Title:

**Mechanism of improved cellulosic bio-ethanol production from alfalfa stems
via ambient-temperature acid pretreatment**

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ABSTRACT

Model compounds and recalcitrant biomass were studied to elucidate the mechanism of ambient-temperature acid pretreatment of cellulosic biomass for bio-ethanol production. Pure cellulose, a pure hemicellulose and alfalfa stems were pretreated with sulfuric acid under ambient temperature with varied acid loading and time. Changes in water-soluble carbohydrates (WSCs) and chemical components of substrates were determined, and ethanol production *via* simultaneous saccharification and fermentation (SSF) was studied. The results showed significant amount of WSCs formed, and the WSCs increased with increasing acid loading and pretreatment time. The ethanol yields from pure cellulose were primarily affected by the added ash. Acid loading showed significant positive effect on ethanol production from alfalfa stems, whereas pretreatment time showed much weaker positive effect. However, non-significant amounts of WSCs were removed by washing of dried substrates. It was hypothesized to be because the WSCs adsorbed onto bulk substrates during the freeze-drying step, as supported by experimental results.

Keywords:

Ambient-temperature sulfuric acid pretreatment;

Water-soluble carbohydrates (WSCs);

Hemicellulose adsorption;

Cellulosic bio-ethanol;

Alfalfa stems

1. Introduction

To fulfill the food and energy demand of the United States, with considerations in energy and economic security, the land use and current cropping systems need to be modified and optimized (ORNL, 2011). Alfalfa, a legume, has excellent characteristics to benefit land and water quality by improving soil and nutrient retention, reducing nitrogen fertilizer input, and increasing other crops' yields (Bolton *et al.*, 1976; Rasse and Smucker, 1999; Dien *et al.*, 2011). Additionally, it is beneficial to the environment by lowering N₂O emissions and nitrate-N leaching as compared to typical corn cropping methods (MacKenzie *et al.*, 1997; González-García *et al.*, 2010).

Alfalfa is a good candidate for both food and biofuel production. Alfalfa has been widely used as animal feed around the world. It has also recently been considered as a feedstock for biorefineries (González-García *et al.*, 2010). Alfalfa leaves have a crude protein content of about 30% **dry matter (DM)** (Dale, 1983), which is a good animal feed, and also a good potential resource for producing protein concentrate products. Alfalfa stems have a high polysaccharide content of around 50% DM (Dien *et al.*, 2011; Zhou *et al.*, 2014), that can be used for liquid fuel (bio-ethanol) and chemical production. If alfalfa leaves and stems can be separated at a farm level, less harvests without decreasing alfalfa yield (Lamb *et al.*, 2003) can be carried out to save harvest energy and labor, and optimize their utilization.

Bio-ethanol production from lignocellulosic biomass is considered as a feasible replacement of fossil fuel for transportation (Kuhad *et al.*, 2011). Lignocellulosic biomass is a complex matrix mainly including cellulose, hemicellulose and lignin, as well as some pectin, protein, and ash, whereas cellulose, which is the useful portion for ethanol production, is mainly surrounded by hemicellulose and lignin (Kumar *et al.*, 2009). The main steps for producing

ethanol from lignocellulosic biomass include substrate production and collection, pretreatment, saccharification (hydrolysis), fermentation, and separation. The pretreatment step, whose goal is to increase cellulose exposure, is considered as one of the costly capital investments (Tao *et al.*, 2011) due to the recalcitrance of lignocellulosic biomass. Some well-known pretreatment techniques include mechanical comminution, steam explosion, ammonia fiber explosion (AFEX), CO₂ explosion, ozonolysis, acid hydrolysis, alkaline hydrolysis, organosolv, and biological pretreatment (Kumar *et al.*, 2009).

Dilute acid pretreatment at elevated temperature (DA) has been widely studied, and considered one of the most promising pretreatment techniques for industrial application (Alvira *et al.*, 2010). It is acid hydrolysis usually carried out with low acid concentration of 0.2% to 2.5% w/w, at elevated temperature between 130 °C and 210 °C (Brodeur *et al.*, 2011). Sulfuric acid is commonly used due to its low cost, though other acids have also been studied (Kumar *et al.*, 2009). It has been theorized that the primary effects of DA pretreatment include hemicellulose removal to create structural changes of the substrates to expose cellulose (Knappert *et al.*, 1980). Though lignin hydrolysis also happened during the DA pretreatment, it would re-condense and form a new lignin polymer (Weil *et al.*, 1994). During the DA pretreatment, almost all of the hemicelluloses can be removed and most of them can be recovered as sugar streams (Kumar *et al.*, 2009; Zhang and Runge, 2012). Severe conditions (high acid charge or temperature, or long time) are less favorable because hydrolyzed hemicellulose can be degraded to inhibitors, which can significantly inhibit enzymatic saccharification (Kumar *et al.*, 2009). Cellulose can also be degraded which is not favorable (Kumar *et al.*, 2009). Carbohydrate degradation reduces sugar recoveries. Thus, low severe conditions were developed to allow hemicellulose removal as well as achieve high xylose yield and recovery for favorable economics (Kumar *et al.*, 2009).

The ambient-temperature pretreatment combined with on-farm storage has been proposed as an alternative of high temperature pretreatment recently (Digman *et al.*, 2010a; Digman *et al.*, 2010b; Zhou *et al.*, 2014). On-farm pretreatments with acid, alkali, ozone and enzymes (xylanases or feruloyl esterase) at ambient temperature were compared, and the cellulose conversion and xylose yields were examined, whereas sulfuric acid and lime pretreatments were found to be promising techniques (Digman *et al.*, 2007). Both chemical loading and pretreatment time showed positive effects on the conversion, whereas the chemical loading was shown to be the main effect (Digman *et al.*, 2010b), for both lab- and farm-scale studies (Digman *et al.*, 2010a; Digman *et al.*, 2010c). Studies on the sulfuric acid or lime pretreatments showed that both of these pretreatments improved the conversion of cell wall cellulose to ethanol, with better results from sulfuric acid pretreatment (Digman *et al.*, 2010c). Recent studies on pretreating alfalfa stems with sulfuric acid at ambient temperature demonstrated improved ethanol yields (Zhou *et al.*, 2014). These studies examined possible substrate property changes and their correlation with ethanol production, and concluded that structural changes to pores, surface area and cellulose crystallinity were the primary changes related to the increase in ethanol production (Zhou *et al.*, 2015), likely due to hemicellulose hydrolysis and removal from the lignocellulosic matrix (Zhou *et al.*, 2014).

However, previous studies indicated there were interferences from added ash (due to acid pretreatment and neutralization), *i.e.*, decreased hemicellulose content might be due to increased added ash (Zhou *et al.*, 2014), raising questions of how were hemicelluloses removed, and how would the chemical components change without ash interference? Previous work also showed that pretreated substrates had lower cellulose content than original substrates (Zhou *et al.*, 2014), which is very important because reduced cellulose would result in lower overall ethanol yield,

raising the question of was the cellulose degraded? Behind these phenomena, there might be a different mechanism other than just hemicellulose removal, which is fundamental and interesting, but has not been reported before. Additionally, interference from ensiling (Zhou *et al.*, 2015) would also affect our investigation of the mechanism. In order to investigate the responsible mechanism under more controllable conditions and to answer these questions, substrates were only pretreated with sulfuric acid at ambient temperature without ensiling in present study. Model compounds including pure cellulose, pure hemicellulose, and real biomass including alfalfa stems were all pretreated with sulfuric acid with varied acid loading and pretreatment time. The water-soluble carbohydrates (WSCs) and chemical components of substrates were determined, and the subsequent ethanol production was investigated by performing simultaneous saccharification and fermentation (SSF). Specific mechanism of hemicellulose adsorption was proposed, which was supported through the experimental data.

2. Materials and methods

2.1 Materials and chemicals

Alfalfa stems were collected at the Arlington Agricultural Research Station (Arlington, WI), University of Wisconsin, on June 25, 2014, transported to laboratory, oven dried at 50 °C according to NREL procedure NREL/TP-510-42620 (Hames *et al.*, 2008), and ground with a Christy & Norris 8" Laboratory Mill (Christy Turner Ltd, UK) to pass a 1 mm screen.

Avicel PH-101 microcrystalline cellulose (~ 50 µm particle size), filter paper (Whatman, qualitative, grade 1, 90 mm ø, Cat No. 1001090), and xylan from beechwood (≥ 90%, cell wall) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Sulfuric acid (96.6% w/w) and sodium hydroxide (50% w/w) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Sulfuric acid (72% w/w) was purchased from Ricca Chemical Company (Arlington, TX, USA). D(-)-arabinose, D(+)-galactose, D(+)-glucose (anhydrous), D(+)-mannose, and D(+)-xylose (all 99+%) were purchased from Acros Organics (Geel, Belgium). Yeast extract (molecular genetics powder), and peptone were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Tetracycline was purchased from Sigma Aldrich (St. Louis, MO, USA).

Cellic[®] CTec3 enzyme, with a filter paper activity of 140 FPU/mL measured in lab according to an NREL procedure (NREL/TP-510-42628) (Adney and Baker, 2008), was from Novozymes (Denmark). Yeast *Saccharomyces cerevisiae* D5a was used for fermentation.

2.2 Acid pretreatment and washing

For each individual experiment, to a 240 mL glass bottle was added 20 g (d.b.) Avicel PH-101 cellulose or alfalfa stem powder, or 5 g (d.b.) xylan. Sulfuric acid solution was then added to reach a sulfuric acid loading of 35, 100, or 200 g/kg DM (dry matter), and moisture content of 50% (w.b.), resulting in sulfuric acid concentration of 35, 100, or 200 g/L in water. The bottle was sealed and shaken to mix well. For acid loading level, the bottles were aged at ambient indoor conditions (~22 °C) to pretreat substrates with different time (4, 10, 30, and 60 days for Avicel PH-101 and alfalfa stems, and 4, 10, and 30 days for xylan). All experiments were run in duplicates. Thus, there were 12 experiments (24 tests) on Avicel PH-101 or alfalfa stems, and there were 9 experiments (18 tests) on xylan.

After acid pretreatments, water (190 mL for Avicel PH-101 or alfalfa stems, and 100 mL for xylan) was added and the samples were neutralized with sodium hydroxide (50% w and 5% w

NaOH). Liquid sample (1 mL) was taken from each bottle for analyzing water-soluble carbohydrates (WSCs). Bottles with solids and liquids were then freeze-dried for component analysis, simultaneous saccharification and fermentation (SSF), and washing.

Washing was carried out in 50 mL Thermo Scientific™ Nunc™ sterile centrifuge tubes with a solid:liquid ratio of about 1:10. Tubes with alfalfa stems and added water were shaken to mix well, then sitting for 20 minutes with shaking every 5 minutes. Then, solids and liquids were separated by an Eppendorf Centrifuge 5810 R (15 amp version) at 2000×g for 5 min. Samples were washed until no obvious sulfate (SO_4^{2-}) was detected by saturated barium hydroxide ($\text{Ba}(\text{OH})_2$) solution. The washed alfalfa stems were then freeze-dried for component analysis, and simultaneous saccharification and fermentation (SSF).

2.3 Water-soluble carbohydrates (WSCs) determination

Water-soluble carbohydrates (WSCs) determination of liquid samples included monosaccharides and soluble oligosaccharides. Monosaccharides in liquid samples were defined as water-soluble sugars (WSSs). To determine the WSSs, liquid samples were initially centrifuged at 16,000×g for 10 min. The supernatant was diluted and analyzed for sugars according to Zhou and Runge (2014).

In order to determine the total soluble monosaccharides and oligosaccharides, which were defined as WSCs, 0.5 mL of the supernatant after centrifugalizing was mixed with 1 mL of 6% w sulfuric acid to result in a 4% w sulfuric acid content, which was then autoclaved at 121 °C for 60 min in an Allen-Bradley PanelView Plus 700 autoclave. The hydrolysate was neutralized with 5% w sodium hydroxide, centrifuged at 16,000×g for 10 min, and diluted for sugar analysis (Zhou and Runge, 2014).

The percentage of water-soluble sugars (WSSs) based on dry substrates was calculated according to:

$$\text{WSSs (\%)} = ((m_{\text{ara}} + m_{\text{xyl}}) \times 0.88 + (m_{\text{gal}} + m_{\text{glu}} + m_{\text{man}}) \times 0.9) / m_{\text{sub}} \times 100\%;$$

where, m_{ara} , m_{xyl} , m_{gal} , m_{glu} and m_{man} were the mass of detected arabinose, xylose, galactose, glucose and mannose, respectively;

0.88 or 0.9 is the molecular weight ratio of five- or six-carbon sugars to their anhydrides, respectively; and

m_{sub} is the dry mass of substrates.

Whereas, for pure cellulose,

$$\text{WSSs (\%)} = m_{\text{glu}} \times 0.9 / m_{\text{sub}} \times 100\%;$$

The percentage of total WSCs after one-step acid hydrolysis was calculated according to:

$$\text{WSCs (\%)} = ((m_{\text{ara}} + m_{\text{xyl}}) \times 0.88 + (m_{\text{gal}} + m_{\text{glu}} + m_{\text{man}}) \times 0.9) / (m_{\text{sub}} \times r_{\text{glu}}) \times 100\%;$$

where, the r_{glu} is the sugar (glucose) recovery during the one-step acid hydrolysis, which was determined by carrying out one-step dilute acid hydrolysis on solution of standard glucose.

2.4 Substrate analysis

The total solids and ash content of freeze-dried substrates were determined according to NREL procedures NREL/TP-510-42621 (Sluiter *et al.*, 2008a) and NREL/TP-510-42622 (Sluiter *et al.*, 2008b). The glucan, hemicellulose, acid soluble lignin (ASL), and Klason lignin contents were analyzed with a modified two-step hydrolysis (Zhou and Runge, 2014). Total nitrogen (TN) was determined on a PerkinElmer Series II CHNS/O Analyzer, and crude protein (CP) was estimated as $\text{TN} \times 6.25$ (Hatfield *et al.*, 1994).

2.5 Simultaneous saccharification and fermentation (SSF)

SSF and ethanol analysis were carried out according to previous report (Zhou *et al.*, 2014). In brief, *S. cerevesiae* D5a was cultured for 24 h at 35 °C. It was then harvested and mixed with a sterile media, Cellic® CTec3 enzyme (Novozyme), citrate buffer (pH 4.8), and tetracycline solution. The resulting enzyme/yeast mixture was added to substrates in vials, resulting in a solid loading of 10%. All vials were sealed and incubated at 35 °C for 72 h with shaking at 160 rpm. The enzyme loading was 5 FPU/g alfalfa stems (d.b.). The ethanol analysis was carried out according to previous report (Zhou *et al.*, 2014).

The ethanol yield (%) was calculated according to following equations, giving an ethanol yield based upon the cellulose composition of substrate when added for SSF.

$$\text{Ethanol (g/g glucan, experimental)} = (\text{Ethanol (g/L, net)} \times V \text{ (L)}) / \text{Glucan (g)};$$

$$\text{Ethanol (g/g glucan, theoretical)} = 0.5684 \text{ g/g glucan};$$

$$\text{Ethanol yield (\%)} = \text{Ethanol (experimental)} / \text{Ethanol (theoretical)} \times 100\%.$$

2.6 Modeling

In order to get optimized ethanol production conditions from our present study, models for predicting ethanol yields were created using JMP Pro 11 (SAS Institute Inc., Cary, NC, USA). The experimental ethanol yields from alfalfa stems at acid loading of 35, 100, and 200 g/kg DM, and pretreatment time of 4, 10, 30, and 60 days during the ambient-temperature sulfuric acid pretreatment were input into the JMP Pro 11 as raw data, linear regression was used to create the models, and a stepwise strategy was carried out to exclude the non-significant quadratic terms. Each model had 12 distributed experimental data points with duplicates for each of the 12

experiments. The models were regenerated into surface plots with MATLAB (MathWorks, Natick, Massachusetts, USA) for improved visualization.

2.7 Adsorbing hemicellulose onto cellulosic substrates

In order to investigate the WSC adsorption during the ambient-temperature acid pretreatment, experiments were carried out at conditions that were very similar to those during the pretreatment, using multiple cellulosic substrates including Avicel PH-101, filter paper and alfalfa stems, and multiple WSCs including xylan and xylose. To 50 mL Thermo Scientific™ Nunc™ sterile centrifuge tube was added 3 g cellulosic substrate. Xylan or xylose (0.1, 0.5, or 1 g) was added, followed by the addition of 30 mL Millipore filtered water and 0.3 mL tetracycline solution (10 mg/mL in ethanol). The tubes were sealed and shaken at 200 rpm and 20 °C in a New Brunswick Scientific Excella E24 incubator for 15 hours, then sitting for 5 hours. Then, the tubes were frozen and freeze-dried for 12 days. Dried substrates were washed with reverse osmosis (R.O.) water 3 times as following. Water (about 50 mL for the first washing, and about 30 mL water for the second and third washing) was added to the tubes with substrates, which were shaken to mix well. Then, the tubes were sitting for 20 min, with shaking every 5 min. After that, solid and liquid were separated with a centrifuge at 2000×g for 5 min. Washed substrates were freeze-dried for carbohydrates analysis following procedures outlined by Zhou and Runge (2014).

The loaded and gained percentages were both calculated based on cellulosic substrates (d.b.), in mass. The adsorption efficiency was the percentage of adsorbed hemicelluloses out of total loaded hemicelluloses.

3. Results and discussion

3.1 Models for ethanol production

Models for predicting ethanol yields from alfalfa stems based on acid loading of 35-200 g/kg DM and pretreatment time of 4-60 days are shown in Table 1 and Fig. 1. Analyzing the factorial response data with an multivariate analysis of variance (Table 1) it may be seen that the variables tested were both statistical significant for influencing the ethanol yield and responsible for much of ethanol yield data variance measured (unwashed model $R^2 = 0.89$ and washed model $R^2 = 0.65$). Although the models did not provide a perfect correlation coefficient, this would not be expected for this type of analysis, as we did not model all variables that could influence the system and would expect some amount of experimental error. Additionally, the models were used to create plots (Fig. 1) to help visualize the interaction between the variables tested.

For unwashed alfalfa stems, acid loading, pretreatment time, and their cross term had a significant contribution. The predicted maximum ethanol yield was 32.1% at an acid loading of 200 g/kg DM and pretreatment time of 4 days (Fig. 1A). We believe the low ethanol yield was primarily because there was a high amount of added ash (from the neutralized acid), which inhibited the ethanol production as discussed in section 3.4. The predictive formula derived was,

$$\text{Yield (\%)} = 0.05352 \times a + 0.05378 \times t - 0.0008034 \times (a - 112.2) \times (t - 25.83) + 19.69;$$

where, a is the acid loading, in g/kg DM; and

t is the pretreatment time, in day.

For washed alfalfa stems, only acid loading and pretreatment time had a significant contribution. The predicted maximum ethanol yield was 51.3% at an acid loading of 200 g/kg DM and pretreatment time of 60 days (Fig. 1B). The predictive formula for yield was,

$$\text{Yield (\%)} = 0.09824 \times a + 0.2491 \times t + 16.67.$$

3.2 Water-soluble carbohydrates (WSCs) after acid pretreatment

According to previous study (Zhou *et al.*, 2014), water-soluble monosaccharides increased, while the hemicelluloses content of alfalfa stems decreased with increasing acid loading during the ambient-temperature acid pretreatment, indicating the acid pretreatment hydrolyzed hemicelluloses into WSCs. To further understand this, water-soluble monosaccharides and oligosaccharides of alfalfa stems as well as pure hemicelluloses after acid pretreatment were determined in present work. The monosaccharides detected in liquid samples just after pretreatment were defined as water-soluble sugars (WSSs). To determine whether the cellulose of substrates was degraded, WSSs of pure cellulose with pretreatment was also analyzed. In order to measure the soluble oligosaccharides, one-step dilute acid (4% H₂SO₄) hydrolysis was carried out on the liquid samples. The monosaccharides detected in liquid samples after one-step dilute acid hydrolysis plus those degraded sugars during one-step dilute acid hydrolysis were defined as total water-soluble carbohydrates (WSCs), representing the total water-soluble monosaccharides and oligosaccharides.

The WSSs and WSCs in pure cellulose, pure hemicellulose, and alfalfa stems after ambient-temperature sulfuric acid pretreatment are shown in Fig. 2. In general, the WSCs and WSSs increased with increasing acid loading and pretreatment time. However, the WSSs in pure cellulose were less than 0.1% of total substrates, thus was negligible. Almost no sugar was detected in liquid samples after the one-step dilute acid hydrolysis, indicating almost no oligosaccharides were produced during the ambient-temperature acid pretreatment. In summary, very little **highly crystalline pure cellulose was hydrolyzed into WSSs**, indicating cellulose was not hydrolyzed during the ambient-temperature acid pretreatment.

For pure hemicellulose, the WSSs increased significantly with increasing acid loading and pretreatment time (Fig. 2B). The WSSs increased almost linearly with pretreatment time, indicating an almost constant hydrolysis rate, probably due to low temperature while water and acid catalyst were uniformly distributed among pure hemicellulose. The WSCs detected in the pure hemicellulose samples were around 24.4%DM, many times higher than WSSs, but did not show certain trends based on acid loading or pretreatment time. We hypothesized this was because the xylan could dissolve in water, thus acid pretreatment was not able to affect significantly. In summary, hemicelluloses were hydrolyzed during the ambient-temperature acid pretreatment, whereas acid loading and pretreatment time showed positive effects on the acid hydrolysis of hemicelluloses.

For alfalfa stems, both WSSs and WSCs increased with acid loading and pretreatment time, whereas the WSCs were 2 to 3 times of WSSs (Fig. 2C, D). The WSSs increased almost linearly with pretreatment time at an acid loading of 35 or 100 g/kg DM, indicating a constant rate of WSS production. However, the WSS production rate decreased with increasing pretreatment time at an acid loading of 200 g/kg DM. This was probably due to there are “two portions” of hemicellulose in biomass cell walls: fast- and slow-hydrolyzing hemicelluloses (Kobayashi and Sakai, 1956; Jacobsen and Wyman, 2000). We hypothesized that the fast-hydrolyzing hemicelluloses are more accessible portion, small fragments, acting like soluble xylan, while the slow-hydrolyzing hemicelluloses are less accessible, large fragments, which are distributed deep in the cell walls. Thus, acid hydrolysis happened mainly on the fast-hydrolyzing hemicelluloses at a relatively low acid loading (35 or 100 g/kg DM), or short time (at the early stage with acid loading of 200 g/kg DM), resulting in linear trends, similar to that of soluble pure hemicelluloses (xylan). At the late stage with a high acid loading (200 g/kg DM), while the fast-

hydrolyzing hemicelluloses were already degraded, the acid hydrolysis were happening mainly on slow-hydrolyzing hemicelluloses, which was limited by hemicellulose accessibility. This accessibility limitation also explained the WSC trends of pretreated alfalfa stems (Fig. 2D). In summary, cell wall hemicelluloses were partially hydrolyzed and dissolved during the ambient-temperature acid pretreatment, which was improved by acid loading and pretreatment time, but was limited by hemicellulose accessibility.

Results showed that the ambient-temperature acid pretreatment hydrolyzed and dissolved as much as 50% of the total hemicelluloses. If these dissolved hemicelluloses were removed, it could improve cellulose exposure significantly, and would result in changes in contents of hemicelluloses as well as other chemical components.

3.3 Substrate properties

Studies on the substrate properties can help us to understand the correlations between substrates and ethanol production, thus to find out strategies to improve ethanol production. For pure cellulose, substrate properties after ambient-temperature acid pretreatment were expected to be the same since the acid hydrolysis of cellulose during pretreatment was negligible. However, sulfuric acid was added during the acid pretreatment, and sodium hydroxide was added to neutralize the mixture, resulting in “added” ash. Results showed that the ash content of original pure cellulose was not detected, whereas the ash contents of acid pretreated cellulose increased with increasing acid loading in general, with an average ash content of 4.37%, 6.25%, or 13.65% for acid loading of 35, 100 or 200 g/kg DM. The pretreatment time did not show an effect on the ash content as expected, except that over neutralization resulted in more added ash.

The substrate properties of original and pretreated alfalfa stems, without and with washing, were summarized in Table 2. The pretreatment time did not show an obvious effect in most cases, thus it was not shown in this table. The ash contents of acid pretreated alfalfa stems without washing increased significantly with increasing acid loading during pretreatment, due to added ash was formed. The glucan and hemicellulose contents of unwashed alfalfa stems decreased significantly with acid loading, was hypothesized mainly due to the added ash. The acid soluble lignin (ASL) of pretreated alfalfa stems was generally lower than that of original alfalfa stems, which was also due to added ash. Thus, the ASL was expected to decrease with increasing acid loading. However, the ASL with 100 g/kg DM acid loading was the same as that with 35 g/kg DM acid loading, while the ASL with 200 g/kg DM was significantly higher than that with 35 or 100 g/kg DM acid loading. This was hypothesized to be because the acid pretreatment hydrolyzed some lignin, thus increased the ASL, which was also supported by the contents of Klason lignin (acid insoluble lignin) data. The Klason lignin content of pretreated alfalfa stems was lower than that of original, and it decreased with increasing acid loading, which were likely both due to the added ash diluting the other components. The crude protein (CP) contents showed similar trends due to similar reasons.

Since ash contents changed significantly due to “added ash”, it might affect other property changes caused by ambient-temperature acid pretreatment. In order to remove this artifact from the compositional analysis, all data were recalculated with the added ash being removed to give a new base for calculations, basically assuming that all samples had the same ash contents as original alfalfa stems. The recalculated component results were summarized in Table 2. All components were close across different acid loadings as expected, because the pretreatment was carried out in sealed bottles, thus ideally nothing was lost. However, acid

pretreatment did result in some small (around 1%DM) but statistically significant changes on the ASL and CP contents. The ASL contents increased a little with increasing acid loading, indicating some hydrolysis happened on lignin. The CP decreased a little with increasing acid loading, indicating acid pretreatment turned some non-volatile nitrogen components into volatiles, which were lost later. However, all these were small changes, which were not likely to cause significant improvement in ethanol production during later SSF.

Since Fig. 2D showed significant amounts of WSCs in alfalfa stems and obvious changes of them, it was reasonable to assume that these WSCs could be removed from the substrates by washing thus resulting in decreased hemicellulose contents. To study this, freeze-dried substrates were well washed, and the component analysis of washed alfalfa stems was also summarized in Table 2. The data indicates the residual ash contents of original alfalfa stems and alfalfa stems pretreated with 35 g/kg DM acid loading were close, while the ash contents of alfalfa stems pretreated with 100 and 200 g/kg DM acid loading were higher. However, these residual ash contents were low and the differences were small, thus the residual ash contents were not able to affect the contents of other components and ethanol yields too much. The glucan content of alfalfa stems with an acid loading of 100 g/kg DM was slightly higher than that with an acid loading of 35 or 200 g/kg DM, was believed to be because of over-estimation. The hemicellulose contents decreased with increasing acid loading during pretreatment on the washed samples, with the hemicellulose content of alfalfa stems with an acid loading of 200 g/kg being significantly lower than others, showing the effects of the acid loading. The ASL of washed alfalfa stems with an acid loading of 35 g/kg DM was a little lower than others, may be due to under-estimation. The Klason lignin (KL) and crude protein (CP) contents did not show significant differences.

3.4 Ethanol production

Simultaneous saccharification and fermentation (SSF) was carried out on pure cellulose (original and acid pretreated), and alfalfa stems (original, acid pretreated, original with washing, and acid pretreated with washing). Original pure cellulose gave the highest ethanol yield of 69.6%, and the ethanol yields decreased with increasing acid loading for acid pretreated pure cellulose, with an average ethanol yield of 66.5%, 63.9% or 61.8% at an acid loading of 35, 100 or 200 g/kg DM. Additionally, ethanol yield was lower at 30 days than other pretreatment times, corresponding to that the ash content was higher at 30 days than other pretreatment times. Thus, we were able to conclude that the ethanol yield from pretreated cellulose was affected mainly by added ash, since acid pretreatment made negligible changes on cellulose substrates (Fig. 2A).

Fig. 3 shows the ethanol yields of alfalfa stems (original, acid pretreated, unwashed and washed). For acid pretreated alfalfa stems without washing (Fig. 3A), the ethanol yields increased significantly with increasing acid loading, while ethanol yields increased significantly with pretreatment time only at acid loading of 35 g/kg DM. This indicated both acid loading and pretreatment time could improve ethanol yields during ambient-temperature acid pretreatment. However, the effect of pretreatment time was not significant at acid loading of 100 or 200 g/kg DM. This could be because of two reasons. One reason could be that the inhibition effect of added ash covered the positive effect of pretreatment time, because there was a very high ash content at an acid loading of 100 or 200 g/kg DM. Another reason could be that pretreatment time had weak effect compared to acid loading, indicating acid loading was the primary effect at the conditions used. Thus, low acid loading with long pretreatment time or high acid loading with short pretreatment time can be used to reach similar ethanol yields, providing potential flexibility to farm operation.

Since ash played an important role in substrate properties and ethanol production, washing was carried out to remove added ash. Table 2 shows that the ash contents of washed substrates were much lower than those of unwashed and original alfalfa stems, due to some original ash was also removed. As a consequence, ethanol yields of all washed alfalfa stems were improved as shown in Fig. 3B. Similar to that of unwashed alfalfa stems, acid loading improved ethanol yields significantly, while pretreatment time also improved ethanol yields significantly at both acid loading of 35 and 100 g/kg DM. This supported the two reasons discussed previously, *i.e.*, the effect of added ash covered the effect of pretreatment time, and pretreatment time had much weaker effect compared to that of acid loading.

3.5 Mechanism

Surprisingly, the alfalfa stem components especially the hemicellulose contents did not decrease much with increasing acid loading and pretreatment time, while the WSCs increased a lot, indicating washing was not able to remove many WSCs (soluble hemicelluloses) other than ash. We hypothesized that this was because the WSCs adsorbed onto bulk substrate surfaces and might formed connections with bulk substrates such as hydrogen-bonds due to complete dehydration during freeze-drying. According to previous reports (Zhou *et al.*, 2009; van Heiningen *et al.*, 2010; Ban and van Heiningen, 2011), pulp (cellulose fiber) could gain as much as 14.5% weight when it was soaking in extraction liquor prepared with hemicellulose and water. In the present study, the cellulose contents of acid pretreated alfalfa stems were at least 32%DM, while the main components except ash and soluble hemicelluloses were at least 65%DM (at acid loading of 200 g/kg DM), which could adsorb as much as 4.6%DM and 9.4%DM of WSCs in maximum, respectively. The maximum WSCs were 6.66%DM (Fig. 2D), which could

completely be adsorbed by bulk substrates in **ideal** case. Another report prepared composite materials from cellulose by only adsorbing modified hemicellulose onto to the cellulose base (Brumer *et al.*, 2004). The adsorption between cellulosic materials and hemicelluloses might be due to hydrogen bonding (Silveira *et al.*, 2013). Another point was, the substrates was freeze-dried in present work without separation, while reported adsorption experiments filtered to separate the bulk pulp and extraction liquor (Ban and van Heiningen, 2011). Freeze-drying might have helped hydrogen-bond formation to improve WSC adsorption, due to its forced dehydration, while there was not such force during filter separation.

Adsorption experiments were carried out in present work to verify this hypothesis, as results shown in Fig. 4. All substrates including Avicel cellulose, filter paper, and alfalfa stems were able to adsorb some WSCs, not only xylan, but also xylose. WSC loading had a positive effect on the adsorption of both xylan and xylose. Avicel had the best adsorption capability, filter paper was the second, while alfalfa stems was worse than both of them, with adsorption efficiency decreased dramatically with increasing WSC loading. In general, alfalfa stems without acid pretreatment was able to gain 1.7-3.3% weight by adsorbing extra WSCs, regarding alfalfa stems themselves had some WSCs (less than 1%DM). Noting that acid pretreated alfalfa stems had higher surface area, larger pore size and higher pore volume than original alfalfa stems (Zhou *et al.*, 2015), which were hypothesized to be able to improve the adsorption capability, thus acid pretreated alfalfa stems could gain weight of more than 3.3% by adsorbing WSCs. In summary, it was highly possible that most of the WSCs were adsorbed onto bulk substrates for either original or acid pretreated alfalfa stems.

Fortunately, the ambient-temperature acid pretreatment with drying process did improve the ethanol yields, and retained most of the WSCs, which is an advantage of this process. The

reasons why the acid loading and pretreatment time improved the ethanol yields were mainly because higher acid loading and longer pretreatment time improved the acid hydrolysis of hemicellulose during the ambient-temperature acid pretreatment, thus cellulose exposure was improved. Thus, we theorized the mechanism of this process included several steps: 1) sulfuric acid hydrolyzed some hemicelluloses (Fig. 2) to form WSCs, and dissolved ash (Table 2), which changed the biomass complex structure and improved the cellulose exposure; 2) most of the water-soluble carbohydrates (WSCs) adsorbed onto the surfaces of bulk alfalfa stems during the freeze-drying, but created surface and improved pores were (partially) kept; 3) washing removed most of the ash, but little WSCs which were adsorbed; and 4) the improved cellulose exposure of alfalfa stems, and decreased ash content, finally improved the ethanol production from alfalfa stems.

4. Conclusions

Model compounds and alfalfa stems were studied to investigate the mechanism of ambient-temperature acid pretreatment. Water-soluble carbohydrates (WSCs), chemical components of substrates, and ethanol production *via* simultaneous saccharification and fermentation (SSF) were determined. Results showed that **no significant amount of** highly crystalline cellulose was hydrolyzed **into water-soluble sugars** during the ambient-temperature acid pretreatment. WSCs formed from hemicellulose hydrolysis during the ambient-temperature acid pretreatment, but hemicellulose contents did not change significantly, which was theorized to be because most WSCs retained by absorbing onto the bulk substrates. The ambient-temperature acid pretreatment improved ethanol production from alfalfa stems by hydrolyzing hemicelluloses and removing ash to improve cellulose exposure.

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Figure Captions

Fig. 1. Prediction model for ethanol yields of unwashed (A) and washed (B) alfalfa stems.

Fig. 2. WSCs of different substrates with ambient-temperature acid pretreatment (95%CI).

(WSSs, water-soluble sugars; WSCs, water-soluble carbohydrates; A, WSSs of pure cellulose; B, WSSs of pure hemicellulose; C, WSSs of alfalfa stems; D, WSCs of alfalfa stems; DM, dry matter)

Fig. 3. Ethanol yields of unwashed (A) and washed (B) alfalfa stems (95%CI).

(0, untreated alfalfa stems; 35, 100, and 200 g/kg, acid loading during the ambient-temperature acid pretreatment)

Fig. 4. Adsorption capability and efficiency of cellulosic substrates (95%CI).

(A, gained weight of different substrates at different xylan loading; xylan was loaded as solid for Avicel, F-Paper, A-Stem, whereas it was loaded as solution for F-Paper-S; B, gained weight of different substrates at different xylose loading; Avicel, Avicel PH-101; F-Paper, filter paper; A-Stem, alfalfa stems)

Tables

Table 1

Parameter estimates of prediction models for ethanol production.

Term	Unwashed ($R^2 = 0.89$)			Washed ($R^2 = 0.65$)		
	Estimate	t Ratio	Prob > b	Estimate	t Ratio	Prob > b
Intercept	19.69	26.59	< 0.0001*	16.67	5.31	< 0.0001*
a	0.05352	11.15	< 0.0001*	0.09824	4.81	< 0.0001*
t	0.05378	3.61	0.0019*	0.2491	3.93	0.0008*
(a-112.2)*(t-25.83)	-0.000803	-3.66	0.0017*	--	--	--

* denotes statistical significance.

Table 2

Component analysis of alfalfa stems (95%CI).

Alfalfa stems	Acid	Ash	Glucan	Hemicellulose	ASL ^a	KL ^b	CP ^c	Recovery
	g/kg DM ^d	%DM						
Untreated	0	5.57±0.01 ^e	38.95±0.40	15.63±0.21	4.16±0.17	19.44±0.08	10.16±0.30	93.92±0.05
Acid treated	35	10.39±0.91	41.05±0.65	14.82±0.37	3.63±0.09	18.39±0.39	8.96±0.21	97.37±0.87 ^f
	100	17.39±0.88	36.95±1.24	13.78±0.57	3.63±0.09	16.64±0.98	7.90±0.13	
	200	27.15±0.68	32.29±1.29	12.29±0.20	3.88±0.13	15.78±0.53	7.16±0.16	
Added ash removed	35	5.57	43.14±0.85	15.58±0.43	3.82±0.10	19.32±0.39	9.41±0.12	96.18±0.96
	100		41.89±1.09	15.63±0.73	4.12±0.13	18.86±1.05	8.96±0.11	
	200		41.19±1.70	15.68±0.33	4.95±0.18	20.12±0.61	9.06±0.14	
Washed	0	2.41±0.14	40.73±0.35	16.39±0.06	3.98±0.02	22.42±0.55	7.31±0.61	93.24±0.02
	35	2.33±0.14	42.98±0.91	16.22±0.27	3.71±0.10	23.64±1.22	6.59±0.31	95.42±1.00 ^f
	100	3.17±0.24	40.95±0.56	16.00±0.16	3.89±0.05	24.01±1.20	7.20±0.47	
	200	2.96±0.27	43.95±1.16	15.52±0.21	3.95±0.09	22.91±0.92	6.30±0.39	

^a acid soluble lignin; ^b Klason lignin; ^c crude protein; ^d dry matter; ^e mean ± (1.96 × standard error); ^f average cross the acid loading of 35, 100 and 200 g/kg DM.

Figures

Fig. 1.

A

B

Fig. 2.

A

B

C

D

Fig. 3.

A

B

Fig. 4.

A

B