PILOT-SCALE ON-FARM PRETREATMENT OF PERENNIAL GRASSES WITH DILUTE ACID AND ALKALI FOR FUEL ETHANOL PRODUCTION

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Abstract. *Switchgrass (Panicum virgatum L.) and reed canarygrass (Phalaris arundinacea L.) were pretreated during anaerobic wet storage by adding sulfuric acid or calcium hydroxide 50 g(kg DM)$^{-1.}$ Experiments were conducted at both the laboratory (250 g DM) and pilot-scale (250 kg DM) for either 60 or 180 days. Pretreated and untreated samples were fermented to ethanol by Saccharomyces cerevisiae in the presence of a commercially available cellulase (Celluclast 1.5L) and β-glucosidase (Novozyme 188). With acid pretreatment, conversion of cellulose to ethanol was 35 and 12 percentage units higher than for untreated controls for reed canarygrass and switchgrass, respectively. Similarly, lime-pretreated reed canarygrass and switchgrass out-yielded controls by 3 and 13 percentage units, respectively. Cellulose and lignin contents were largely unaffected by pretreatment and anaerobic storage, but hemicelluloses were lower for both pretreatments. Chemical costs for biomass pretreatment applied at a rate of 50 g(kg DM)$^{-1}$ were estimated to be as low as $4.05 and $5.20 per Mg DM for calcium hydroxide and sulfuric acid, respectively.*

Keywords. pretreatment, biomass, perennial grass, switchgrass, reed canarygrass, ethanol

INTRODUCTION

It is becoming increasingly evident that the current system of providing fuel in America is not sustainable. Consequently, 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 our country's impact on global climate change.

Fuel ethanol production is currently limited to sugar and starch crops, such as corn and sugar cane. It is expected that further expansion of the fuel ethanol market will depend upon conversion of lignocellulose, which include agricultural residues and herbaceous perennials, such as corn stover and switchgrass. These feedstocks are advantageous to traditional feedstocks in that they could have a much lower carbon footprint and a higher net energy return (Farrell et al., 2006).

Despite the lower carbon footprint, the amount of biomass that will be needed to produce biofuels will be immense. Thus, the logistics of harvesting, storing, and transporting the immense quantities of biomass needed to supply cost-competitive biorefineries is a great challenge (Hess et al., 2007). Most research on biomass conversion assumes the crops will be harvested and stored dry. Such dry harvesting and packaging requires many operations that do not add value to the biomass when considering most conversion processes will ultimately require the material to be processed wet (Hess et al., 2007; Shinners et al., 2003; Shinners and Boettcher, 2006).

Recently, a more cost-effective wet-storage alternative has been proposed (Richard et al., 2001). Wet storage methods for feedstock preservation and on-farm storage of perennial grass and corn stover biomass have been purported to reduce harvesting costs, lower dry matter (DM) losses during storage, increase product uniformity, improve feedstock susceptibility to enzymatic hydrolysis, and lower risk of fire.

Also, as the biomass is already wet, it may be possible to pretreat it at the same time for increased degradability (Digman et al., 2007; Ren et al., 2004; Ren et al., 2006; Ren et al., 2007). While in-storage pretreatments will be limited to ambient temperature and pressure conditions, reaction times can be on the order of months. Significantly

63 increasing the degradability of the biomass while in storage is expected to add value by either allowing milder, or
64 possibly need for, pretreatment at the biorefinery, thereby, providing better return for farmers.
65
66 Biomass needs to be pretreated prior to further processing because the carbohydrates are largely contained in
67 complex cell wall structures that impede their enzymatic conversion into fermentable sugars. Biomass recalcitrance
68 also limits digestion in ruminants and, as such, there is a long history of chemically treating forages to increase their
69 energy values because ruminants only recover half of the carbohydrates when fed untreated forage (Hatfield et al.,
70 1999). In fact, many of the same pretreatment strategies investigated for biofuel applications also have been
71 evaluated on livestock forages (e.g. ammonia fiber expansion (AFEX)). Previous research on chemical treatment of
72 forages is an excellent resource for developing an on-farm biomass pretreatment system. First, systems have already
73 been developed for applying some of the milder types of pretreatments on the farm. Second, many similarities exist
74 between ruminant utilization of forages and biochemical conversion of biomass into ethanol. As such, researchers
75 have correlated in vitro gas production, a widely accepted assay of ruminant forage digestibility, to ethanol yields by
76 simultaneous saccharification and fermentation (SSF) (Weimer et al., 2005).
77
78 There has been extensive prior work in applying chemical methods to forage to improve animal digestibility
79 (Sundstol and Owen, 1984). Chemical treatments of forages often rely on wet-storage methods. This means that to
80 improve digestibility these methods need to both preserve the biomass and avoid dry matter losses. Storing biomass
81 in a wet form necessitates storing it anaerobically in order to avoid excessive heating and respiration by
82 microorganisms. Long-term stability of anaerobically stored silages depends upon ensuring appropriate amounts of
83 readily fermented carbohydrates, moisture content, and acidity(McDonald, 1981a).
84
85 In areas of the world (e.g. Northern Europe) where wilting forages to moisture contents low enough to promote
86 desirable fermentation by LAB is challenging, mineral and organic acids have been added to lower the pH and
87 restrict undesirable fermentation by clostridia (Muck, 1988). The before-mentioned acid treatments provide the basis
88 for, yet are quite different from, the ensuing work exploring pretreating forages by adding acids. In this work, much
89 higher chemical loadings were explored to produce a fiber digestibility response. As such, pretreatment of forages
90 and plant residues with sulfuric acid was conducted at orders of magnitude higher than when used as a preservative:

