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Optimizing on-farm pretreatment of perennial grasses for fuel ethanol production

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ABSTRACT

Switchgrass (*Panicum virgatum* L.) and reed canarygrass (*Phalaris arundinacea* L.) were pretreated under ambient temperature and pressure with sulfuric acid and calcium hydroxide in separate experiments. Chemical loadings from 0 to 100 g (kg DM)$^{-1}$ and durations of anaerobic storage from 0 to 180 days were investigated by way of a central composite design at two moisture contents (40% or 60% w.b.). Pretreated and untreated samples were fermented to ethanol by *Saccharomyces cerevisiae* D5A in the presence of a commercially available cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme 188). Xylose levels were also measured following fermentation because xylose is not metabolized by *S. cerevisiae*. After sulfuric acid pretreatment and anaerobic storage, conversion of cell wall glucose to ethanol for reed canarygrass ranged from 22% to 83% whereas switchgrass conversions ranged from 16% to 46%. Pretreatment duration had a positive effect on conversion but was mitigated with increased chemical loadings. Conversions after calcium hydroxide pretreatment and anaerobic storage ranged from 21% to 55% and 18% to 54% for reed canarygrass and switchgrass, respectively. The efficacy of lime pretreatment was found to be highly dependent on moisture content. Moreover, pretreatment duration was only found to be significant for reed canarygrass. Although significant levels of acetate and lactate were observed in the biomass after storage, *S. cerevisiae* was not found to be inhibited at a 10% solids loading.

1. Introduction

There is considerable interest in developing a transportation fuel economy based on locally produced, renewable feedstocks. These fuels promise to promote rural development, reduce dependence on non-renewable energy sources and, most importantly, reduce human impact on global climate change. In the United States, the biofuel economy is based primarily on corn grain ethanol, but next generation biofuels feedstocks will likely be based on cellulosic plant residues and dedicated energy crops. Cellulosic biofuels further promote efforts to reduce greenhouse gas emissions and stabilize production costs while enabling a diverse and robust feedstock (Farrell et al., 2006).

Despite its lower carbon footprint, the amount of cellulosic biomass that will be needed to produce biofuels will be immense. A 75 ML yr^{-1} ethanol production facility will require approximately 408–870 Mg day^{-1} of biomass, depending on conversion efficiency and type of feedstock (NREL, 2009). Supplying this amount of feedstock will require developing a supply network rivaling that of the current corn and soybean networks. Unfortunately, research on feedstock harvesting and storage logistics is lacking in comparison to research related to biomass processing. Without a coherent, effective system in which to produce, process and deliver such biomass feedstocks, the expansion of the ethanol market into cellulosic ethanol will be limited.

One challenging area of logistics is developing methods of long-term storage. Prior research and economic analysis of biomass conversion emphasize dry storage. Such dry harvesting and packaging requires operations that do not add value to the biomass because biological conversion processes will ultimately require the material to be processed in a liquid slurry. These current dry harvesting operations may include, but are not limited to, windrowing, merging, baling, collecting and staging, and finally storing either indoors or outdoors. Furthermore, delivery to the biorefinery requires loading, transporting, staging, de-twining, size reduction and finally rehydration (Hess et al., 2007; Shinners et al., 2003; Shinners and Boettcher, 2006; Sokhansanj et al., 2009).

Recently, a potentially more cost-effective alternative has been proposed: wet storage of biomass. Wet storage methods for feedstock preservation and on-farm storage of perennial grass and corn stover biomass have been purported to reduce harvesting costs,

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lower dry matter (DM) losses during storage, increase product uniformity, improve feedstock susceptibility to enzymatic hydrolysis and reduce risk of fire (Richard et al., 2001; Shinners et al., 2007). Wet storage systems may also present an opportunity to add value to the feedstock by integrating a chemical or biological pretreatment (Digman et al., 2007; Ren et al., 2004, 2006, 2007; Richard et al., 2001). In-storage pretreatments at ambient temperature and pressure but with prolonged reaction times (e.g. months) may lower total pretreatment costs and provide better return for producers.

Forages are commonly stored wet by a process known as ensiling. Ensiled crops are packed so as to exclude oxygen. The absence of oxygen promotes the growth of lactic acid-producing bacteria and other related genera. Conversion of the soluble sugars present in the wet biomass into a mixture of organic acids and alcohols lowers the pH until bacterial growth ceases, thereby preserving the remaining biomass. Typical biomass losses are between 1% and 10%, which is lower than observed for dry stored biomass (Shinners et al., 2007; Shinners and Boettcher, 2006). Exclusion of oxygen is necessary to prevent growth of fungi and the possibility of spontaneous ignition from respiration-generated heat.

Wet storage of biomass for production of ethanol will most likely operate on a slightly different basis than the process currently used for forages. Organic acids generated during ensiling are at sufficient concentrations to adversely affect fermentative microbes used to produce ethanol. Therefore, we propose an ensiling process utilizing chemical stabilizers that retard ensiling and may also promote enzymatic conversion of plant carbohydrates into fermentable sugars at the biorefinery. In fact, chemicals have long been added to forages to improve animal digestibility (Sundstol and Owen, 1984). Addition of sufficient sulfuric acid to silage has been demonstrated to block bacterial growth and promote animal digestion by as much as 30% (Owen et al., 1984). Likewise, adding alkali to raise the pH will also promote digestibility. Typical alkaline agents include NaOH, NH_3 and $Ca(OH)_2$. In general, NaOH is the most popular for applying to wet biomass and has a rapid reaction time. While $Ca(OH)_2$ may take weeks to react, it has other advantages compared to NaOH, including lower cost, handling ease and avoidance of residual sodium salts.

Prior work on wet storage of biomass for conversion to biofuels has been limited to a few treatment conditions and crops, and generally has not included ethanol conversion data. In this work, wet storage was applied to the energy crops switchgrass (*Panicum virgatum* L.) and reed canarygrass (*Phalaris arundinacea* L.). Furthermore, each feedstock was treated with either sulfuric acid or calcium hydroxide. While all experiments were at laboratory scale, pretreatment conditions were carefully selected to allow for on-farm use during wet storage. Therefore, in each case the wet-harvested biomass was treated and stored under ambient temperature and pressure. The experimental design was selected to explore those factors across a range that could be manipulated, albeit to a limited degree, by the producer. Factors investigated in the design included: chemical loading, storage duration and substrate moisture content. Following pretreatment and storage, the biomass was fermented to ethanol using the yeast *Saccharomyces cerevisiae* in the presence of a commercially available cellulase. Pretreatment effects on silage acids, cell wall carbohydrates and cellulose conversion to ethanol are presented. Our goal was to determine those conditions that preserved the biomass cell wall carbohydrates, minimized in-storage formation of organic acids during storage from soluble sugars and promoted its conversion to ethanol.

2. Methods

The following sections outline the methods used to characterize each fresh substrate and the influence of both storage and pretreatment. The cell wall of the substrate is of considerable interest as it contains the carbohydrates not immediately available for conversion to fuel ethanol. Our approach was to assess not only those carbohydrates present at harvest but also those after pretreatment to better understand pretreatment effects. Consequently, we defined conversion as the amount of initial (at harvest) cell wall glucose measured as ethanol after simultaneous saccharification and fermentation. This definition is conservative considering that the only way all of these carbohydrates could be utilized would be through direct conversion after harvest. Alternative definitions of conversion would ignore losses of carbohydrates from plant respiration or microbial degradation during storage.

2.1. Experimental design

While pretreatment conditions were carefully selected for applicability on the farm during wet storage, the experimental design was selected to explore a range of factors that could be manipulated by the producer. Factors investigated in the design included: chemical loading, storage duration and substrate moisture content (Table 1). Each chemical and substrate combination was investigated as a separate experimental design, yielding four experiments: reed canarygrass treated with sulfuric acid (acid), reed canarygrass treated with calcium hydroxide (lime), switchgrass treated with acid and finally switchgrass treated with lime.

Chemical loadings and durations were arranged using a central composite design (CCD) (Box et al., 1978). The center point of the chemical loading and duration (50 g (kg DM)$^{-1}$ and 105 days) was highly replicated, as shown in Table 1, to achieve equal precision throughout the inferential range of the response surface. Two levels of moisture content were investigated in a complete factorial arrangement with the chemical loading and duration treatments. Each experiment was replicated three times as completely randomized blocks.

2.2. Substrate

Biomass substrates – switchgrass and reed canarygrass – were obtained from four-hectare plots established in the spring of 2004 at the University of Wisconsin Arlington Agricultural Research Station located in Arlington, WI (Shinners and Boettcher, 2006). The studied substrate was harvested with a direct-cut forage harvester at a theoretical length of cut of 5 mm in July and August 2007 for reed canarygrass and switchgrass, respectively. After harvest, each substrate was homogenized with a reel type mixer (Model 3115, Kuhn North America, Brodhead, WI) and transferred to sealed 158 L plastic bags. Half of the substrate was immediately stored (−20 °C) and the remainder was dried on screens to a moisture content of ~30% wet basis (w.b.). During the wilting process the material was intermittently hand-mixed and monitored to

Table 1
Response surface utilizing a central composite design (CCD) in which the CCD treatments were arranged in factorial combination for each biomass species, pretreatment chemical and moisture level combination.

Duration days	Loading g (kg DM)$^{-1}$	Replicates
30	50	3
52	14.6	3
52	85.4	3
105	0	3
105	50	15
105	100	3
158	14.6	3
158	85.4	3
180	50	3

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ensure the target moisture content was met, at which time the material was bagged and stored at −20 °C. The substrate was removed from storage one-day prior to use, transferred to a refrigerator (5 °C) and allowed to thaw. Moisture was determined for both reed canarygrass and switchgrass as loss on drying in a forced air oven; the temperature and drying time were 103 °C and 24 h, respectively per ASABE S358.2 (ASABE, 2008). Mean particle size was also determined at this time using an ASABE standard particle size separator (ASABE, 2007).

2.3. Pretreatment

Sulfuric acid (acid) and calcium hydroxide (lime) were applied as an 18 N solution and as a dry powder, respectively. First, substrate was rehydrated prior to pretreatment to meet the ~40% or ~60% wet basis (w.b.) targets, as rehydration has also been utilized in related ensiling work (Ren et al., 2007). Using moisture determined prior to start of hydration, as previously described, specific rehydration prescriptions were determined for both reed canarygrass and switchgrass substrates. Both low- and high moisture samples were initially amended with the same amount of water to ensure that level of moisture content could be considered as a separate effect from the influence of the hydration process. Individual sample moisture was modified by applying distilled water with a spray bottle and mixing by hand, ensuring water was well-absorbed and thereby minimizing surface moisture.

Next, pretreatment for both acid and lime was applied by hand for individual samples. Both acid and lime were applied on a substrate dry matter (DM) basis. Each sample consisted of 250 g DM of rehydrated substrate. Chemical was applied while mixing the sample by hand in a dish tub. To ensure adequate coverage, acid was applied using a spray bottle and lime was carefully distributed from pre-weighed aluminum weigh boats.

Upon completion of pretreatment, samples were densified by hand into "mini-silos" (1-l borosilicate glass canning jars; J. Weck GmbH and Co., Oflingen, Germany) consistent with the method of Deutsche Landwirtschafts-Gesellschaft (DLG) (Pflaum et al., 1996). Storage conditions were anaerobic, 22 °C and 200 kg DM m^{-3} density, similar to that of on-farm ensiling (Muck and Holmes, 2000, 2006; Pitt and Muck, 1993). Finally, after each mini-silo had been stored for the duration prescribed by the experimental design, it was frozen at −20 °C until it could be sampled and processed as described in the following sections.

2.4. Sample preparation for analysis

After anaerobic storage, a representative 40 g subsample was collected from each silo. Each aliquot was suspended in 150 ml of distilled water and ground using a laboratory grinder (Model B-400, Büchi Labortechnik AG, Flawil, Switzerland). Next, the samples were individually titrated to neutral pH using 4 M NaOH or 18 N sulfuric acid, after which each suspension was frozen and subsequently freeze-dried. Finally, dried samples were ground in a vortex mill (Udy Corporation, Fort Collins, CO) through a 1 mm screen. These sub-samples were used for all subsequent analysis, except for determination of anaerobic storage fermentation products.

2.5. Dry matter and ash

Dry matter was determined by loss on drying as previously described. Ash content was determined as residue remaining after combustion at 500 °C for 4 h. Each of the following analytical methods utilized these dry matter and ash values to report constituents on an organic matter basis. Ash and dry matter for each freeze-dried and ground sample were determined gravimetrically.

2.6. Compositional analysis

A modified version of the Uppsala Total Dietary Fiber Method was used to determine fermentable carbohydrates of the freshly harvested substrate (Theander et al., 1995). This process included preparation of a starch-free, alcohol-insoluble residue that was subsequently dissolved in 12 M sulfuric acid at room temperature for 3 h. The next step included complete cell wall hydrolysis by first diluting with distilled water to yield 0.3 M sulfuric acid, followed by heating the residue in an autoclave at 121 °C for 1 h. After filtration, neutral sugars, including glucose, xylose, arabinose, mannose, galactose, rhamnose and fucose, were quantified by gas chromatography as alditol acetate derivatives. Total uronic acids were measured prior to heating via colorimetry, using glucuronic acid as a standard (Ahmed and Labavitch, 1977). The insoluble Klason lignin residue recovered by filtration was corrected for ash content by combustion in a muffle furnace for 6 h at 450 °C.

To better understand the influence of pretreatment and storage on the cell wall, 104 post-pretreatment and storage samples were also assayed via the modified Uppsala method to develop a near infrared reflectance spectroscopy (NIRS) model to predict the 208 remaining samples. Freeze-dried and ground samples were scanned in duplicate with a spectrophotometer (Model 6500, FOSS NIRSystems Inc., Laurel, MD). The resulting calibration, outlier detection, data transformation and sample prediction was automated using WinISI (Version 1.5, Infrasoft International LLC, State College, PA), a commercial chemometrics package.

2.7. Post-storage fermentation (ensilage) products

A 1 ml aliquot was taken before titration, frozen at −20 °C and used for measuring fermentation products (Muck and Dickerson, 1988). Fermentation acids (lactic, acetic, propionic and butyric) and ethanol were determined by high-performance liquid chromatography (Varian ProStar, Varian Inc., Palo Alto, CA) with a refractive index detector. Samples were injected (20 µl) onto an organic acid column (Aminex HPX-87H Column, Bio Rad Laboratories Inc., Hercules, CA) and eluted with 0.015 N H_2SO_4 in 0.0034 M ethylenediamine tetraacetic acid (EDTA) free acid at 0.7 ml min^{-1} and 45 °C.

2.8. Simultaneous saccharification and fermentation (SSF)

Ethanol and pentose sugar yields were determined using a modified simultaneous saccharification and fermentation (SSF) method (Dowe and McMillan, 2001). First, a 1 g DM aliquot of freeze-dried substrate was added to a 25 ml media bottle (Corning Glass, Corning, NY) along with 8 ml of sterile, distilled water and 0.04 g l^{-1} tetracycline as antibiotic. Then the samples were allowed to rehydrate overnight at 5 °C. The fermentation was buffered using sodium citrate buffer (0.5 ml, stock: 1 M, pH 5). Additional sterile, distilled water was added to give a final solids loading of 10%, which took into account differences in moisture content and additions.

Next, Celluclast 1.5 L (Novozymes, Bagsvaerd, Denmark) was added at 5 FPU(g DM)$^{-1}$ substrate, Novo188 ß-glucosidase (Novozymes, Bagsvaerd, Denmark) was added at 15 IU(g DM)$^{-1}$ substrate and 10× YP (2 ml, stock: 100 g l^{-1} yeast extract and 200 g l^{-1} peptone) was added to each bottle. Enzymes and YP stock were sterilized by 0.2 µm filtration and autoclaving, respectively.

Each bottle was inoculated with *S. cerevisiae* D5A to an O.D. of 1.0 at 600 nm. The inoculum was prepared by growing the yeast overnight in YPD (10 g l^{-1} yeast extract, 20 g l^{-1} peptone and 50 g l^{-1} dextrose) at 35 °C and 200 rpm. The cells were harvested by centrifugation (3200*g* for 15 min) and resuspended in a dilute

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peptone solution (1 g l^{-1} peptone). Fermentation was conducted for 72 h at 35 °C with gentle shaking (100 rev min^{-1}).

Monosaccharide and ethanol concentrations were measured by a HPLC system equipped with a refractive index detector (Spectra-SYSTEM, Thermo Electron Corporation, CA). Samples were injected (20 μl) onto an organic acid column (Aminex HPX-87H Column, Bio Rad Laboratories Inc., Hercules, CA) and eluted with 0.010 N H_2SO_4 at 0.6 ml min^{-1} and 65 °C.

2.9. Analysis

Effects of chemical loading, duration and moisture were tested using the GLM procedure of SAS (SAS, Cary, NC). The lsmeans statement was used to separate significant effects. Statistical significance was recognized for $P < 0.05$. Cellulose conversion was modeled using the stepwise procedure for data best described by linear and quadratic models. The nlin procedure was used for data that were better described by non-linear models.

3. Results

3.1. Substrate composition and physical properties

Composition and physical properties of fresh reed canarygrass and switchgrass are summarized in Table 2. Mean particle size was similar to or lower than theoretical length of cut: 5 and 3 mm for reed canarygrass and switchgrass, respectively. This may be attributed to substrate homogenization and handling. The moisture content measured after harvest for reed canarygrass was 45% w.b. and the harvested material was wilted to 29% as previously described. Similarly, switchgrass moisture content at harvest was 53% and was wilted to 28%. Table 2 also presents lignin as Klason lignin and cell wall carbohydrates as individual sugars, as determined by the modified Uppsala method.

3.2. Post-storage composition

Cell wall carbohydrates and lignin were also assayed after pretreatment and storage to better understand the preservative or detrimental effects of pretreatment and storage. The number of samples assayed with the Uppsala method was reduced using NIRS techniques as outlined in the methods section. A robust NIRS calibration ensures accurate prediction results, maximizing the experiment's resolution to describe the response of cell wall composition to pretreatment variables. Calibration performance for each carbohydrate and lignin is presented in Table 3 and is consistent with that found in previous work (Lamb et al., 2007).

Except for arabinose, chemical loading and duration did not lead to significant differences in cell wall component and therefore, the mean compositional data for post-storage samples are shown in Table 2. Xylan and glucan contents varied less than 10% (relative basis) from the untreated materials, which suggests that the major cell wall carbohydrates were preserved; earlier controls demonstrated insignificant loss of biomass dry matter in laboratory silos (data not shown). Similarly, Klason lignin also varied little, suggesting that it was resistant to the chemical pretreatments. Arabinose contents were lowered by the dilute-acid, but not the lime, pretreatment. It must be noted that in the Uppsala assay any carbohydrate that is soluble, in this case as a result of pretreatment and/or storage, is lost in the initial steps of the assay that are responsible for solubilizing and extracting non-cell-wall storage (e.g. starch) and soluble carbohydrates. Therefore, with the exception of arabinose, wet storage appeared to have had little influence on cell wall chemical composition.

Table 3
Predicted data range and near infrared reflectance spectroscopy (NIRS) model fit for prediction of cell wall components.

	Range (g (kg DM)$^{-1}$)	RMSECV	R^2
Xylose	106–230	9.0	0.89
Glucose	208–313	7.7	0.93
Klason lignin	89–152	6.8	0.69
Arabinose	4.0–38	1.9	0.95
Uronic acid	13–26	1.7	0.65
Galactose	4.0–15	0.49	0.97
Mannose	4.4–9.1	0.35	0.58
Rhamnose	0.0–2.9	0.24	0.88

RMSECV – root mean standard error of cross-validation.

3.3. Post-storage fermentation (ensilage) products

Fermentation products in pretreated and stored substrates (before SSF conversion) showed similarities regardless of lime or acid pretreatment. Butyrate, propionate, ethanol and formate concentrations were observed at low levels and were not well-described by models that included moisture and loading as variables. Because of their low concentrations, these acids were not included in subsequent plots. Another observation used to simplify the presentation of the data was that, compared to moisture levels, duration had a relatively minor influence on production of acetate, lactate and pH. Consequently, the effect of duration was not reported by itself but rather treatment data were pooled across all durations, adding any variability from duration to the standard error. A final observation resulted in descriptive models for lactate, acetate and pH plotted to highlight trends resulting from both moisture content and chemical loading.

The trends for post-storage fermentation products for reed canarygrass treated with acid are depicted by the treatment means and regression models in Fig. 1. First, pH fell as more acid was added, decreasing from 4.0 to 1.3 and 3.5 to 1.4 for low and high moisture contents, respectively. Adding more sulfuric acid also retarded production of lactic acid, whose levels decreased from 5.0 to

Table 2
Physical properties and cell wall composition of fresh reed canarygrass and switchgrass substrates after direct-cut harvest and following wet storage/pretreatment.

