PERACETIC ACID PRETREATMENT OF ALFALFA STEM AND ASPEN BIOMASS

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Alfalfa stems and ground aspen were exposed to peracetic acid (0.5 to 9% on biomass) at temperatures ranging from 40 to 100° C and reaction times from 1 to 5 hours. Glucose release as a percentage of total cellulose content was determined using subsequent standard enzymatic hydrolysis. Statistical analysis confirmed that aspen showed a strong response to peracetic acid addition rate. 9% peracetic acid removed 14% of the original lignin and increased the rate of glucose release from 23% to 44%. Temperature and reaction time played a less significant role. For alfalfa stems, low levels of peractic acid (0.5%) increased glucose release from 30 to 47%. The addition of larger doses of peracetic acid did not show any significant improvement; this effect appears to be closely related to rate of lignin removal. While peracetic acid effectively removed lignin from aspen, 98% of the original lignin was still present in alfalfa after higher level peracetic acid treatments; the yield loss observed during pretreatment of alfalfa stems originates from other biomass components.

Keywords: Peracetic acid; Alfalfa, Aspen; Biomass pretreatment; Cellulose conversion

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INTRODUCTION

Due to considerations such as climate change and instability of the petroleum producing regions, it is important to develop alternative energy sources to support the world energy consumption and decrease environmental pressures. Lignocellulosic biomass is considered to be one of the most promising sources of fermentable sugars and therefore is a promising feedstock for bioenergy sources such as bio-ethanol or bio-butanol (Perlack et al. 2005; Wiselogel et al. 1996). As pointed out by a study performed by DOE and USDA, 1.3 billion dry tons of biomass is expected to be available annually for bioenergy industries. Utilizing that biomass to produce bioenergy could displace more than 30% of our current fuels consumption (Perlack et al. 2005). About 998 million dry tons of this biomass is agricultural byproducts (Perlack et al. 2005).

However, conversion of lignocellulosic material to fermentable sugars still has considerable limitations due to the natural recalcitrance of plant cell walls to chemical, physical, and enzymatic degradation. The encrusting material, lignin, is partially linked to the polysaccharides in the cell walls, restricting access to the polysaccharides and unproductively binding cellulases (Berlin et al. 2006; Chernoglazov et al. 1988; Converse et al. 1990; Wiselogel et al. 1996). In addition, part of the cellulose exists in crystalline form in plant material, restricting complete sugar conversion. To achieve high yields of

fermentable sugars, the biomass must be exposed to effective pretreatments (Gharpuray et al. 1983; Myung and Kennelly 1992; Toyama and Ogawa 1975; Zhu, et al. 2008). Over the past few decades, a large number of pretreatment technologies have been considered. These pretreatment processes can be grouped into physical, chemical, and biological methods. Combination processes which utilize at least two of the methods above in order to increase pretreatment effect have also been developed (Wiselogel et al. 1996).

This study examines the possible use of low concentrations of peractic acid as a biomass pretreatment option. Previous studies using peracetic acid in the pretreatment of biomass before conversion are limited and have mainly focused on high concentrations of peracetic acid under either high temperature for a short period of time (Rodriguez-Vazquez 1993) or ambient temperature for seven days (Teixeira et al. 2000). For example Zhao et al. (2007, 2008) used up to 50% peracetic acid (based on dry material) to pretreat sugar cane bagasse to enhance enzymatic digestability. Currently the price of peracetic acid ranges from $1.5 to $10 /kg, making high level applications in biomass pre-treatment difficult to justify (see sites http://peraceticacidsystems.com/prices.html and http://www.alibaba.com/product-gs/233191654/peracetic_acid.html). Our group (De Lu et al. 2011; Duncan et al. 2010) explored the use of enzymatically generated peracetic acid as a pretreatment option for aspen biomass. This method, while being very effective, used expensive enzymes and chemicals and will need further development to make it economically feasible. To reduce peracetic acid charge required for good enzymatic digestability, Teixeira et al. (1999) and Zhao et.al (2009) used an additional caustic treatment before the peracetic acid pre-treatment step. Zhao (2009) used 10% NaOH pretreatment (based on dry biomass) for 1.5 h at 90 °C followed by a 2.5 h peracetic acid application (10% on dry biomass) at 75°C for bagasse. As of this time, no studies have been performed on one-step, short term, low concentration peracetic acid pretreatments of biomass to enhance enzymatic hydrolysis.

The other focus of our current study is on the comparison of pretreatment agents' effect on plant species with different lignin structures and composition. Specifically, we are considering the differences in pretreatment response of a common hardwood (aspen) and an agricultural residue (alfalfa stems).