0 to 160 g(kg DM)$^{-1}$ compared to 0.04 g(kg DM)$^{-1}$. Sulfuric acid pretreatment of straw was demonstrated to increase dry matter digestibility of barley straw *in vivo* as much as 30 percentage points after a 60 g(kg DM)$^{-1}$ treatment followed by neutralization with NH_3 (Owen et al., 1984). However, low pH, the need for neutralization, and concern about animal intake led to little further study of acid as a pretreatment for increasing digestibility of animal feedstuffs.

As an alternative to acid pretreatment, alkali chemicals have been the most studied and widely applied forage treatments (Sundstol and Coxworth, 1984). The more commonly applied chemicals include sodium hydroxide (NaOH), anhydrous ammonia (NH_3), aqueous ammonia (NH_4OH) and urea. In wetter conditions, such as ensiling, NaOH is the most widely investigated alkali pretreatment (Jackson, 1977; Wilkinson, 1984). Pretreatment success was variable depending upon feedstock composition and moisture content. In general, it was found that low moisture and initial digestibility had a negative influence on alkali pretreatment success.

Calcium hydroxide ($Ca(OH)_2$) has been proposed as an alternative to NaOH. The benefits of $Ca(OH)_2$ are that it is less expensive, safer to handle and avoids Na residue (Hadjipanayiotou, 1984; Owen et al., 1984). The main disadvantage is that $Ca(OH)_2$ reacts much more slowly than does NaOH. NaOH reacts in minutes whereas $Ca(OH)_2$ has been reported to take 10 to 14 days at room temperature (Owen et al., 1984). Additionally, it has been reported that the moisture range for successful treatment and anaerobic storage of $Ca(OH)_2$ is very narrow (50 to 60% w.b.). Higher moistures reportedly lead to fermentation before chemical reaction and lower moisture contents lead to molding (Hadjipanayiotou, 1984; Owen et al., 1984). Nevertheless, $Ca(OH)_2$ has not only been proven to be an effective stabilizer of silage, it also has been shown to improve *in vitro* digestibility (Hadjipanayiotou, 1984).

Most of the aforementioned pretreatments have been investigated for biochemical conversion of lignocelluloses to biofuels (Chandra et al., 2007; McMillan, 1993; Mosier et al., 2005; Sun and Cheng, 2002; Wyman et al., 2005). In each case, the pretreatments were designed to be carried out at the biorefinery where short processing times become essential. As a consequence, biomass was reacted at high temperatures and/or pressures that would preclude their use on farms.

Prior work on wet storage of biomass for conversion to ethanol has been limited to a few treatment conditions and crops and generally does not include ethanol fermentation data. In this work, switchgrass (*Panicum virgatum* L.) and reed canarygrass (*Phalaris arundinacea* L.) were pretreated with both sulfuric acid and calcium hydroxide, in separate experiments at both the laboratory (250 g DM) and pilot-scale (250 kg DM) and for two storage durations, 60 and 180 days. Pretreatment conditions were anaerobic at ambient temperature and pressure. Following pretreatment and storage, the biomass was fermented to ethanol using the yeast *Saccharomyces cerevisiae* in the presence of commercial cellulase. Pretreatment effects on silage acids, cell wall carbohydrates and cellulose conversion to ethanol are presented.

MATERIALS AND 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.

SUBSTRATE

Biomass substrates -- switchgrass (*Panicum virgatum* L.) and reed canarygrass (*Phalaris arundinacea* L.) -- were obtained from two 4 ha 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 substrates studied were harvested with a direct-cut forage harvester with a theoretical length of cut of 19 mm on 2 July and 19 August 2007 for reed canarygrass and switchgrass, respectively.