Sample	Treatment	Ash (g (kg DM)$^{-1}$)	MPS (mm)	Cell wall components (g (kg DM)$^{-1}$)							
				KL	Glc	Xyl	Ara	Gal	Man	Rha	UA
Reed canarygrass	Untreated	78	5	130	250	150	35	14	9.6	1.3	21
	Ensiled			120	240	150	32	13	6.2	0.9	18
	Lime			120	240	150	33	12	6.9	0.8	16
	Dilute-acid			130	230	150	22	12	6.1	0.7	18
Switchgrass	Untreated	80	3	150	300	200	29	8.9	6.8	2.5	25
	Ensiled			140	290	190	29	7.8	6.4	2.1	21
	Lime			130	290	190	27	7.2	6.7	1.8	18
	Dilute-acid			130	290	190	16	7.1	6.5	1.9	22

MC, moisture; MPS, mean particle size; KL, Klason lignin; Glc, glucose; Xyl, xylose; Ara, arabinose; Gal, galactose; Man, mannose; Rha, rhamnose; UA, uronic acids.

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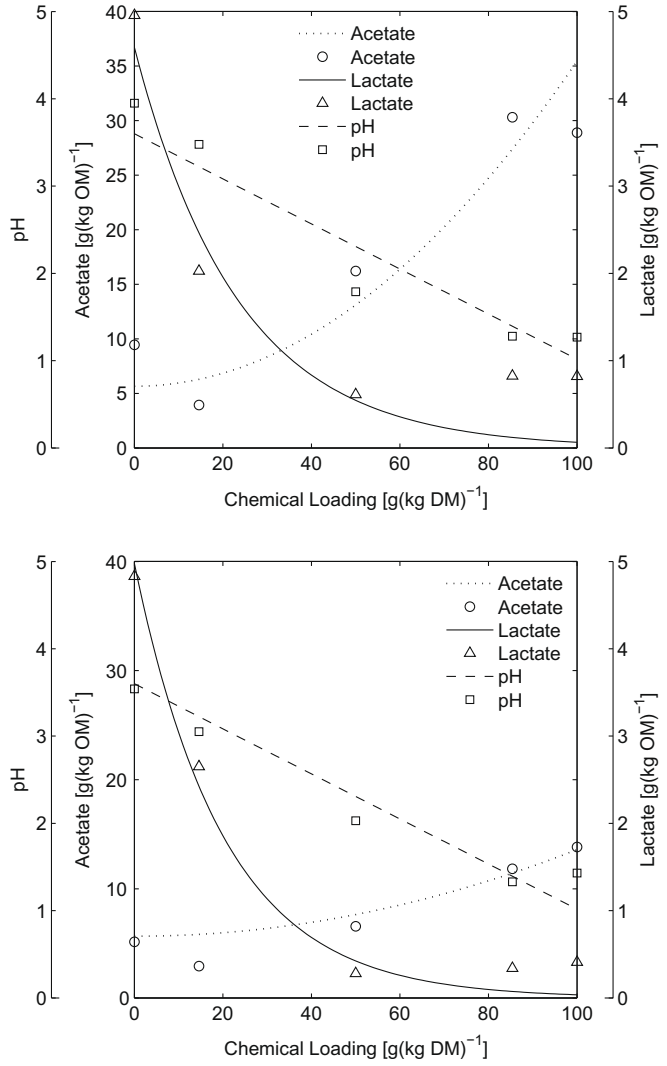

Fig. 1. Post-storage fermentation products for reed canarygrass at two moisture levels, 39% (top) and 59% w.b. (bottom) treated with varying levels of sulfuric acid. The pooled standard errors were 0.08, 0.29 and 1.45 for pH, lactate and acetate, at the low moisture level and 0.10, 0.13 and 0.56 at the high level, respectively.

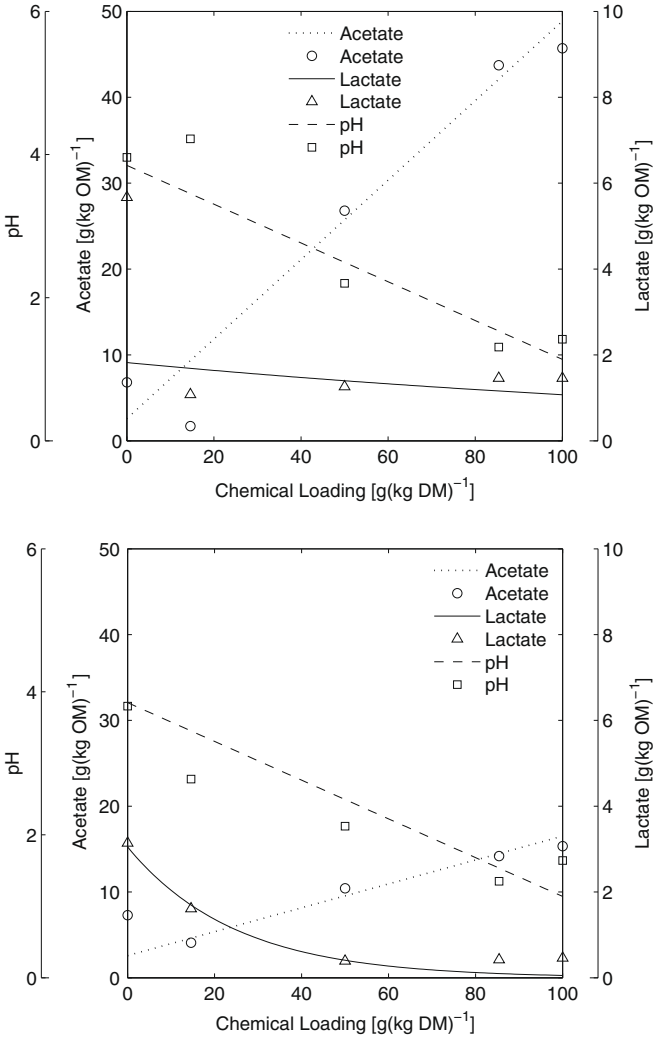

Fig. 2. Post-storage fermentation products for switchgrass at two moisture levels, 39% (top) and 67% w.b. (bottom) treated with varying levels, of sulfuric acid. The pooled standard errors were 0.20, 0.16 and 2.33 for pH, lactate and acetate, at the low moisture level and 0.15, 0.04 and 0.76 at the high level, respectively.

0.82 and 4.8 to 0.41 g(kg OM)$^{-1}$ for low and high moisture substrates. On the other hand, adding more sulfuric acid promoted acetate formation. Acetate concentration increased with chemical loading from 9.5 to 29 and 5.1 to 14 g(kg OM)$^{-1}$ for low and high moisture contents.

Switchgrass treated with acid yielded similar fermentation profiles to those of reed canarygrass (Fig. 2). As in the case of reed canarygrass, pH fell with sulfuric acid addition, decreasing from 4.0 to 1.4 and 3.8 to 1.6 for low and high moisture contents, respectively. Similarly, lactate levels decreased as more sulfuric acid was added, from 5.7 to 1.5 and 3.1 to 0.46 g(kg OM)$^{-1}$ for low and high moisture substrates, respectively. The positive response of acetate to sulfuric acid loading was linear for both moisture levels but was more pronounced for the lower moisture substrate, ranging from 6.8 to 46 g(kg OM)$^{-1}$. Sulfuric acid's influence on acetate was substantially less at the high substrate moisture level. Over the range of loadings studied, acetate increased from 7.3 to 15 g(kg OM)$^{-1}$.

Fig. 3 depicts the fermentation product trends observed for reed canarygrass treated with lime. First, pH increased with lime loading, ranging from 4.0 to 10 and 3.6 to 9.6 for low and high moisture levels, respectively. Conversely, levels of lactic acid decreased from 4.5 to 1.3 and 3.8 to 0.58 g(kg OM)$^{-1}$ with increasing loadings of lime for low and high moisture substrates, respectively. Acetate levels increased with lime loading and peaked with 85.4 g (kg DM)$^{-1}$ lime at levels of 27 and 38 g (kg OM)$^{-1}$ for low and high moisture contents, respectively. Otherwise, levels of acetate increased with chemical loading from 10 to 38 and 6 to 20 g(kg OM)$^{-1}$ for low and high moisture substrates, respectively.

In-storage fermentation product trends observed for switchgrass treated with lime are shown in Fig. 4. Similar to the lime-treated reed canarygrass, as more lime was added pH increased from 4.1 to 11 and 3.8 to 10 for low and high moisture substrates, respectively. Additionally, adding more lime retarded lactic acid production with final lactate concentrations decreasing from 6.3 to 2.5 and 2.9 to 0.98 g(kg OM)$^{-1}$ for low and high moisture substrates, respectively. Acetate's response to chemical loading varied depending on substrate moisture. At the low moisture level, acetate increased with lime loading from 11.5 to 57 g(kg OM)$^{-1}$. In the high moisture substrate, the acetate model reached an inflection point at a chemical loading of approximately 60 g (kg DM)$^{-1}$.

3.4. Cellulose conversion

In this study, cellulose conversion (C) was not determined directly (i.e., as the percentage of cellulose C_{CW} disappearing), but

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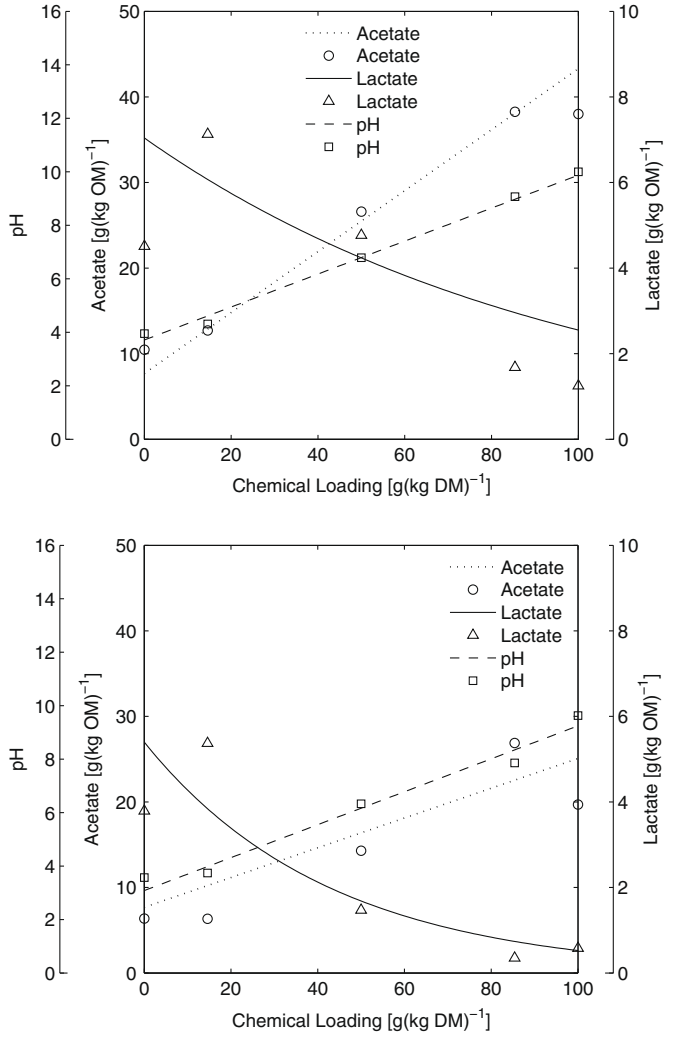

Fig. 3. Post-storage fermentation products for reed canarygrass at two moisture levels, 36% (top) and 57% w.b. (bottom) treated with varying levels of calcium hydroxide. The pooled standard errors were 0.16, 0.41 and 1.55 for pH, lactate and acetate, at the low moisture level and 0.17, 0.30 and 1.34 at the high level, respectively.

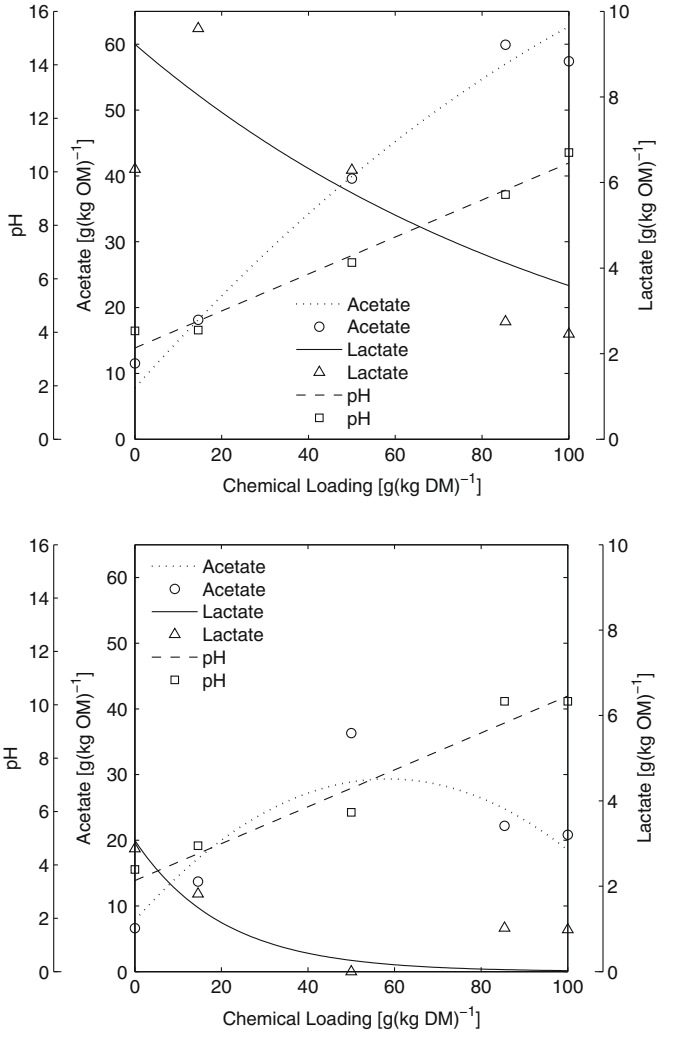

Fig. 4. Post-storage fermentation products for switchgrass at two moisture levels, 36% (top) and 66% w.b. (bottom) treated with varying levels of calcium hydroxide. The pooled standard errors were 0.10, 0.36 and 1.72 for pH, lactate and acetate, at the low moisture level and 0.44, 0.23 and 2.73 at the high level, respectively.

was calculated based on ethanol yield (E) following pretreatment, anaerobic storage, SSF and HPLC quantitation.

In Eq. (1), the percentage of cell wall glucose G in the original feedstock that was converted to ethanol C_{EtOH} was calculated assuming a stoichiometry of 2 mol ethanol per mol of glucose consumed and an increase in glucose mass upon cellulose hydrolysis of 1.11-fold (180 g glucose/162 g anhydroglucose G_a in cellulose; Eq. (2)).

Thus:

$$C = \frac{C_{EtOH}}{C_{CW}} \tag{1}$$

where

$$C_{EtOH} = E[g]\left(\frac{1E[mol]}{46.1E[g]}\right)\left(\frac{1G[mol]}{2E[mol]}\right)\left(\frac{180G[g]}{G[mol]}\right)\left(\frac{1G[mol]}{162G_a[g]}\right). \tag{2}$$

Results from four separate experiments in which reed canarygrass and switchgrass were treated with either lime or acid are presented. Although each experiment was conducted separately, the following trends were observed across experiments. First, xylose measured after SSF was low. Xylose levels were consistently less than 10% of xylose measured by the Uppsala method. Glucose measured in the SSF hydrolysate was low (less than 0.5 g l^{-1}), suggesting its nearly complete fermentation. Finally, post-SSF lactate and acetate were similar to that measured after anaerobic storage suggesting the absence of microbial contamination.

In the first experiment, in which reed canarygrass was treated with acid, a non-linear model was determined to provide best fit of the trends observed (Fig. 5). Because only two levels of moisture were investigated, the equations were specific for both high (59%) and low (39%) moisture contents.

$$C(L,D) = 91(1 - 0.82e^{-0.024L - 0.00056D}) \tag{3}$$

$$C(L,D) = 102(1 - 0.88e^{-0.017L - 0.00047D}) \tag{4}$$

Eqs. (3) and (4) represent the low and high moisture models, respectively. Here the dependent variable (C) conversion is a function of chemical loading (L), from 0 to 100 g (kg DM)$^{-1}$ and duration (D) for 0–180 days. Conversion of cell wall glucose to ethanol was well-described by the models, which had an average standard error of predicted values (SEP) of 6.9 and a R^2 of 0.89 for high moisture samples and a SEP 5.9 of and a R^2 of 0.88 for low moisture samples. Exploring the treatment means revealed conversions ranging from 22% for 14.6 g (kg DM)$^{-1}$ loading,

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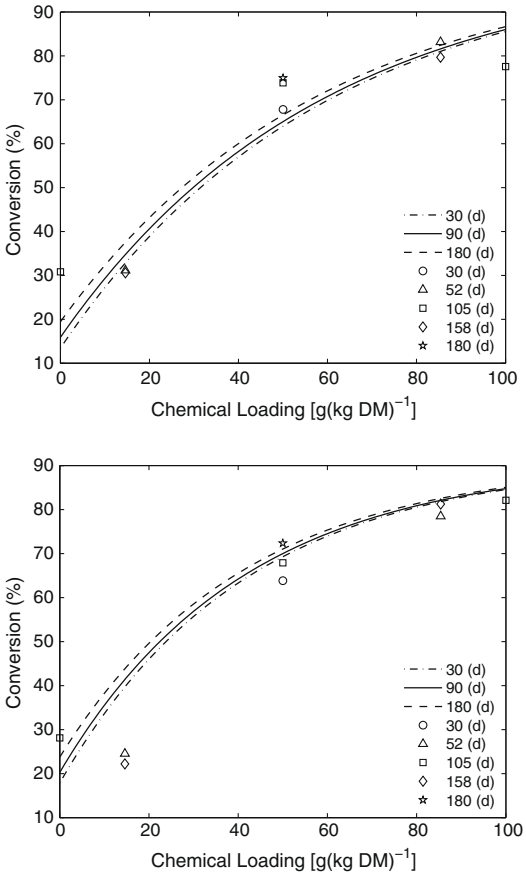

Fig. 5. Conversion of cell wall glucose to ethanol for reed canarygrass at two moisture levels, 39% w.b. (top) and 59% w.b. (bottom) treated with varying levels of sulfuric acid. Data points represent means of individual treatment combinations. Regression lines represent predicted values for specific moisture content and stated duration (D) based on computations from Eq. (3) (top) or Eq. (4) (bottom). The SEP for high- and low moisture samples is 6.9 and 5.9, respectively.

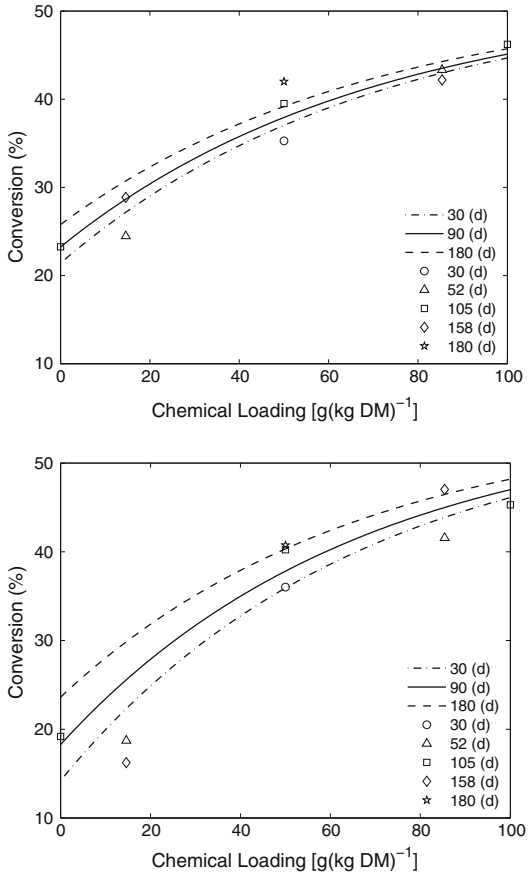

Fig. 6. Conversion of cell wall glucose to ethanol for switchgrass at two moisture levels, 39% w.b. (top) and 67% w.b. (bottom) treated with varying levels of sulfuric acid. Data points represent means of individual treatment combinations. Regression lines represent predicted values for specific moisture content and stated duration (D) based on computations from Eq. (5) (top) or Eq. (6) (bottom). The SEP for high- and low moisture samples is 4.3 and 4.9, respectively.

158 day duration and high moisture level to 83% for substrate treated at the 100 g (kg DM)$^{-1}$ level, 105 days and low moisture. The effect of duration on glucose conversion was subjugated by increased chemical loading.

The response of switchgrass to acid pretreatment was similar to that of reed canarygrass (Fig. 6). As such, a non-linear model was found to best describe the trends in the treatment means.

$$C(L,D) = 52(1 - 0.61e^{-0.014L - 0.00103D}) \quad (5)$$

$$C(L,D) = 55(1 - 0.78e^{-0.015L - 0.00174D}) \quad (6)$$

Eqs. (5) and (6) represent the low and high moisture models, respectively. Conversion of cell wall glucose to ethanol was well-described by the models which had a SEP of 4.3 and a R^2 of 0.73 for high moisture samples and a SEP of 4.9 and a R^2 of 0.73 for low moisture samples. Conversion treatment means ranged from 16% for 14.6 g (kg DM)$^{-1}$ loading, 158 day duration and low moisture level to 47% for substrate treated at the 85.4 g (kg DM)$^{-1}$ level, 158 days and low moisture. Moisture had less of an effect on conversion than was observed for reed canarygrass. The influence of duration on glucose conversion was inconsistent.

In the third experiment, conversion results for reed canarygrass treated with calcium hydroxide (lime) was best described by a quadratic model depending on L, D and moisture content (M) for 36% or 57% w.b. (Fig. 7; Eq. (7)).

$$C(L,D,M) = 21 - 0.000053LD^2 + 0.0028DL^2 + 0.0025M^2 \quad (7)$$

With an SEP of 8.0 and an R^2 of 0.88, the model accurately described the data, revealing that the efficacy of lime pretreatment increased with substrate moisture and duration as measured by increased conversion of cell wall glucose to ethanol. Additionally, conversions ranged from 21% for low moisture substrate treated at the 14.6 g (kg DM)$^{-1}$ level to nearly 55% for high moisture substrate treated at the 85 g (kg DM)$^{-1}$ level and stored for 158 days. This conversion performance was not maximized given the range of variables explored. Lastly, the experiment revealed that low levels of lime treatment did not improve glucose conversion.

In the final experiment, the response of switchgrass treatment with calcium hydroxide was different than for reed canarygrass. A linear model depending on L and M for 36% or 66% w.b. most accurately described the results observed over the range of variables explored (Fig. 8; Eq. (8)).

$$C(L,M) = 22 + 0.054LM \quad (8)$$

With an SEP of 7.2 and an R^2 of 0.79, the model revealed that the efficacy of lime pretreatment increased with substrate moisture as in reed canarygrass treated with lime. Here, however, it was found that the loading's effect was not independent of moisture. Additionally, conversions ranged from 18% for untreated, anaerobically stored, high moisture substrate to nearly 54% for high moisture substrate treated at the 85.4 g (kg DM)$^{-1}$ level and stored for

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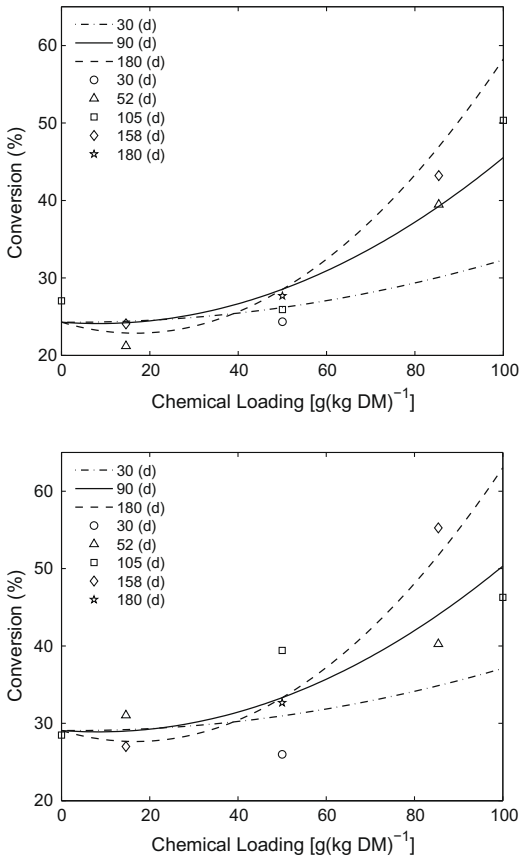

Fig. 7. Conversion of cell wall glucose to ethanol for reed canarygrass at two moisture levels, 36% w.b. (top) and 57% w.b. (bottom) treated with varying levels of calcium hydroxide. Data points represent means of individual treatment combinations. Regression lines (SEP = 8.0) represent predicted values for specific moisture content and stated duration (D) based on computations from Eq. (7).

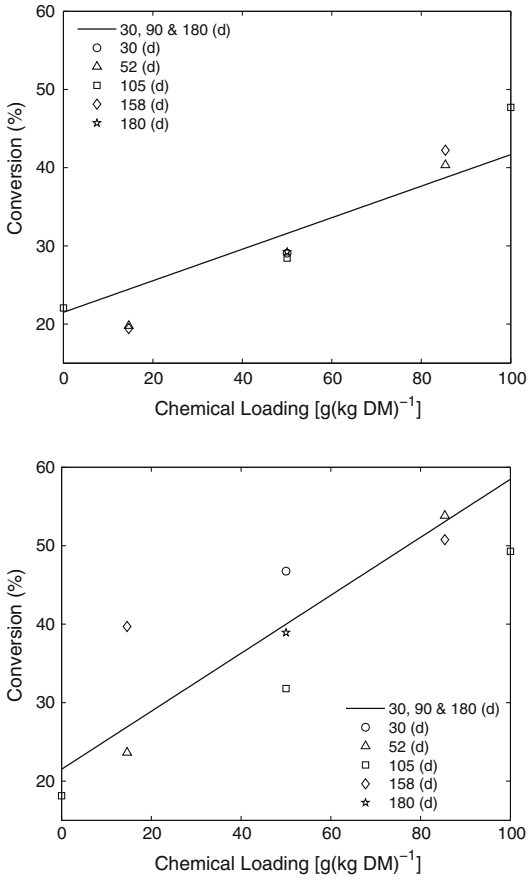

Fig. 8. Conversion of cell wall glucose to ethanol for switchgrass at two moisture levels, 36% w.b. (top) and 66% w.b. (bottom) treated with varying levels of calcium hydroxide. Data points represent means of individual treatment combinations. Regression lines (SEP = 7.2) represent predicted values for specific moisture content and stated duration (D) based on computations from Eq. (8).

52 days. This conversion performance was well correlated with chemical loading but was not maximized given the range of variables explored. Duration was not found to significantly influence conversion.

4. Discussion

4.1. Composition

Fresh reed canarygrass was found to have lignin and cell wall levels consistent with reed canarygrass in the "ripe seed" stage of maturity (Dien et al., 2006). Similarly, the composition of switchgrass indicates a maturity near to anthesis when considering level of lignification, yet cell wall carbohydrates were slightly higher, suggesting a more mature crop (Dien et al., 2006). These differences might be explained by agronomic or varietal differences: the reference values were for Cave-in-Rock variety grown in east-central Nebraska and harvested in 2003, whereas our switchgrass was Shawnee grown in south-central Wisconsin and harvested in 2007.

All wet storage conditions were successful in preserving cell wall-associated glucose and xylose intact, and the small changes that were observed were not attributable to the selected pretreatment conditions. In contrast, arabinose was removed from the cell wall when treated with sulfuric acid and, furthermore, the effect increased with greater acidification. Arabinose occurs as a side-group on the xylan chain of arabinoxylan and should be more easily hydrolyzed than xylose during dilute-acid pretreatment. Stepwise regression was used to relate level of residual level of arabinose in the cell wall after pretreatment and storage to conversion of cell wall glucose to ethanol. Level of arabinose was highly correlated with conversion, with a root mean standard error (RMSE) of 4.6% total cellulose converted and an R^2 of 0.95 for reed canarygrass. Similar performance was observed in switchgrass with a RMSE of 4.0% conversion and an R^2 of 0.78. Prior literature suggests that increased xylan hydrolysis is highly correlated to greater enzymatic conversion of cellulose (Yang and Wyman, 2004).

4.2. Post-storage fermentation (ensilage) products

Production of fermentation products during wet storage showed similarities regardless of lime or acid pretreatment. First, pH was strongly influenced by chemical loading independent of moisture content or storage duration. Both pretreatment methods demonstrated a strong relationship between chemical loading and the measured concentrations of lactate and acetate.

Lactate was suppressed by increased chemical loading regardless of pretreatment (i.e., for low and high pH [acid- and lime-pretreated substrates, respectively]). Suppression of lactate was more pronounced at lower substrate moistures when the substrate was treated with acid. Conversely, higher substrate moistures resulted in lower levels of lactate production for lime-pretreated substrates. Suppression of lactate may be attributed to the inhibition of lactic acid bacteria. Given the pH values observed, this result is consis-

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tent with previous work in anaerobic storage and ensiling of forages (McDonald, 1981a; Muck, 1988).

Acetate concentration increased with addition of either acid or lime. There are two possible sources for acetate formation: *de novo* production by *in situ* fermentation of soluble sugars and chemical hydrolysis of acetyl side-chains from arabinoxylan. As it is doubtful that fermentation activity was responsible for acetate formation at higher acidic or alkaline pHs because the extreme pHs would have suppressed microbial activity, acetate most likely originated from chemical de-acetylation of arabinoxylan. Previous work has documented release of acetyl groups from hemicellulose constituents resulting from alkali pretreatment of crop residues to improve digestibility (Chesson, 1981). Switchgrass treated with lime at the high moisture level showed a more complex pattern of acetate production. The acetate model reached an inflection point at approximately 60 g (kg DM)$^{-1}$ with a pH of 7.5. This may indicate that fermentative production of acetate was promoted by lower lime loadings. Neutral pH values are known to be favorable to both clostridia and enterobacteria, which are responsible for production of acetic and butyric and acetic acid, respectively (McDonald, 1981b,c). This explanation is consistent with the butyric acid measurements at medium levels of lime pretreatment but cannot be substantiated because no measurements of microbial populations were performed.

At appropriate chemical loadings, both acid and lime were successful in limiting microbial activities, as monitored by production of lactate. Furthermore, none of the stored biomass samples contain sufficient lactate and acetate concentrations to inhibit subsequent fermentation of glucose by *S. cerevisiae* at 10% wt/wt solids, as evidenced by the very low accumulation of residual glucose during SSF. At a 10% solids loading, acetate levels would range from 1.5 g l^{-1} for reed canarygrass treated with acid at high moisture to almost 6 g l^{-1} for switchgrass treated with lime at low moisture. It has been reported that acetate levels as low as 0.5 g l^{-1} can cause stress on some yeasts (Almeida et al., 2007). However, at a buffered pH of 5, yeast can tolerate considerably higher levels of both acetic (pK_a 4.74) and lactic acid (pK_a 3.86) as the acids will be mostly in the dissociated form, which is not inhibitory to growth (Graves et al., 2006). This may explain why inhibition was not observed in our fermentations, but it still is of concern under other scenarios, such as in fermentations at higher solids loading or at lower pH. Therefore, for wet storage of biomass, sufficient chemical loadings to retard microbial activity are still warranted for optimizing subsequent biological conversion into ethanol.

4.3. Cellulose conversion

Cellulose was considered to be the primary substrate for ethanol production in this study because mature grasses contain low amounts of other glucose sources (free glucose, sucrose and starch) and only limited amounts of cell wall hexoses (galactose and mannose) and because most cell wall glucose is present as cellulose (Dien et al., 2006). Conversion of cell wall glucose to ethanol was enhanced by acid pretreatment and storage for both reed canarygrass and switchgrass substrates. The variable with the strongest influence on conversion was chemical loading. Additionally, duration was observed to have a positive impact on conversion although this effect decreased as acid loading increased. On the other hand, increased moisture had a negative effect on conversion for both switchgrass and reed canarygrass substrates, particularly at low chemical loadings. Although chemical loading was determined on a dry matter basis, higher levels of moisture would decrease the concentration of acid and consequently, its effectiveness. Finally, the response of reed canarygrass to acid pretreatment was greater than switchgrass. Although not statistically comparable because they were determined from different experiments, there were considerable conversion differences between acid-treated substrates: reed canarygrass conversions ranged from 22% to 83% whereas switchgrass conversions ranged from 16% to 46%. This observation is consistent with the known greater recalcitrance of C_4 grasses relative to C_3 grasses.

Lime pretreatment and anaerobic storage increased conversion of cell wall glucose to ethanol under most conditions, but results were not always consistent between crops and substrate moistures. The lime experiments also were less repeatable as indicated by higher standard errors for treatment means. Contributing factors might be the low-solubility of lime and its application as a dry powder, though every effort was made to ensure homogeneous application. Lime pretreated substrate did, however, respond to chemical loading. The influence of moisture was strong for both substrates and inseparable from the effect of loading in the switchgrass substrate. Low levels of pretreatment reagent resulted in inconsistent glucose conversion results for both reed canarygrass and switchgrass. Duration was a factor in both high and low moisture reed canarygrass but was not significant in switchgrass. To summarize, substrate pretreated with lime at high moistures and chemical loading resulted in conversions significantly better than untreated substrate. Reed canarygrass conversions ranged from 21% to 55% of total cellulose and switchgrass conversions ranged from 18% to 54%. Even though the conversion performance was well correlated with chemical loading, it was not maximized given the range of variables explored.

Ethanol yields were optimal for reed canarygrass pretreated with dilute sulfuric acid and the maximum ethanol conversion efficiency of 83% compares favorably with those reported in literature. For example, glucose yields for corn stover pretreated with a wide-variety of pretreatments were 86–96% (Wyman et al., 2005). In another study (Tucker et al., 2003), corn stover was pretreated with dilute-acid at 160–190 °C and the washed solids were converted to ethanol using SSF. The ethanol conversion efficiencies were 72–92%. However, these studies included much more intensive pretreatments and processing, including a washing step following pretreatment. In contrast, results from on-farm pretreatment of switchgrass suggest that further pretreatment will be required at the ethanol facility to increase its conversion efficiency.

That reed canarygrass and switchgrass should yield different results is not surprising because the former is a cool season and the latter a warm season grass and, therefore, the two are anatomically quite different. Furthermore, reed canarygrass contains less lignin than does switchgrass (130 vs. 150 g (kg DM)$^{-1}$). Prior comparison of these two energy crops also revealed that reed canarygrass was significantly more amenable to conversion than switchgrass (Dien et al., 2006). However, this is not to suggest that wet storage is not a feasible option for switchgrass. Switchgrass will arrive at the ethanol facility with the acid or base still intact and, therefore, probably would only require a thermal treatment. Whether or not on-farm pretreatment acts synergistically with the additional thermal pretreatment remains to be tested. Regardless, as suggested before, chemical adjustment of pH is still warranted to control microbial activity during storage.

5. Conclusions

Ensiling with and without chemical addition was evaluated for wet storage of switchgrass (SWG) and reed canarygrass (RCG) prior to conversion into ethanol. Adding either dilute sulfuric acid (acid) or calcium hydroxide (lime) was found to suppress microbial activity during storage as evidenced by reduced organic acid formation and upon subsequent cellulase addition, the wet-stored biomass was fermentable by *S. cerevisiae* into ethanol. Acid was most effective for RCG where the cellulose to ethanol conversion efficiency

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was 22–83%; for SWG it was only 16–46%. Lime was less effective for RCG (21–55%) and similar to acid for SWG (18–54%).

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