Alfalfa (*Medicago sativa*) is a perennial flowering plant cultivated as an important forage crop (Putnam et al. 2001; Putnam 2004). For our study we used alfalfa stems only, a byproduct after removal of the protein-rich alfalfa leaves for cattle feed. Utilization of the byproduct alfalfa stems for industrial use has just started to attract researchers' attention. Alfalfa stems have been considered for fuel through combustion and for biological conversion to ethanol and other chemicals (Koegel and Straub 1996; Chen et al. 2007; Sreenath et al. 1999, 2001a,b).

Aspen (*Populus tremuloides*) is a hardwood species commonly used in industrial applications, such as paper manufacturing. Besides whole wood, waste materials such as saw dust and branches are expected to be readily available. Aspen lignin and alfalfa lignin are known to have considerable differences in cell wall and in lignin structure. For example, it is known that aspen lignin has a Syringyl/Guaiacyl (S/G) ratio of around 2 (Hou and Li 2011), while the S/G ratio for alfalfa lignin is around 0.5 (Baucher et al. 1999).

EXPERIMENTAL

Materials

Alfalfa stems were obtained from a field study performed at the University of Minnesota's Southern Field and Outreach Center in Waseca, MN. The aspen wood chips were commercial chips received from the SAPPI mill in Cloquet, MN. The raw materials were air dried, then ground using a Wiley mill. The fraction passing through a 40 mesh screen was designated for experimental use.

Methods

The composition of biomass was determined according to NREL standard method TP-510-42618 (Sluiter et al. 2008). For this analysis, ground biomass samples were hydrolyzed in a water bath for 60 minutes at 30°C using 72% H_2SO_4. The samples were diluted with deionized water and heated in an autoclave at 120°C for 60 minutes. After cooling to room temperature, the acid-insoluble lignin was filtered off (Klason lignin), dried at 105°C, and weighed. The filtrate obtained after Klason lignin removal was used to determine the carbohydrate content. The contents were determined by preparing calibration solutions from pure sugar monomers, dimers, and polymers purchased from Sigma-Aldrich (D-cellobiose, Cellulose, , D-(+)-xylose, D-(+)-galactose, D-(+)-mannose and D-(-) arabinose). The HPLC used was a Waters system equipped with a Bio-Rad De-ashing cartridge in line with a VARIAN MetaCarb 87P analytical column. The operating pressure was 541 psi at a flow rate of 0.3 mL/min and a column temperature of 80 ºC.

Biomass digestibility was determined using NREL standard procedure TP-510-42629 (Selig et al. 2008). Cellulase used in the experiment was obtained from Sigma-Aldrich Co. The enzyme activity expressed as "filter-paper units" (FPU) was determined using the NREL procedure proposed by Adney and Baker (1996). β-glucosidase (Novozyme 188), which was obtained from Sigma-Aldrich Co., was assayed by the method recommended by Herr using pNPG (p-nitrophenyl-p-D-glucosid) (Herr 1979). β-glucosidase activity is expressed in pNPGU. Combined cellulase and Novozyme 188 with a ratio of 60 FPU and 64 pNPGU were added to each gram of cellulose suspension at a solid to liquid ratio of 1:200. Enzymatic digestion was performed in a 50^{o}C shaking incubator for up to seven days at 150 rpm in 0.05M sodium citrate buffer (pH 4.8).

For all samples, pretreatments were performed in 250 mL beakers using a sample size of ten grams of biomass and sixty grams of reaction mixture (biomass-to-liquid ratio of 1:6). This was the highest consistency allowing efficient mixing and complete wetting of the material. No pH adjustments were made; starting pH values for aspen varied from 4.45 (water only) to 1.81 (9% PAA) and for alfalfa from 6.08 (water only) to 2.5 (9% PAA). The beakers were covered and heated in a water bath. Pretreatment was carried out using different reactants, including peracetic acid, acetic acid, and 2% hydrogen peroxide. All chemicals were obtained from Sigma-Aldrich and used as received. Concentrations of the purchased peracetic acid and hydrogen peroxide were determined through iodometric and permanganometric titrations as proposed by Bodiroga and Ognjanović (2002).

After pretreatment, the residues were washed with deionized water until neutrality and dried at room temperature for one day. The dried samples were stored in bags for

subsequent composition analysis and enzymatic hydrolysis. Liquids from pretreatments were collected.

RESULTS AND DISCUSSION

The chemical composition of aspen wood and alfalfa stems used in this study are listed in Table 1. As was expected, a distinct difference was observed in the chemical composition between the aspen wood chips and the alfalfa stems. Alfalfa stems have considerably lower lignin content (15.2 % vs. 21.1%) but significantly higher ash content (8.6% vs. 0.5%). In addition, 27.9 % of the alfalfa biomass consists of a mixture of components, listed here as "others". While we did not test for all of these compounds, we know from literature that "others" includes proteins (~ 13%), extractives (~1%), uronic acids (~ 7.5%), and acetyl groups (~5%) (Wiselogel et al. 1996; Dien et al. 2011). The high ash content and high level of other components results in a lower total amount of fermentable sugars for alfalfa stems than for aspen.

In this study we are exploring the potential use of low concentration peracetic acid as pretreatment chemical. Industrial-scale production of peracetic acid is performed by using acetic acid and hydrogen peroxide. Small amounts of hydrogen peroxide are added to commercial peracetic acid stock solutions to support stability (Teixeira et al. 1998). During our pretreatment with commercial peracetic acid, we need to consider that the chemicals present in the aqueous phase of the pretreatment solution include acetic acid, peracetic acid, and hydrogen peroxide. Prescreening experiments for aspen and alfalfa with hot water, acetic acid, and acetic acid-hydrogen peroxide pretreatment were carried out and compared to pretreatments with commercial peracetic acid.

Table 1. Chemical Composition of Aspen and Alfalfa Stems

Component	Aspen Wood % of dry total	Alfalfa Stem % of dry total
Glucan	44.5	30.2
Xylan	17.7	9.7
Mannan	1.7	1.5
Arabinan	0.5	3.8
Galactan	1.3	3.0
Lignin	21.1	15.2
Ash	0.5	8.6
Others	12.7	27.9

The results shown in Fig. 1 indicate the effect of different pretreatments on final glucose release. A control set of aspen that was treated with water only at 65°C resulted in a glucose release of 23.1% (based on total cellulose content in biomass) under the standard enzymatic hydrolysis conditions used. A pretreatment with acetic acid (330g/L) did not significantly change lignin content, but it increased the conversion rate of cellulose to glucose to about 37%. This effect did not change significantly when using a combination of 2% hydrogen peroxide (equal to 3.3g/L) and acetic acid (330g/L), although hydrogen peroxide is an oxidant and has the potential to react with acetic acid to form peracetic acid. Treatment with hydrogen peroxide/acetic acid in an aqueous system

released approximately the same amount of glucose during the subsequent enzymatic hydrolysis than acetic acid treatment alone (Fig. 2). It is known that in aqueous systems the reaction equilibrium is on the side of the peroxide and acetic acid, generating only small amounts of peracetic acid (Saha et al. 2003). For aspen, the commercial peracetic acid pretreatment (10% on o.d. biomass) resulted in lignin loss (from 21.1 % to 17.1%) and a higher cellulose to glucose conversion rate of 51.2% (Figs. 1 and 2).

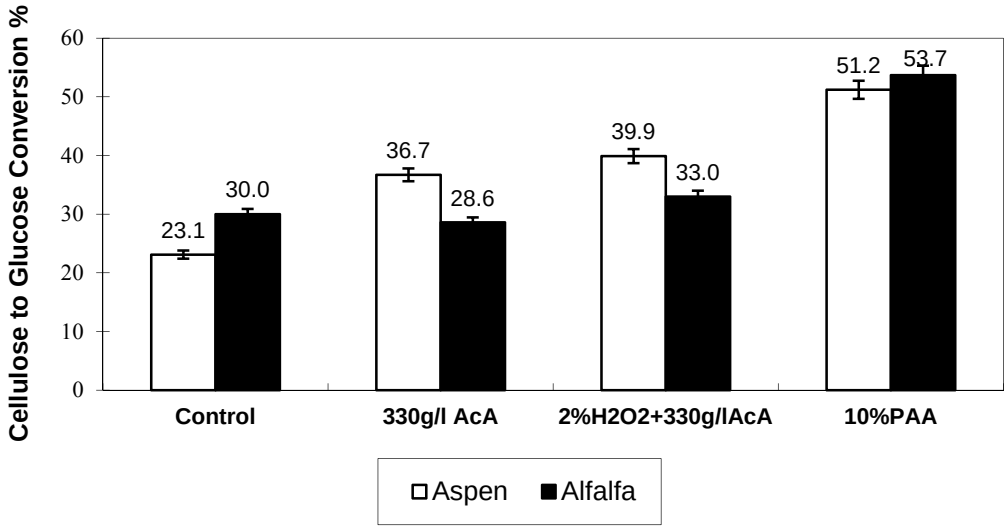

Fig. 1. Pretreatment prescreening experiment for aspen wood and alfalfa stems; reaction temperature 65°C*, AcA= Acetic acid, PAA = Peracetic Acid*

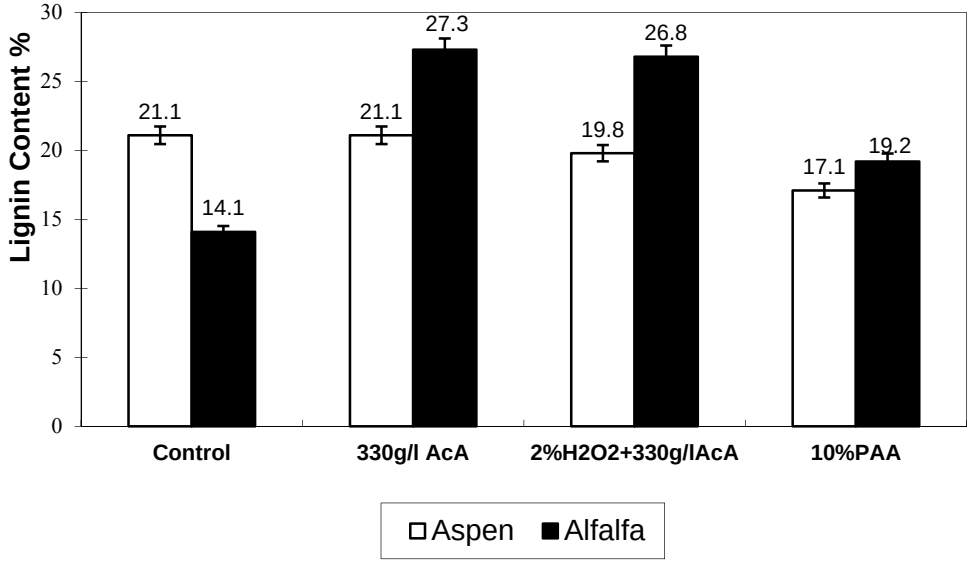

Fig. 2. Pretreatment prescreening experiments for aspen wood and alfalfa stem, reaction temperature 65°C, *AcA=Acetic acid, PAA= Peracetic Acid*

For alfalfa stems, hot water pretreatment (control) was able to achieve a cellulose-to-glucose yield of around 30% after enzymatic hydrolysis. Adding acetic acid, with or without hydrogen peroxide, increased the yield of glucose only marginally. Once again, peracetic acid pretreatment stood out among the rest of the pretreatments. Interestingly, the lignin content for all pretreatments increased for the alfalfa stems. As will be discussed later in this paper, the increase was due to an overall yield loss caused by easily dissolvable components of alfalfa stems.

To study the impact of different peracetic acid treatments, the effect of three factors: peracetic acid concentrations (0.5-9 weight %), reaction times (1 to 5 hours), and reaction temperatures (40-100°C) were evaluated by a statistical 3^4 fractional factorial design, which includes three variables at four levels. Triplicate experiments were performed for each sample. In an attempt to reduce experimental runs, needed interactions among variables were not quantified. Analysis of variance (ANOVA) of cellulose to glucose yield for aspen powder and alfalfa stems under different peracetic acid pretreatment conditions was performed using Statistics Analysis System (SAS) software. Results are shown in Table 2 (Aspen) and Table 3 (Alfalfa Stems).

Table 2: ANOVA Table for Peracetic acid pretreatment of Aspen

Source	DF	SS	MS	F-Value	P>F	Significant
Model	9.00	1427.23	475.74	18.55	0.00	
Error	6.00	153.86	25.64			
Corrected Total	15.00	1581.10	105.41			
Temperature	3.00	385.81	128.60	5.02	0.04	Yes
Time	3.00	153.75	51.25	2.00	0.22	No
PAA Concentration	3.00	887.67	295.89	11.54	0.01	Very

Table 3: ANOVA Table for Peracetic acid pretreatment of Alfalfa Stem

Source	DF	SS	MS	F-Value	P>F	Significant
Model	9.00	645.46	215.15	4.72	0.001	
Error	22.00	1002.24	45.56			
Corrected Total	31.00	1647.70	53.15			
Temperature	3.00	36.19	12.06	0.26	0.85	No
Time	3.00	568.27	189.42	4.16	0.02	Yes
PAA Concentration	3.00	41.01	13.67	0.30	0.82	No

For aspen (Table 2), peracetic acid concentration as a variable had the smallest P value. It was clearly the most significant among the three factors tested. As expected, changing the peracetic acid concentration during pretreatment had the largest impact on improving glucose yield after enzymatic hydrolysis. Temperature, which had a slightly bigger P value (0.04), had some, but less significant impact in modifying peracetic acid pretreatment procedures, whereas, reaction time did not affect pretreatment procedures.

Since our peracetic acid concentrations were fairly low, peracetic acid was consumed rapidly, either through reaction with lignin or decomposition reactions originated by transition metals in the biomass. Additional reaction time did not affect the outcome.

Contrarily, during peracetic acid pretreatment of alfalfa stems, reaction time had the smallest P value (0.02) (Table 3). In this case, reaction time was the most significant. Surprisingly, peracetic acid concentration with a P value of 0.82 and reaction temperature (P value of 0.85) did not seem to be significant under the selected conditions.

Figure 3 takes a closer look at the impact of peracetic acid concentration during pretreatment for both biomass types. Peracetic acid concentration, where most of the cost comes from, is shown to be significant for aspen wood powder but not for alfalfa stems. For aspen, the cellulose to glucose conversion rate increased significantly when the peracetic acid concentration was increased from 0.5% to 9% based on wood weight. A pretreatment with 9% peracetic acid resulted in a cellulose to glucose conversion rate of around 44%. The reason for this yield enhancement is expected to be the degradation and modification of lignin and some dissolving of hemicelluloses, While a 44% release rate is an improvement over the original 23%, it is clearly is not high enough to justify the addition of 9% more peracetic acid. It also has to be pointed out that the overall percentage of glucose release under the given standard hydrolysis conditions was significantly lower for aspen than that for alfalfa stems. For pretreated alfalfa stems, all data points were quite close to each other, clustering around values of 42 to 47% glucose release (Fig. 3). 0.5 % peracetic acid addition resulted in significant improvement in the release of glucose, where an additional increase in peracetic acid charge did not significantly change the glucose yield. High levels of peracetic acid were clearly not beneficial for alfalfa saccharification. This trend was completely different than the one observed for aspen wood meal. As will be discussed, we hypothesize that this difference is based on the different response to peracetic acid, specifically to the observed changes in lignin concentrations.

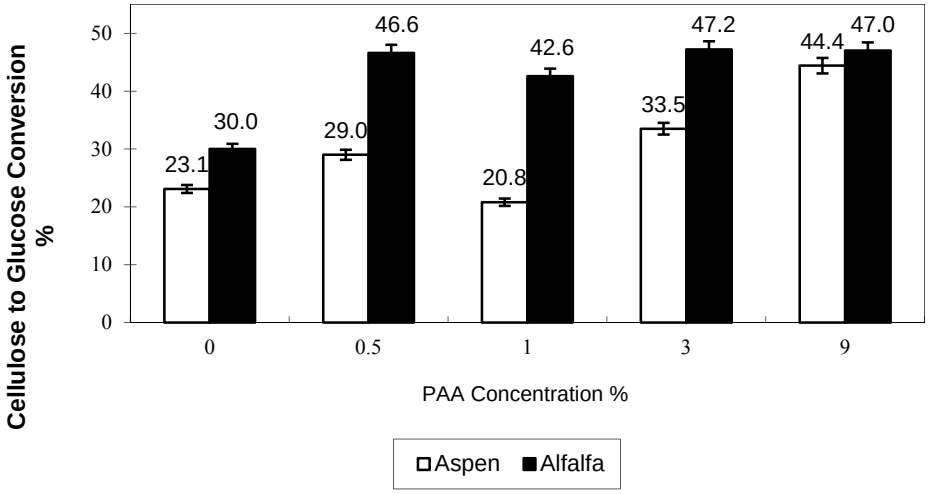

Fig. 3. Effect of peracetic acid concentration on glucose yield after enzymatic hydrolysis (reaction time 120 min, reaction temperature 100°C)

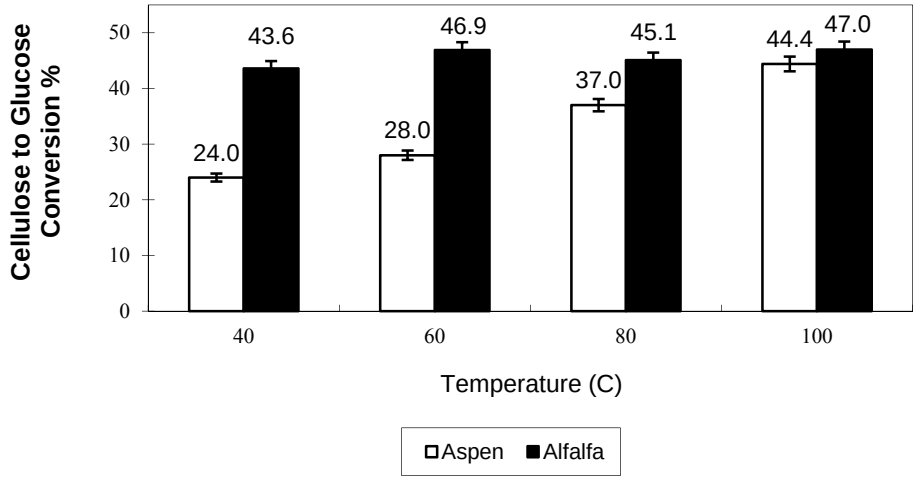

Fig. 4. Effect of reaction temperature on glucose yield after enzymatic hydrolysis (reaction time 120 min, peracetic acid addition 9%)

While temperature played some role in aspen pretreatment with peracetic acid (Fig. 4), the impact was less significant than peracetic acid concentrations. The yield of glucose after enzymatic hydrolysis reached the highest value at 100°C. For aspen pretreated with 9 % peracetic acid and a reaction time of 120 minutes, glucose yield after enzymatic hydrolysis increased from 24 to 44% as temperature was increased from 40°C to 100°C. For alfalfa, the glucose yield increased from 43 to 47% under the same conditions. These results are in agreement with previous studies considering high temperature peracetic acid pretreatments (Teixeira et al. 1998).

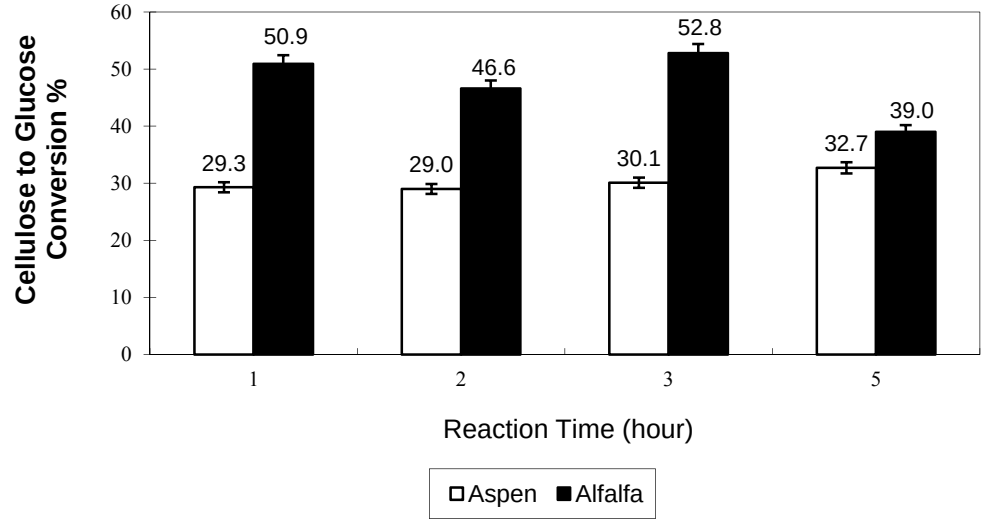

Fig. 5. Effect of reaction time on glucose yield after enzymatic hydrolysis (peracetic acid concentrations = 0.5%, reaction temperature = 100°C)

According to our statistical analysis, reaction time had a heavier impact than other factors on alfalfa stem pretreatment. A time of two hours appeared to be the best reaction time for aspen, three hours being the optimum time for alfalfa stems.
For alfalfa, when reaction times exceed these optimum points, glucose yield drops as time increases. For aspen powder, however, glucose conversion rate remains essen-tially the same at increased reaction times.

Optimal Pretreatment Condition of Current Study

The ANOVA table shows that the important variable for aspen pretreatment was peracetic acid concentration, while the most important factor for alfalfa stems was the reaction time. However, the other two variables still need to be considered, since the differences among their P values were close. Although the interactions between the factors have been ignored in this experiment design to emphasize the effect of single factors, these three factors are expected to interact with each other during the pretreatment process. The optimum condition for aspen was determined to be 100°C, 9% peracetic acid for 2 hours. For alfalfa stems, 100°C, 0.5% peracetic acid for 3 hours was the best combination. Under optimized conditions, around a 10% enhancement in glucose conversion rate was observed. These conversion rates, although being an improved enzymatic hydrolysis rate, are still too low to be commercially attractive and will need further improvement. Alternatively, combinations with low final glucose conversion rate and high yield/cost ratio can still be chosen as long as the remaining material can be used for other applications.

Relationship between Lignin Content and Enzymatic Glucose Release

As lignin is considered to be a critical barrier for converting pretreated biomass into glucose (Gharpuray et al. 1983), lignin content was examined closely in our study. The glucose conversion rate after hydrolysis versus Klason lignin content is shown in Figs. 6 and 7. As mentioned before, in alfalfa stems the lignin content (in % of total mass) increased after peracetic acid pretreatment. Figure 6 appears to suggest an increased glucose release at higher lignin content, which differs from data commonly found in literature, but this is misleading. The increased lignin content was caused by dissolution of material other than lignin during the pretreatment, resulting in a changed overall composition. A significant yield loss (up to 23%) could be observed during peracetic acid pretreatment of alfalfa stems. Interestingly, polysaccharides, and lignin were not removed to any extent. Even with 9% peracetic acid at 100 °C treatment, 99.7 of the originally present cellulose, 91% of hemicellulose, and 98% of the original lignin were retained (Table 4). The majority of material loss originated from the mixture of other components (labeled “others”). Aspen’s peracetic acid pretreatment showed a different response. The overall yield loss was only 2.3% and the lost material consisted mainly of lignin; about 11% of the originally present lignin was dissolved (Table 5). As expected, the amount of lignin decreased after peracetic acid pretreatment as the yield of glucose after enzymatic hydrolysis increased. This can be explained by the generally accepted fact that delignification can improve yield of enzymatic hydrolysis through increased accessibility of enzymes to carbohydrates (Oehgren et al. 2007).

The different response of aspen lignin and alfalfa lignin to peracetic acid is based on their different cell wall and lignin structure. Peracetic acid, as a strong oxidizing agent, has been shown to react with β-aryl bonds in lignin through several pathways, effectively cleaving these important lignin linkages. In addition, it is capable of hydroxylating the aromatic ring of phenolic lignin units to form hydroxyquinones, which in turn undergo ring openings and form organic acids, thereby introducing highly polar units (Lawrence et al. 1980).

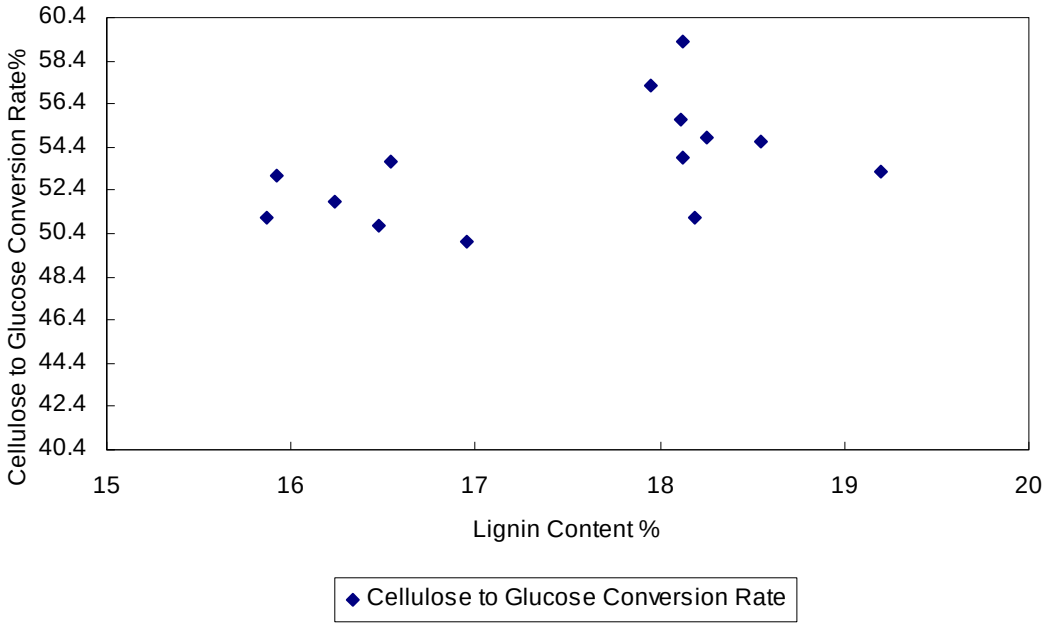

Fig. 6. Glucose conversion rate in correlation to lignin content (% of total) for alfalfa stems

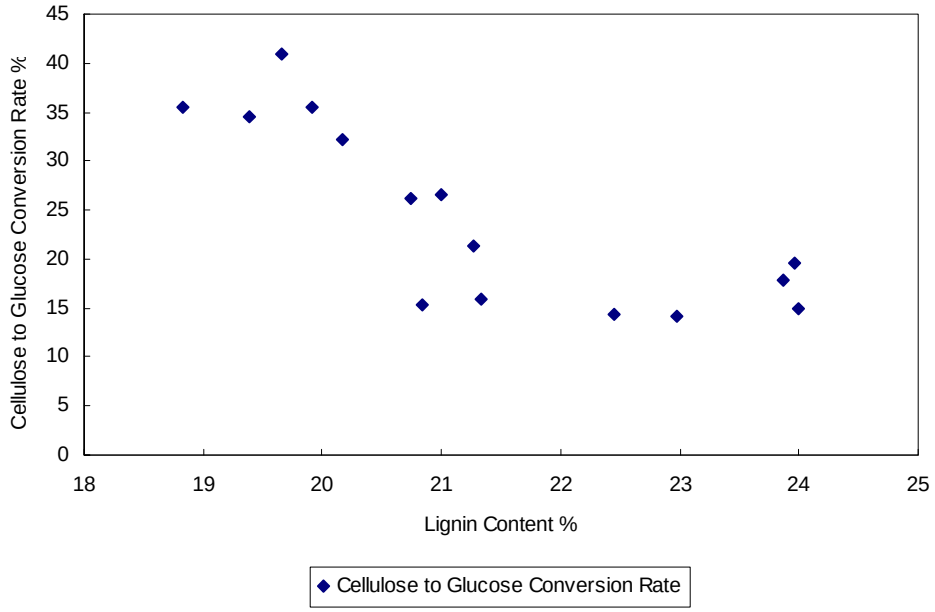

Fig. 7. Glucose conversion rate in correlation to lignin content (% of total) for aspen wood

Table 4. Components of Pretreated Alfalfa Stem (composition normalized for yield loss*)

Component [%]	Original Alfalfa	3h 100°C 0%PAA	3h 100°C 3%PAA	3h 60°C 9%PAA	3h 100°C 9%PAA
Glucan	30.2	31	29.8	28.6	30.1
Xylan	9.7	9.6	9.3	7.6	9.0
Galactan	3.0	2.8	2.5	1.8	2.4
Arabinan	3.8	2.0	1.3	1.5	2.6
Mannan	1.5	1.6	1.4	1.2	2.3
Klason Lignin	15.2	15.2	15	15.2	14.9
Others	36.6	29.2	26	23.9	15.9
Yield	-	91.4	85.3	79.8	77.2

Table 5. Components of Pretreated Aspen (composition normalized for yield loss*)

Component [%]	Original Aspen	3h 100°C 0%PAA	3h 60°C 9%PAA	3h 100°C 9%PAA
Glucan	44.5	46.2	45.6	46
Xylan	17.7	16.3	15.8	16.4
Galactan	1.3	2.3	1	1.4
Arabinan	0.5	0.4	0.3	0.2
Mannan	1.7	1.5	1.4	1.3
Klason Lignin	21.1	21.3	20.1	18.8
Others	13.2	11.5	15.2	13.6
Yield	-	99.5	99.4	97.7

* Normalized for yield loss: the concentrations in this table reflect % of material based on original starting material; e.g. Klason lignin content of alfalfa 3h, 100 C, 9% PAA was 19.3% concentration based on total material at end of reaction. With a yield loss of 22.8%, that translates to a Klason lignin content of 14.9 % based on original material

Alfalfa lignin, which is known to be highly condensed, appears to respond less favorably to oxidative treatments as several other lignin types (Jung et al. 1992). It has to be noted that peracetic acid addition on our experiments was limited to up to 10% on biomass due to cost considerations. Higher application levels are capable of removing lignin from alfalfa stems.

CONCLUSIONS

1. Pretreatment effectiveness of aspen biomass is strongly dependant on peracetic acid addition rate. Reaction temperature and reaction time have a less significant impact.
2. Pretreatment of alfalfa stems appears to be independent from peracetic acid addition rate in the range tested. 0.5 % peracetic acid increases rate of glucose release from 30 to 47%; additional peracetic acid (up to 9% on dry biomass) does not significantly improve this response.
3. As expected, the rate of glucose release is closely related to the presence of lignin. Peracetic acid actively removes lignin from aspen, thereby increasing sugar release, but appears to not significantly remove alfalfa lignin.
4. Although glucose release was increased through low level peracetic acid pretreatment (from 23 to 44% for aspen and from 30 to 47% for alfalfa stems), these rates are still too low to be commercially attractive.

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