PRETREATMENT AND STORAGE

After harvest, the substrate was staged on a cement pad until treatment and storage. Next, material for one 500 kg experimental silo was loaded with a skid-loader into a reel type feed mixer (Model 3115, Kuhn North America, Brodhead, WI) equipped with a four point weighing system and digital indicator (Digi-Star, Model 2400, Fort Atkinson, WI). The mixer was used to homogenize the substrate for each pilot-scale silo. After mixing for 5 minutes, the substrate was subsampled three times to assay initial carbohydrates. Initial carbohydrate samples were sealed in 950 ml plastic bags and stored at -20°C until assayed.

For each substrate (reed canarygrass or switchgrass) and chemical (sulfuric acid, calcium hydroxide or untreated) combination, an equal number of pilot-scale silos were produced and stored for either 60 or 180 days. Thus, two pilot-scale silos for each grass, chemical treatment, and duration were made each of which had three corresponding laboratory silos resulting in a total of 32 pilot-scale silos and 96 laboratory silos. Before chemical application,

153 substrate dry matter content was estimated using a microwave oven per ASABE S358.2 (ASABE, 2008).
154 Accounting for substrate and moisture weight in the mixer, the amount of acid or lime was determined and applied
155 at a rate of 50 g(kg DM)$^{-1}$ to a continuously mixing substrate. Calcium hydroxide (lime) pretreatment was applied as
156 a dry powder whereas sulfuric acid (acid) was applied as an 18 *N* solution. Lime was pre-weighed and slowly
157 distributed by hand over the blending auger. Acid was applied using a peristaltic pump that supplied a low-pressure
158 nozzle array consisting of six quarter-circle irrigation nozzles (Model 54011, Orbit Irrigation; Bountiful, UT). The
159 nozzle array was located above the blending auger of the mixer to ensure uniform substrate coverage. Application
160 time was approximately 10 minutes. After pretreatment, the substrate was homogenized in the mixer for 10 minutes.
161 Three 300 g laboratory silos were produced from each mixer load (pilot-scale silo) in vacuum-sealed 305 x 406 mm,
162 80 μm nylon-polyethylene vacuum pouches. Each subsample was vacuum-sealed, placed in another bag and
163 vacuum-sealed again. These silos were stored in the laboratory at approximately 22°C.

164 Pilot-scale silos were made using the following process. First, the substrate was unloaded from the mixer into a skid-
165 loader bucket and transferred to a custom-made compacting device used to compress the material to a density
166 similar to that found in a on-farm forage bunker or bag silo, approximately 200 kg DM m^{-3}. The compacting
167 machine consisted of a 1.2 x 1.2 x 2.4 m chamber with a movable platen that was powered by a 0.15 m diameter
168 telescoping cylinder. As each load (0.5 m^3) was transferred from the mixer, it was subsequently compressed using
169 this machine. After all of the material had been transferred from the mixer, it was held to the dimensions necessary
170 to meet the density target, constrained using nylon straps and ejected. After the pilot-scale silo was removed from
171 the chamber, a coring device was used to implant a temperature data logger near the center of the silo (Model: UA-
172 001-64, Onset Computer Corp., Bourne, MA). Finally, each pilot-scale silo was wrapped with six layers of 25 μm
173 polyethylene film (Up North Plastics Inc., Cottage Grove, MN) using a commercial bale wrapper (Model BW40,
174 AGCO Corp., Hesston, KS). Finally, pilot-scale silos were weighed and stored outdoors on wood pallets.

175 An untreated silo was made immediately after each treated (acid or lime) silo so that composition could be
176 compared after storage. The untreated silos were processed using the before-mentioned method. Replicate silos and
177 their paired untreated silo were stored outdoors for either 60 or 180 days. After the silo had been stored for the
178 duration prescribed by the experimental design, the silos were opened, weighed, broken apart and three random 300

g samples were taken and frozen at -20°C along with their corresponding laboratory silos and processed as described in the following sections.

Sample Preparation for Analysis

After anaerobic storage, 40 g representative samples were taken from each laboratory silo and before-mentioned pilot-scale silo subsamples. 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 subsamples were used for all subsequent analysis, except for determination of anaerobic storage fermentation products.

Dry Matter, Ash and Mean Particle Size

Moisture was determined for both fresh reed canarygrass and switchgrass as loss on drying in a forced air oven; the temperature and drying time were 103ºC and 24 hours per ASABE S358.2 (ASABE, 2008). Ensiled samples (post-storage) require a different procedure as to not drive off volatile fermentation acids. Here, samples were dried at 60ºC for 72 hours. Mean particle size was determined for both fresh and ensiled samples using an ASABE standard particle size separator (ASABE, 2007).

Ash and dry matter for each freeze-dried and ground sample were determined gravimetrically. Dry matter was determined by loss on drying; the temperature and drying time were 103ºC and 24 hours. Ash content was determined as residue remaining after combustion at 500ºC for 4 hours. These data were used to present all data on an organic matter (OM) basis.

Composition Analysis

Initial cell wall composition of the substrate -- reed canarygrass or switchgrass -- was determined using detergent fiber analysis. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were assayed using the Ankom filter bag method (Ankom Technology Corp., Fairport, NY) (Vogel et al., 1999). NDF and sequential ADF and ADL samples were corrected for ash after combustion at 500ºC for 4 hours. Cellulose was estimated by subtracting ADL from ADF, and hemicelluloses by the difference of NDF and ADF.

The number of post-storage samples for composition totaled 192, 96 from pilot-scale silos (3 subsamples each) and 96 from laboratory silos (1 subsample each). To reduce the amount of samples for detergent fiber analysis, Near Infrared Reflectance Spectroscopy (NIRS) was used to select samples encompassing the spectral variability, and therefore, compositional variability of the samples. Sixty-four samples were used to develop a NIRS model, which was used to subsequently predict the fiber content of the remaining 128 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.

POST-STORAGE FERMENTATION PRODUCTS

A 1 ml aliquot was taken before titration, frozen at -20ºC 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* EDTA free acid at 0.7 ml min^{-1} and 45ºC.

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 0.004% tetracycline as antibiotic. Then the samples were allowed to rehydrate overnight at 5ºC. 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 1.5L (Novozymes, Bagsvaerd, Denmark) was added at 5 FPU$(g\ DM)^{-1}$ substrate, Novo188 β-glucosidase (Novozymes, Bagsvaerd, Denmark) was added at 15 IU $(g\ DM)^{-1}$ substrate and 10x 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 or autoclaving, respectively.

Each bottle was inoculated with *Saccharomyces 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 x g for 15 min) and resuspended in a dilute peptone solution (1 g l^{-1} peptone). Fermentation was conducted for 72 h at 35°C with gentle shaking (100 rpm).

Monosaccharide and ethanol concentrations were measured by HPLC (SpectraSYSTEM, Thermo Electron Corporation, CA). Samples were injected (20 µl) onto an organic acid column (Aminex HPX-87H Column, Bio Rad Laboratories Inc., Hercules, CA), eluted with 5 m*M* H_2SO_4 at 0.6 ml min^{-1} and 65°C, and detected using a refractive index detector.

ANALYSIS

Each chemical and substrate combination was analyzed as a separate experiment: reed canarygrass treated with acid, reed canarygrass treated with lime, switchgrass treated with acid, and finally switchgrass treated with lime. Effects of pretreatment, duration and type of silo (laboratory or pilot-scale) 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 $P < 0.05$.

RESULTS

Composition and physical properties of fresh reed canarygrass and switchgrass are summarized in Table 1.

Table 1. Physical properties and cell wall composition of fresh reed canarygrass and switchgrass after direct cut harvest and homogenization.

	MC	Ash	MPS	Density	Cellulose	Hemicelluloses	Lignin
	%w.b.	g(kg DM)$^{-1}$	mm	(kg DM)m^{-3}		g(kg OM)$^{-1}$	
Reed Canarygrass	59	99	14	220	300	230	88
Switchgrass	67	81	16	200	340	270	96

Moisture Content, MC; Mean Particle Size, MPS

Detergent fiber components were assayed after pretreatment and storage to better understand both the preservative and detrimental effects of pretreatment and storage. The number of samples needed to be assayed with the Ankom

method was reduced using Near Infrared Reflectance Spectroscopy (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 (Table 2).

Table 2. Near Infrared Reflectance Spectroscopy (NIRS) model performance for detergent fiber.

	RMSECV g(kg DM)$^{-1}$	r^2
NDF	19.5	0.93
ADF	16.2	0.83
ADL	9.5	0.68

Neutral Detergent Fiber, NDF; Acid Detergent Fiber, ADF; Acid Detergent Lignin, ADL

Post-anaerobic storage cell wall composition for acid- and lime-treated switchgrass and reed canarygrass is presented in Table 3. Duration (60 or 180 days) and type of storage (laboratory or pilot-scale silos) had only a minor influence on cell wall composition when compared to the influence of pretreatment. Consequently, the effect of duration and storage are not reported separately, but rather the treatment means were averaged across storage type and duration, adding any variability from duration or type of storage to the standard error.

The following trends were observed in comparison with control samples that were ensiled without adding either acid or lime Levels of cellulose measured by detergent fiber analysis were not significantly affected by acid treatment of switchgrass or reed canarygrass but were significantly higher for lime-treated substrates. Conversely, hemicellulose levels were lower for both acid- and lime- treated substrates. In general, lignin content was not consistently affected by pretreatment, but was lower in acid-treated reed canarygrass and higher in lime-treated switchgrass.

Table 3. Post storage cell wall composition for reed canarygrass and switchgrass after on-farm treatment with calcium hydroxide or sulfuric acid at a rate of 50 g(kg DM)$^{-1}$ and anaerobic storage compared to control.

	Cellulose	Hemicelluloses	Lignin
		g(kg OM)$^{-1}$	
Reed Canarygrass			
Acid	303	220*	107*
Control	313	262	120
Switchgrass			
Acid	362	287	123
Control	356	295	119
Reed Canarygrass			
Lime	360*	229*	120
Control	339	247	119
Switchgrass			
Lime	375*	269*	120*
Control	359	288	113

*Indicates significant deviation from control at the $\alpha = 0.05$ level.

Dry matter loss was monitored for each pilot-scale and laboratory silo (Table 4). Laboratory dry matter losses showed similar trends but were consistently higher than pilot-scale losses. Dry matter loss differences were significantly higher for untreated substrates compared to acid-treated substrate in reed canarygrass but similar losses between treatments were observed in switchgrass. Alternatively, lime treatment did not significantly affect dry matter losses in reed canarygrass but resulted in higher losses in switchgrass compared to controls. Dry matter losses were consistently higher after 180 days of storage compared to 60 days in acid-treated substrates, but were similar for lime treatment. A negative dry matter loss was observed for reed canarygrass treated with lime. Negative dry matter losses could be the result of scale precision or bias introduced by the two oven methods employed to estimate moisture content for fresh and ensiled samples. Recall from the methods section that dry matter estimates for fresh and ensiled materials were conducted at two different oven temperatures. Although this is necessary as to not drive off volatile fermentation acids (VFAs) in ensiled material, a bias could be introduced resulting in a positive increase in dry matter weight, and therefore negative dry matter loss.

Table 4. Columns 1-2: Dry matter loss comparison between untreated and acid-treated and untreated and lime-treated reed canarygrass and switchgrass silos. Columns 3-4: Loss comparisons for 180-60 days for reed canarygrass and switchgrass. Columns 5-6: Loss comparisons for laboratory and pilot-scale silos.

	Untreated	Treated	60d	180d	Lab	Pilot
	%DM		%DM		%DM	
Acid						
Reed Canarygrass	3.7*	1.9	1.4*	4.4	3.7*	-0.15
Switchgrass	3.3	3.8	1.9*	5.2	4.4*	0.61
Lime						
Reed Canarygrass	0.30	0.43	1.4*	-3.5	1.6*	-1.4
Switchgrass	1.2*	2.1	1.7	1.5	2.3*	0.58

*Indicates difference is significant at the $\alpha = 0.05$ level.

Internal pilot-scale silo temperatures for both acid- and lime-treated switchgrass silos are presented in Figure 1. Initially, silo temperatures were significantly higher for lime-treated switchgrass compared to controls whereas acid-pretreated silos were lower than corresponding controls. However, after 45 days both acid- and lime-treated substrates began to track their associated untreated pilot-scale silo temperatures.

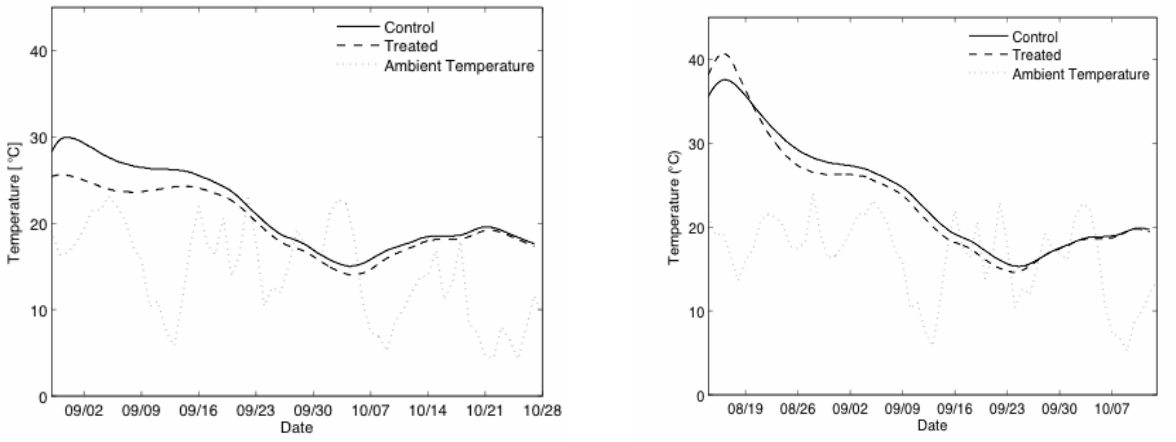

Figure 1. Internal pilot-scale silo temperature for sulfuric acid (left) and calcium hydroxide (right) treated and anaerobically stored switchgrass throughout a 60 day storage period compared to an untreated control.

299 Post-storage fermentation products for acid- and lime-treated switchgrass and reed canarygrass compared to controls
300 are presented in Table 5. As in cell wall data, duration and type of storage had only a minor influence on
301 fermentation products when compared to the influence of pretreatment and consequently, are not reported
302 separately, but rather as treatment means.
303

Table 5. Post storage fermentation product profile after on-farm treatment with calcium hydroxide or sulfuric acid at a rate of 50 g(kg DM)$^{-1}$ followed by anaerobic storage compared to control.

	pH	Lactate	Acetate	Butyrate	Ethanol
			g(kg OM)$^{-1}$		
Reed					
Acid	2.1*	2.2*	10*	0.01*	0.54*
Control	4.0	35	4.1	1.7	50
Switch					
Acid	2.2*	0.12*	15*	0.00	1.1*
Control	4.2	40	10	0.15	10
Reed					
Lime	7.0*	16*	17*	21*	23*
Control	3.9	51	6	0.02	59
Switch					
Lime	6.2*	0.07*	45*	13*	21*
Control	4.2	42	10	0.54	14

*Indicates significant deviation from control at the $\alpha = 0.05$ level.

304

305 Before this simplification, however, a few observations were made (Appendix A1-2). Duration had statistically
306 significant effects on various fermentation acids depending on grass and storage type, but actual quantities differed
307 by less than 5 g(kg OM)$^{-1}$ leading us to question the biological significance of these findings. However, acetate and
308 butyrate were quite different under certain situations. In acid-treated substrates, acetate was 2 and 7 g(kg OM)$^{-1}$
309 higher at the 180 day storage period for reed canarygrass and switchgrass, respectively. Also, in lime-treated
310 substrates butyrate was measured at higher levels with increased storage duration ranging from 7 to 14 and 5 to 9
311 g(kg OM)$^{-1}$ for reed canarygrass and switchgrass substrates, respectively. This was coupled with a significant
312 reduction in pH from 5.6 to 5.2 and 5.6 to 4.7 g(kg OM)$^{-1}$ for reed canarygrass and switchgrass, respectively.
313

Less significant was the type of storage. As with duration statistically significant effects were limited to actual differences of less than 5 g(kg OM)$^{-1}$. The only exception was ethanol in reed canarygrass. Here, ethanol was measured at higher levels for pilot-scale silos over laboratory silos from 17 to 33 and 27 to 55 g(kg OM)$^{-1}$ for acid- and lime-treated substrates, respectively.

First, pH significantly decreased for acid pretreatment whereas it was increased for lime pretreatment. Treatment with acid decreased levels of lactate, butyrate and ethanol while increasing acetate for both reed canarygrass and switchgrass. All changes in fermentation products were statistically significant except for butyrate in switchgrass where concentrations were low for both treated and untreated substrates. Lime treatment yielded significantly higher levels of acetate and butyrate.

Table 6 summarizes the amount of cellulose converted to ethanol for reed canarygrass and switchgrass substrates during both 60 and 180 day storage and for acid and lime pretreatment. According to the results, acid pretreated substrates outperformed controls by 35 and 12 percentage units for reed canarygrass and switchgrass, respectively. Also when comparing in-field, pilot-scale silos to laboratory silos, the in-field silos performed similar to or better than laboratory silos. A final analysis revealed cellulose conversion to ethanol was higher for 180 than 60 days.

Table 6. Cellulose converted to ethanol by SSF after on-farm treatment with sulfuric acid or calcium hydroxide at a rate of 50 g(kg DM)$^{-1}$ and anaerobic storage. Comparisons between treated and untreated substrates as well as type and duration of storage.

	Cellulose Conversion (% of Total)			
	Reed Canarygrass		Switchgrass	
	Acid	Lime	Acid	Lime
In-field	41*	21*	19	23*
Laboratory	34	16	19	18
Treated	56*	20*	25*	27*
Control	21	17	13	14
180	39*	15*	20*	19*
60	37	22	18	22

*Indicates significant difference between treatment means at the $\alpha = 0.05$ level.

Lime pretreatment was not as effective in reed canarygrass as acid, but was equally effective between pretreatments in switchgrass. Lime pretreated substrates outperformed controls by 3 and 13 percentage units for reed canarygrass and switchgrass, respectively. In-field, pilot-scale silos performed better than laboratory silos. Contrary to acid pretreatment, cellulose conversion to ethanol was lower for 180 than 60 days.

DISCUSSION

Mean particle size was lower than theoretical length of cut: 14 and 16 mm for reed canarygrass and switchgrass, respectively. This may be attributed to size-reduction during substrate homogenization and handling. The moisture content measured after harvest was 59 and 67% w.b. for reed canarygrass and switchgrass, respectively. Densities for both reed canarygrass and switchgrass pilot-scale silos were similar to that seen in previous assessments of on-farm ensiling of forage crops (Muck and Holmes, 2000, 2006; Pitt and Muck, 1993).

Temperatures for acid-treated switchgrass were lower than untreated switchgrass whereas lime-treated switchgrass temperatures were initially higher. This result may indicate a preservation regime in which acid treatment limited biological activity compared to lime treatment where increased temperatures may indicate increased biological activity. This hypothesis is founded given two observations. Acid-pretreated substrate pHs were approximately 2.0, which is known to greatly limit activity of microorganisms known to populate silages (McDonald, 1981a; Muck, 1988) but lime-treated substrates pHs were nearly neutral. Neutral pHs are known to be favorable to both clostridia and enterobacteria, which are responsible for production of butyric and acetic acid, respectively (McDonald, 1981b, c). This explanation is consistent with the elevated butyric and acetic acid levels with lime pretreatment but cannot be substantiated as these organisms were not assayed. Decreased concentrations of lactate were observed for both acid and lime pretreated substrates. Given the pH values of these treatments, this observation may be attributed to the inhibition of lactic acid bacteria (McDonald, 1981d; Muck, 1988).

Cell wall carbohydrates were largely unaffected by pretreatment and anaerobic storage, with the exception of hemicelluloses. Hemicellulose content after storage was significantly lower for both acid- and lime-treated reed canarygrass and lime-treated switchgrass. This result is not surprising, considering hemicelluloses in grasses are susceptible to degradation even under normal (untreated) ensiling conditions (McDonald, 1981a).

Cellulose was observed at higher levels in lime-treated substrates, but this is thought to be assay-related, as lime may have influenced cellulose availability to the detergent fiber technique. Previous work describes a treatment effect where cellulose is more digestible due to swelling of cellulose fibers (Tetlow et al., 1987).

Lignin levels were lower in acid-treated reed canarygrass, but higher in lime-treated switchgrass. These findings are inconsistent between substrates and pretreatment and are not consistent with that found in the literature. Initially it was thought that alkali treatment decreased lignin (Jackson, 1977; Klopfenstein, 1978; Sundstol and Coxworth, 1984). However, it was later discovered that acetic acid produced in NaOH-treated substrates was the result of the release of acetyl groups from hemicellulose constituents (Chesson, 1981). This is in agreement with work purporting that digestibility occurs as a result of chemical changes in the complexes of phenolic compounds and hemicelluloses in the cell walls (Tetlow et al., 1987). This is not surprising, however, as it has been demonstrated that ester linkages between uronic acid and either lignin or hemicellulose were destroyed in hydroxide solution with no change to lignin (Tarkow and Feist, 1969).

Cellulose conversion to ethanol was improved by both acid and lime pretreatment. Acid-pretreated substrates converted more cellulose to ethanol than controls by 35 and 12 percentage units for reed canarygrass and switchgrass, respectively. In-field, pilot-scale silos performed similar to, or better than, laboratory silos. Cellulose conversion to ethanol was higher for 180 than 60 days, suggesting that acid pretreatment not only aids in preservation but also continues to improve the substrate's susceptibility to hydrolysis over time. Lime pretreatment was not as effective in reed canarygrass as was acid, but equally effective results were observed for switchgrass. Lime pretreated substrates out-yielded controls by 3 and 13 percentage units for reed canarygrass and switchgrass, respectively. In-field, pilot-scale silos performed better than laboratory silos. Contrary to acid pretreatment, cellulose conversion to ethanol was lower for 180 than 60 days.

Organic acids produced by pretreatment and storage did not inhibit fermentation to ethanol by *Saccharomyces cerevisiae* in a SSF at 10% solids as measured by glucose remaining in hydrolysate. Finally, xylose measured in the hydrolysate was low and was not measured at levels greater than 10% of the total hemicelluloses in fresh substrate by the detergent fiber method.

In all cases, pilot-scale silo conversions were similar to or better than laboratory silos indicating that laboratory studies are appropriate for predicting field studies one thousand times their scale. This is consistent with previous scale comparisons in calcium hydroxide (Petersen et al., 1981). Stability of the laboratory silos over time, however, was not similar to that of the pilot-scale silos. Although similar types and thickness of plastics were used, the surface area-to-volume ratio of laboratory silos is significantly higher than pilot-scale silos. This increases the opportunity for oxygen diffusion for a given mass of substrate and, therefore, degradation by aerobic respiration. This was confirmed by the high dry matter losses observed in laboratory silos. To summarize, laboratory silos are a conservative estimate of pilot-scale silos, but long-term studies may not be as representative.

As a result of our experiments the value of on-farm pretreatment can be estimated. First, buffering capacity curves were used to determine the extent pH could be adjusted with both sulfuric acid (acid) and calcium hydroxide (lime) pretreatment (Appendix B). These data, along with reported estimates of the value that pretreatment, were used to determine feasible rates of on-farm pretreatment. Two methods were explored to estimate the value of pretreatment. The first calculation was based on the assumption that pretreatment is approximately one-third of the cost of ethanol (Wyman, 1999). Next, we assumed that on-farm pretreatment could replace pretreatment at the biorefinery with substrate yields of 265 l ethanol $(Mg\ DM)^{-1}$ valued at \$0.32 l^{-1} (Wyman, 1999). This scenario would mean that on-farm pretreatment's value would be an estimated \$28 $(Mg\ DM)^{-1}$. The second estimate was determined using the same assumptions except pretreatment costs were valued at \$0.08 l^{-1}, as suggested by Mosier et al. (2005) yielding an approximate value of on-farm pretreatment of \$21 $(Mg\ DM)^{-1}$ (Mosier et al., 2005). Therefore by our estimates the maximum value for on-farm pretreatment would be between \$21 and \$28 $(Mg\ DM)^{-1}$. Bulk calcium hydroxide and virgin sulfuric acid are reported to be valued at \$81 and \$104 Mg^{-1} (ICIS, 2006; Miller, 2002). As a result, biomass pretreatment applied at a rate of 50 $g(kg\ DM)^{-1}$ would result in a chemical costs of approximately \$4.05 and \$5.20 $(Mg\ DM)^{-1}$ for lime and sulfuric acid, respectively.

We believe these results show promise for the on-farm acid pretreatment system. Acid pretreatment would not only preserve the substrate by limiting microbial activity but would also begin to degrade the cellulose-hemicellulose-lignin cell wall matrix, thereby enhancing accessibility for enzymatic degradation. Additionally, this method was found to not inhibit fermentation of glucose to ethanol by *Saccharomyces cerevisiae* at a relatively high solids

loading. Acid pretreatment at the rates explored in this study did not yield complete conversion of available cellulose, but residual pretreatment chemical could be exploited at the biorefinery through an additional thermal process (Digman et al., 2008).

CONCLUSION

Pretreatment with calcium hydroxide and sulfuric acid followed by anaerobic storage significantly enhanced enzymatic degradability and subsequent fermentation for both reed canarygrass and switchgrass. In acid-pretreated substrates, conversion of cellulose to ethanol was 35 and 12 percentage units higher than controls for reed canarygrass and switchgrass, respectively. Similarly, lime-pretreated substrates out-yielded controls by 3 and 13 percentage units for reed canarygrass and switchgrass. Cellulose and lignin were largely unaffected by pretreatment and anaerobic storage, but hemicelluloses were lower.

In-field, pilot-scale silos performed similar to or better than laboratory silos. Cellulose conversion to ethanol was higher for 180 than 60 days in acid-pretreated substrate, but lower at the longer duration in lime-pretreated substrates. Biomass pretreatment applied at a rate of 50 g(kg DM)$^{-1}$ would result in chemical costs estimated at $4.05 and $5.20 per Mg DM for calcium hydroxide and sulfuric acid, respectively.

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APPENDIX A

Table A1. Post-storage fermentation product profile comparison of in-field and laboratory silos after on-farm treatment with calcium hydroxide or sulfuric acid at a rate of 50 g(kg DM)$^{-1}$ followed by anaerobic storage.

	pH	Lactate	Acetate	Butyrate	Ethanol
			g(kg OM)$^{-1}$		
Reed - Acid					
In-field	3.0*	18	7.9*	0.65	33*
Laboratory	2.9	19	6.7	1.0	17
Switch - Acid					
In-field	3.2	17*	14*	0.15	6.2*
Laboratory	3.1	22	11	0.00	4.5
Reed - Lime					
In-field	5.3*	35	13*	12*	55*
Laboratory	5.5	32	10	10	27
Switch - Lime					
In-field	4.7*	23*	28	7.1	19
Laboratory	5.6*	18	28	6.3	17

*Indicates significant deviation from control at the $\alpha = 0.05$ level.

Table A2. