a lower minimum detectable change(0.1%) was calculated. This figure was calculated when xylan content was extremely low(1.4% w/w), yet even with this low minimum detectable change, many of the runs are indistinguishable from one another. When the xylan content of the PHS increases to the higher levels, as seen in milder pretreatments, the minimum detectable change increases as well, so that many of the runs are indistinguishable with respect to weight percent xylan.

Also graphed is the percent of original dry matter recovered in the prehydrolyzed solids(Figures 4 and 5). The weight percent polysaccharide of the PHS is multiplied by the dry matter recovered in the PHS to obtain the percent of original polysaccharide added to the reaction vessel which was

Table 7
Composition of Switchgrass PHS and PH

Pretreatment conditions	% of original dry matter in PHS	Wt% glucan of PHS	% of Original glucan in PHS	% of Original glucan in PH	Wt% xylan of PHS	% of Original xylan in PHS	% of Original xylan in PH	Total % Xylan Recovered
0 min., 140°C,1.2%	74.5	38.4	89.0	nd	21.8	80.0	nd	
0.5 min., 180°C, 0.6%	56.1	50.6	88.2	16.6	5.0	13.8	78.5	92.3
0.5 min., 180°C, 0.9%	54.7	51.5	87.5	17.8	5.0	13.4	84.7	98.1
0.5 min., 180°C, 1.2%	56.1	50.6	88.2	16.6	5.0	13.8	78.5	92.3
1 min., 160°C, 0.6%	62.9	43.9	85.9	nd	11.8	36.6	nd	
1 min., 160°C, 0.9%	59.7	46.1	85.6	nd	8.6	25.3	nd	
1 min., 160°C, 1.2%	59.1	46.1	84.8	nd	8.1	23.5	nd	
1 min., 160°C, 1.2%	55.8	50.3	87.3	16.4	4.4	12.1	81.3	93.4
1 min., 180°C, 0.6%	53.0	50.4	84.0	16.2	4.3	11.4	80.2	91.6
1 min., 180°C, 0.9%	52.7	51.3	84.1	nd	3.0	7.7	nd	
1 min., 180°C, 0.9%	51.6	52.5	84.4	17.1	1.7	4.5	88.3	92.8
1 min., 180°C, 1.2%	51.5	51.6	82.6	nd	2.2	5.5	nd	
2 min., 160°C, 0.9%	56.8	47.0	83.1	nd	6.8	19.0	nd	
2 min., 160°C, 1.2%	55.3	48.3	83.1	nd	4.9	13.3	nd	
2 min., 180°C, 0.6%	54.3	51.2	87.5	16.4	5.2	13.3	72.5	85.8
2 min., 180°C, 0.9%	50.0	52.3	82.1	19.2	2.3	5.7	77.1	82.8
2 min., 180°C, 1.2%	49.2	51.7	79.9	21.3	1.9	4.7	72.2	76.9
3 min., 140°C, 0.6%	66.1	41.3	84.8	nd	17.7	57.7	nd	
3 min., 140°C, 0.9%	62.6	45.6	88.7	nd	12.9	39.6	nd	
3 min., 140°C, 1.2%	62.0	44.5	85.8	nd	12.0	36.6	nd	
3 min., 140°C, 1.2%	58.8	46.7	85.5	nd	10.8	31.4	nd	
3 min., 160°C, 0.6%	59.2	45.5	83.8	nd	10.0	29.1	nd	
3 min., 160°C, 0.9%	55.0	49.4	84.6	nd	5.3	14.3	nd	
3 min., 160°C, 0.9%	55.3	51.9	89.2	15.9	3.3	9.1	83.6	92.7
3 min., 160°C, 0.9%	54.5	51.5	87.2	16.9	3.6	9.6	84.9	94.6
3 min., 160°C, 0.9%	55.8	51.5	89.3	18.1	3.8	10.4	89.4	99.8
3 min., 160°C, 1.2%	59.0	47.0	86.2	nd	9.0	26.1	nd	
3 min., 180°C, 0.6%	53.3	51.8	85.9	nd	3.8	9.9	nd	
3 min., 180°C, 0.9%	50.6	52.7	82.9	nd	2.4	5.9	nd	
3 min., 180°C, 1.2%	48.4	52.4	79.6	22.1	1.5	3.7	71.2	74.8
5 min., 140°C, 0.6%	64.4	42.4	84.8	nd	15.0	47.7	nd	
5 min., 140°C, 0.9%	59.8	46.7	86.8	nd	10.7	31.4	nd	
5 min., 140°C, 1.2%	56.5	48.6	85.4	nd	8.3	23.2	nd	
5 min., 160°C, 0.6%	55.3	48.9	84.0	nd	5.5	14.9	nd	
5 min., 160°C, 0.9%	52.6	52.9	86.5	16.7	2.3	6.0	84.7	90.7

Table 7, continued

Composition of Switchgrass PHS and PH, continued

Pretreatment conditions	% of original dry matter in PHS	Wt% glucan of PHS	% of Original glucan in PHS	% of Original glucan in PH	Wt% xylan of PHS	% of Original xylan in PHS	% of Original xylan in PH	Total % Xylan Recovered
5 min., 160°C, 0.9%	55.4	48.6	83.8	nd	5.5	15.0	nd	
5 min., 160°C, 1.2%	51.7	51.4	82.6	nd	2.8	7.2	nd	
5 min., 180°C, 0.6%	49.5	51.4	79.1	nd	2.4	5.8	nd	
5 min., 180°C, 0.9%	49.1	49.0	74.8	nd	1.6	3.9	nd	
5 min., 180°C, 1.2%	49.1	45.0	68.7	nd	4.4	10.7	nd	
15 min., 140°C, 0.6%	58.8	46.6	85.2	nd	10.3	29.7	nd	
15 min., 140°C, 0.9%	56.2	49.9	87.3	15.8	5.1	14.1	78.2	92.3
15 min., 140°C, 0.9%	58.6	45.7	83.4	nd	9.5	27.3	nd	
15 min., 140°C, 1.2%	53.3	46.3	76.7	nd	10.8	28.3	nd	
30 min., 140°C, 0.6%	57.1	48.4	86.7	16.5	8.4	24.0	72.1	96.0
30 min., 140°C, 0.9%	61.3	44.3	85.3	14.7	10.8	32.9	58.9	91.8
30 min., 140°C, 1.2%	54.4	49.6	83.9	nd	4.6	12.3	nd	
60 min., 140°C, 0.6%	55.7	49.1	85.9	16.4	6.9	19.0	71.5	90.5
60 min., 140°C, 0.9%	54.4	49.9	84.4	nd	3.9	10.4	nd	
60 min., 140°C, 1.2%	53.3	50.3	83.4	nd	3.4	9.0	nd	

Table 8

Compositon of Poplar PHS and PH

Pretreatment Conditions	% of Original dry matter in PHS	Wt% glucan of PHS	% of Original glucan in PHS	% of Original glucan in PH	Wt% xylan of PHS	% of Original xylan in PHS	% of Original xylan in PH	Total % Xylan Recovered
0.5 min., 170°C, 1.2%	72.1	52.6	95.3	4.4	2.5	12.1	75.3	87.4
0.5 min., 180°C, 0.9%	69.4	54.3	94.8	5.1	1.9	8.8	80.2	89.1
0.5 min., 180°C, 1.2%	66.9	54.9	92.2	6.4	1.3	5.8	78.9	84.8
0.53 min., 180°C, 1.1%	67.6	53.8	91.4	5.6	1.6	7.5	80.3	87.8
0.56 min., 180°C, 1.0%	73.0	54.9	100.7	5.8	1.5	7.2	82.7	89.9
0.56 min., 180°C, 1.0%	74.9	54.8	103.2	5.7	1.5	7.4	81.1	88.5
0.6 min., 180°C, 0.9%	75.3	54.5	103.1	5.7	1.5	7.7	81.8	89.5
0.6 min., 180°C, 0.9%	75.4	53.3	101.1	5.0	1.8	9.4	77.1	86.5
1 min., 160°C, 0.6%	82.2	48.4	100.0	2.1	9.5	52.6	45.1	97.8
1 min., 160°C, 0.6%	78.3	51.8	101.9	1.8	4.4	23.4	50.9	74.2
1 min., 160°C, 0.9%	75.6	53.0	100.8	1.8	3.5	18.0	56.7	74.7
1 min., 160°C, 0.9%	76.0	55.4	105.8	3.1	5.0	25.5	64.1	89.7
1 min., 160°C, 1.2%	73.2	55.1	101.4	1.8	2.3	11.4	44.2	55.6
1 min., 160°C, 1.2%	75.7	54.1	102.9	3.1	4.9	25.2	62.6	87.8
1 min., 180°C, 0.6%	68.8	56.1	97.0	6.6	0.9	4.2	90.6	94.8
1 min., 180°C, 0.9%	64.6	55.4	90.0	6.6	1.2	5.1	83.0	88.2
1 min., 180°C, 0.9%	66.2	58.3	97.0	7.2	0.3	1.3	78.8	80.1
1 min., 180°C, 1.2%	64.4	56.4	91.3	8.5	0.2	0.9	76.0	76.9
1.2 min., 170°C, 1.2%	67.6	54.0	91.7	5.7	1.4	6.5	82.7	89.2
1.3 min., 170°C, 1.1%	69.7	53.1	93.1	5.4	1.3	5.9	80.4	86.3
2 min., 160°C, 0.6%	74.6	53.8	100.9	0.0	2.8	13.8	64.5	78.3
2 min., 160°C, 0.9%	72.1	54.3	98.5	3.6	2.1	10.1	70.4	80.5
2 min., 160°C, 1.2%	70.5	54.9	97.3	4.2	1.4	6.7	72.7	79.4
2 min., 180°C, 0.6%	67.3	57.1	96.5	4.5	0.6	2.8	72.9	75.7
2 min., 180°C, 0.9%	65.9	56.3	93.2	7.5	0.4	1.8	62.6	64.5
2 min., 180°C, 1.2%	61.9	57.2	89.1	5.3	0.0	0.0	72.2	72.2
2 min., 170°C, 1.2%	67.2	54.4	91.9	6.2	1.3	5.8	81.1	86.9
3 min., 160°C, 0.6%	74.1	53.1	98.8	3.4	2.4	11.8	69.0	80.8
3 min., 160°C, 0.9%	69.5	54.3	94.8	4.7	1.3	6.1	76.1	82.1
3 min., 160°C, 0.9%	71.1	53.5	95.7	3.9	3.2	15.5	71.3	86.8
3 min., 160°C, 1.2%	68.1	54.0	92.5	1.8	0.8	3.6	76.5	80.1
3 min., 180°C, 0.6%	64.9	57.4	93.6	5.9	0.4	1.8	68.1	69.9
3 min., 180°C, 0.9%	63.0	56.7	89.9	9.8	0.0	0.0	69.5	69.5
3 min., 180°C, 1.2%	61.2	54.5	83.8	11.7	0.0	0.0	57.7	57.7

Table 8, continued

Compositon of Poplar PHS and PH, continued

Pretreatment Conditions	% of Original dry matter in PHS	Wt% glucan of PHS	% of Original glucan in PHS	% of Original glucan in PH	Wt% xylan of PHS	% of Original xylan in PHS	% of Original xylan in PH	Total % Xylan Recovered
5 min., 140°C, 0.6%	87.8	44.1	97.2	1.2	11.2	66.0	24.7	90.7
5 min., 160°C, 0.6%	73.2	52.8	97.1	3.4	3.5	17.3	66.9	84.2
5 min., 160°C, 0.9%	69.1	54.8	95.1	4.6	2.0	9.1	73.3	82.4
5 min., 160°C, 0.9%	69.5	53.8	94.0	4.8	1.9	8.9	77.9	86.8
5 min., 160°C, 1.2%	68.1	55.3	94.8	5.2	2.0	9.1	70.2	79.3
5 min., 180°C, 0.6%	63.5	56.5	90.2	7.7	0.5	2.2	55.1	57.3
5 min., 180°C, 0.9%	59.1	54.0	80.2	13.4	0.0	0.0	42.3	42.3
5 min., 180°C, 1.2%	57.7	51.5	74.7	19.4	0.0	0.0	43.5	43.5
15 min., 140°C, 0.6%	79.1	49.0	97.6	1.9	7.8	41.6	48.9	90.5
15 min., 140°C, 0.9%	74.0	51.2	95.3	3.5	5.5	27.3	64.2	91.5
15 min., 140°C, 0.9%	74.4	51.7	96.8	3.0	4.3	21.4	65.0	86.4
15 min., 140°C, 1.2%	71.2	52.6	94.1	3.0	4.4	21.0	66.8	87.8
30 min., 140°C, 0.6%	73.5	53.7	99.2	1.7	3.0	14.7	34.4	49.1
30 min., 140°C, 0.9%	70.6	56.6	100.4	3.1	2.2	10.4	55.7	66.1
30 min., 140°C, 1.2%	69.5	55.2	96.4	4.3	1.6	7.6	69.9	77.5
60 min, 140°C, 0.6%	71.4	54.3	97.4	2.8	2.8	13.5	64.8	78.3
60 min., 140°C, 0.9%	67.7	54.8	93.3	5.0	1.3	6.0	68.0	73.9
60 min., 140°C, 1.2%	67.1	56.0	94.4	5.2	1.3	5.9	66.8	72.7

Figure 2

Weight Percent Xylan vs. Percentage of Original Dry Matter Recovered in Poplar PHS

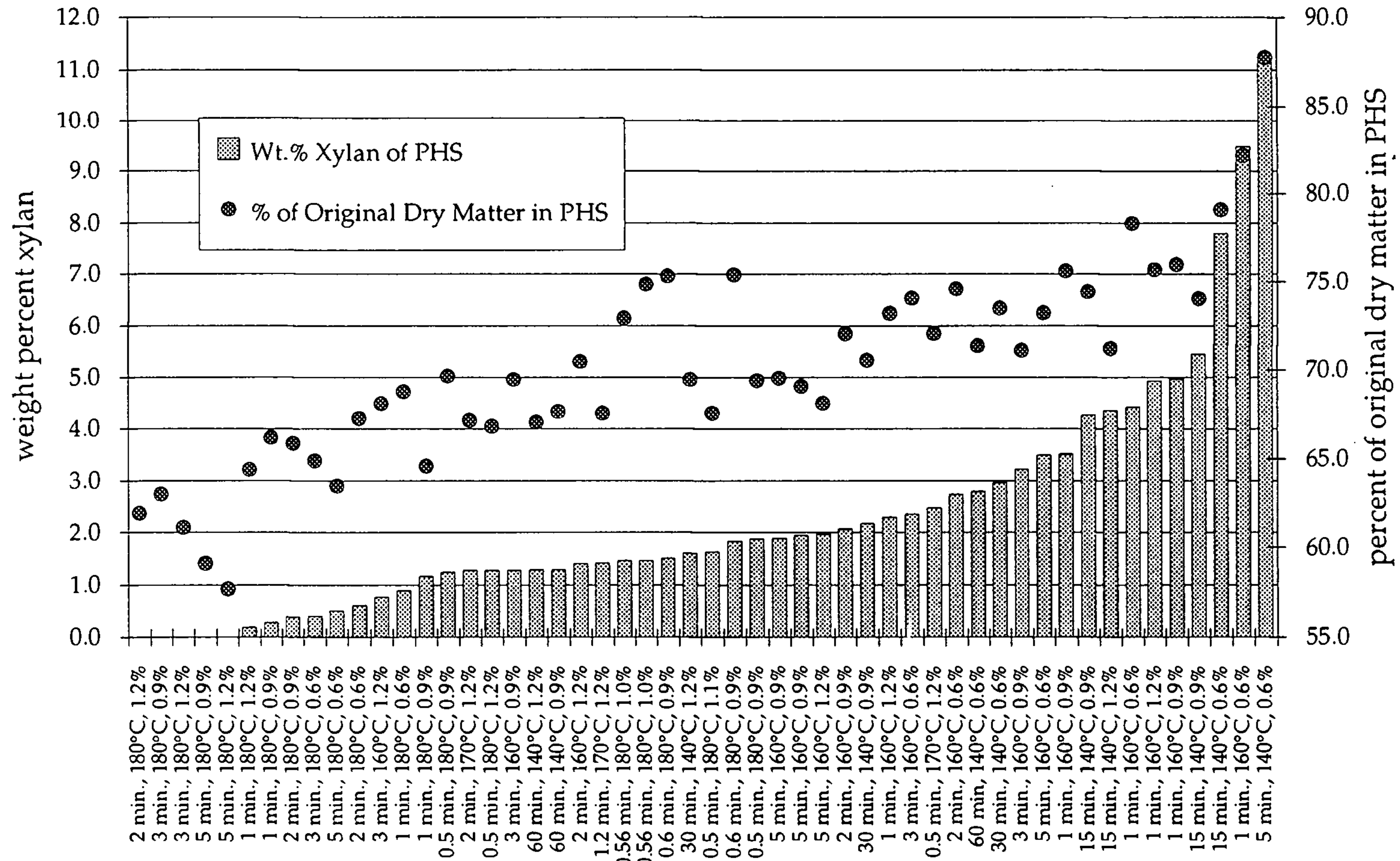


Figure 3

Weight Percent Xylan vs. Percent of Original Dry Matter Recovered in Switchgrass PHS

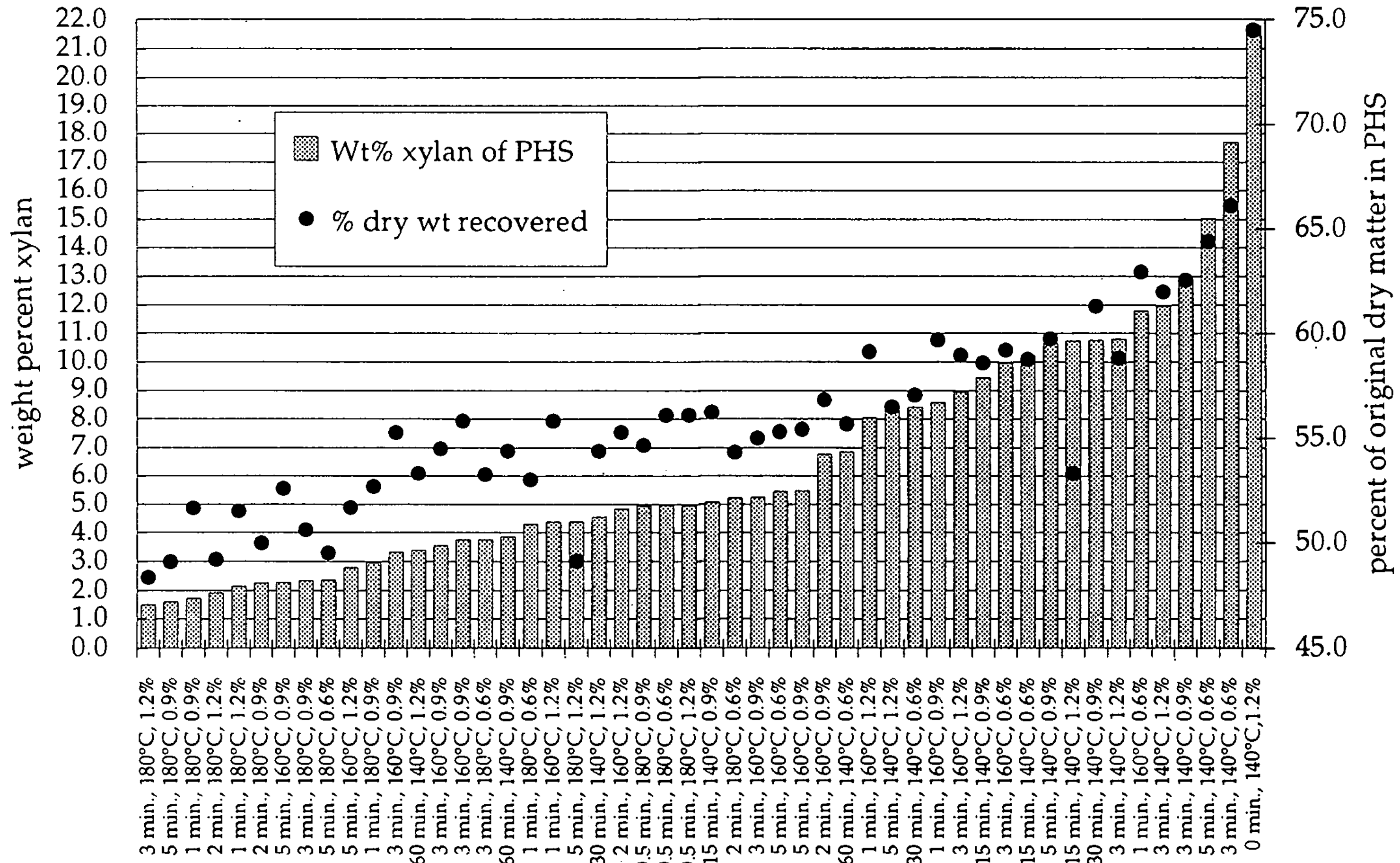


Figure 4

Weight Percent Xylan vs. Percent of Original Dry Matter Recovered in Prehydrolyzed Switchgrass Solids

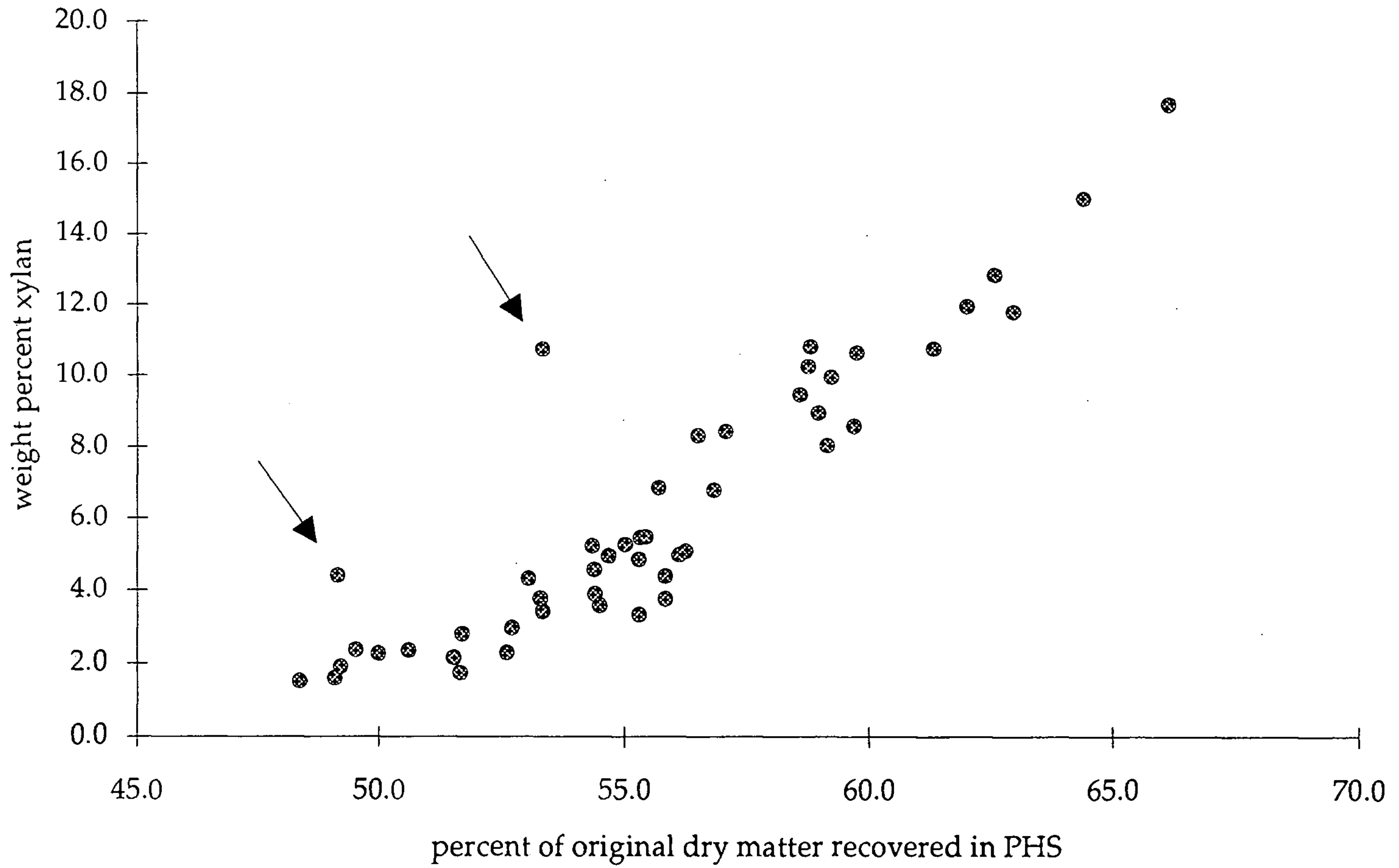
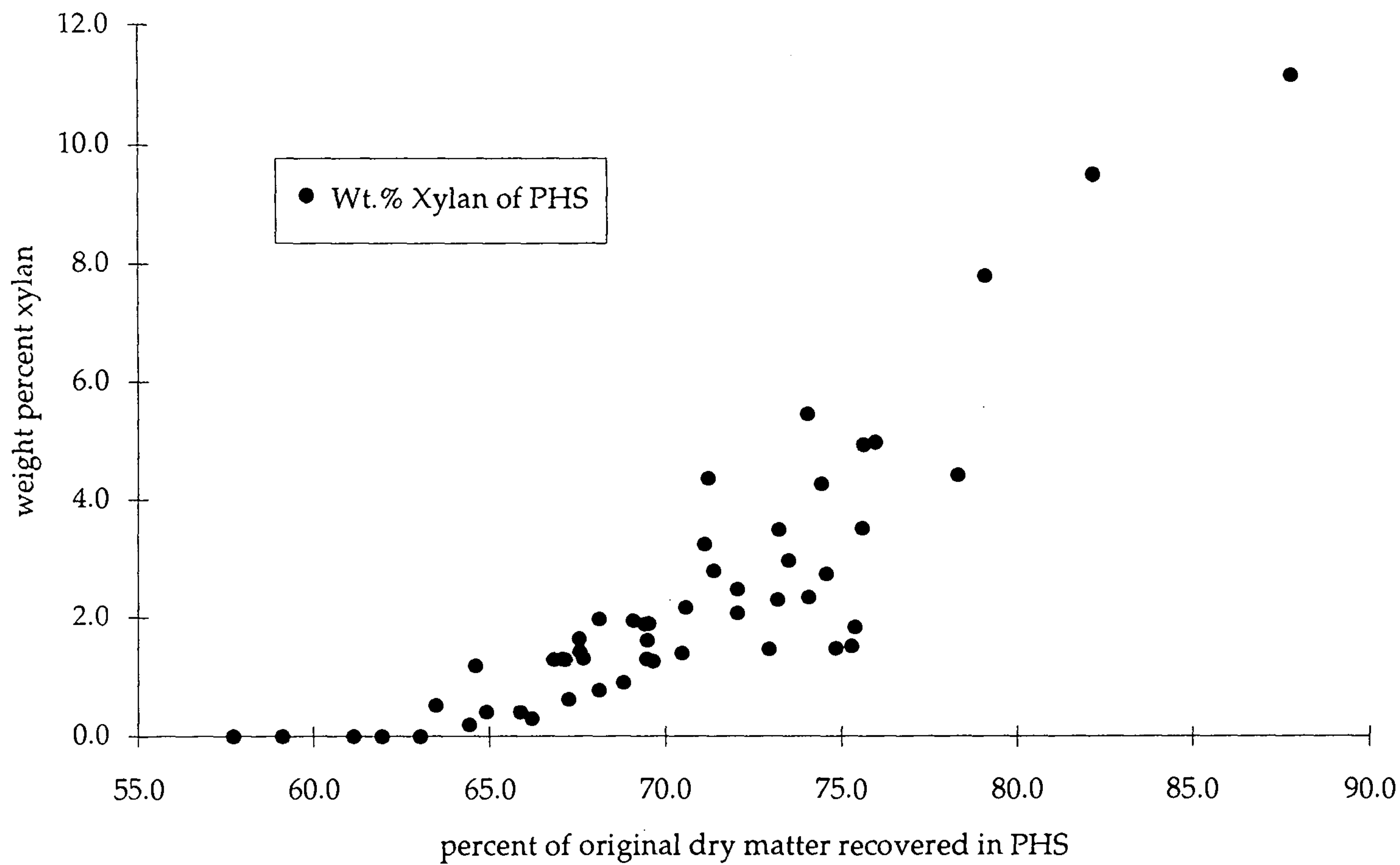


Figure 5

Weight Percent Xylan vs. Percent of Original Dry Matter Recovered in Prehydrolyzed Poplar Solids



recovered in the PHS . When the solids are indistinguishable from one another by polysaccharide analysis, the amount of dry matter recovered takes on added significance, because when multiplied by the weight percent polysaccharide, it can make a difference where there may be none. The dry matter recovery varies by more than would seem warranted by the amount of xylan and glucan remaining in the solids(Figures 2 and 3). Some of the potential sources of variability in the measurement of dry matter recovered are discussed below.

Transfer of biomass from reactor to Buechner funnel

The quantitative transfer of biomass to Buechner funnel is difficult. The reactor has an agitator, acid injection tube, and cooling coil which must be rinsed free of biomass. Also, some of the biomass clings tenaciously to the sides of the reactor. While mass not transferred to the funnel should not directly affect the weight percent polysaccharide analysis of the biomass, it will definitely affect a calculation of overall sugar yield when the weight percent sugar yield is multiplied by the dry matter recovered. Given the low variability of saccharide analysis of dry matter, it seems the graph of the switchgrass data (Figure 4) shows two clear examples of runs where dry matter was lost between pretreatment and polysaccharide analysis. This would have the effect of showing an unusually low percentage of xylose recovered in the solids. The poplar graph (Figure 5) does not show any particular outliers.

Drying of two-piece Buechner funnel

Moisture determinations on the 45°C dried biomass do not appear to introduce much variability. The moisture contents of the samples are

remarkably stable. The values ranged between 97 and 99.5 % solids as determined at 105°C. This range represents a small source of error in determining the dry matter and saccharide recovered. For example, if the 45°C-dried, prehydrolyzed solids in the Buechner funnel are determined to be 97% or 99% solids(w/w) and there are 20 g of 45°C-dried solids in the funnel, the 20g are adjusted to either 20.62g or 20.20g, which represents a range of 69.8% to 71.2% dry matter recovery. The dry matter recovered is then multiplied by the weight percent xylan to determine the percent of original xylan present in the prehydrolyzed solids. For example 7%(w/w) xylan could be determined to represent 1.44 g or 1.42 g of xylan in the Buechner funnel. For poplar, this range covers 0.5% of the xylan added to the reaction vessel, and for switchgrass, it represents 0.3% of the initial xylan present.

The solids content of raw sample was much more variable. However, by doing many replicate measurements(n=6) for raw sample, and weighing the 30 g samples for pretreatment on a single day, the variability due to moisture analyses was sufficiently small.

Miscellaneous sources of error

Uniform, representative sample

For PHS samples it is necessary to leave some sample on the filter paper when transferring the sample from the Buechner funnel to the mortar for grinding. If the filter is scraped too hard, paper contaminates the sample and increases the apparent glucose concentration. It could be that the sample is not completely representative.

Volume occupied by lignin in volumetric flask

After two-stage acid hydrolysis of lignocellulosic biomass, solid lignin remains. It comprises 20-35% of the dry weight. If 0.3 g of biomass are used for the analysis, a maximum of 0.15 g lignin is transferred into the 100 mL volumetric flask along with the hydrolyzed sugars. The lignin is denser than water, and therefore occupies less than 0.15 cubic centimeters. The tolerance of a Class A volumetric flask is 0.08 mL(VWR,1994), therefore the lignin introduces very little error in volume measurements, if any at all.

Recommendations for future work

A suitable internal standard for biomass analyses was not found until after the pretreatments were well under way. Part of the difficulty was that when using the manufacturer-recommended guard column, there is a large 'solvent-ion' sloping baseline which coelutes with the sugars. This sloping baseline dropped precipitously at slightly variable times. The internal standards tested(mannitol, erythritol, and other sugar alcohols) eluted near this drop-off point, and were thus hard to integrate. A de-ashing guard column eliminates this sloping baseline, and makes the choice of an internal standard easier.

Bibliography

Banerjee N, Bhatnagar R, and Viswanathan L. Inhibition of glycolysis by furfural in *Saccharomyces cerevisiae*. *European Journal of Applied Microbiology and Biotechnology*. 11, pp. 226-228, (1981).

Blakeney AB, Harris PJ, Henry RJ, and Stone BA. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research*. 113, pp. 291-299, (1983).

Boyer LJ, Vega JL, Klasson KT, Clausen EC, and Gaddy JL. The efffects of furfural on ethanol production by *Saccharomyces cerevisiae* in batch culture. *Biomass and Bioenergy*. 3(1), pp. 41-48, (1992).

Coughlan MP and Hazlewood GP. Hemicellulose and Hemicellulases. London, England, (1992).

Fox DJ, Gray PP, Dunn NW, and Marsden WL. Comparison of Alkali and Steam(acid) pretreatments of lignocellulosic materials to increase enzymic susceptibility: evaluation under optimised pretreatment conditions. *Journal of Chemisty and Technological Biochemisrty*. **44**, pp. 135-146, (1989).

Gey M, Rietzschel A, and Nattermueller W. Characterization of biotechnological processses and products using high-performance liquid chromatography(HPLC)-determination of organic compounds in sulphite liquor. *Acta Biotechnologia*. 11(5), pp. 511-519, (1991).

Grethlein HE, Allen DC, and Converse A. A comparitive study of the enzymatic hydrolysis of acid-pretreated white pine and mixed hardwood. *Biotechnology and Bioengineering*. 26, pp. 1498-1505, (1984).

Grohmann K, Torget T, and Himmel M. Optimization of dilute acid pretreatment of biomass. *Biotechnology and Bioengineering Symposium*. 15, pp. 59-80, (1985).

Kaar WE and Brink DL. Summative analysis of nine common north american woods. *Journal of Wood Chemistry*. 11(4), pp. 479-494, (1991).

Kaar WE and Brink DL. Simplified analysis of acid-soluble lignin. *Journal of Wood Chemistry*. 11(4), pp. 465-477, (1991).

Kaar WE, Cool LG, Merriman MM, and Brink DL. The complete analysis of wood polysaccharides using HPLC. *Journal of Wood Chemistry*. 11(4), pp. 447-463, (1991).

Knappert D, Grethlein H, and Converse A. Partial acid hydrolysis of poplar wood as a pretreatment for enzymatic hydrolysis. *Biotechnology and Bioengineering Symposium*. 11, pp. 67-77, (1980).

Lee DP and Bunker MT. Carbohydrate analysis by ion chromatography. *Journal of Chromatographic Science*. 27, pp. 496-503, (1989).

Mes-Hartree M, Dale BE, Craig WK. Comparison of steam and ammonia pretreatment for enzymatic hydrolysis of cellulose. *Applied Microbiology and Biotechnology*. **29**, pp. 462-468, (1988).

NREL. Chemical analysis and testing procedure #002. Two stage sulfuric acid hydrolysis for determination of carbohydrates. Golden, Colorado (1992).

Schwald W, Concin R, Bonn G. Analysis of oligomeric and monomeric carbohydrates from hyrothermal degradation of cotton waste materials using HPLC and GPC. *Chromatographia*. **20**(1), pp. 35-40, (1985).

Sjöström E. Wood Chemistry. San Diego, California, (1993).

TAPPI. Official Test Methods, Technical Association of the Pulp and Paper Industry(TAPPI). Atlanta, Georgia, (1993).

Torget R, Himmel M, Wright JD, and Grohmann K. Initial design of a dilute sulfuric acid pretreatment process for aspen wood chips. *Applied Biochemistry and Biotechnology*. **17**, pp. 89-104, (1988).

Wayman M and Parekh S. Biotechnology of Biomass Conversion. London, England, (1990).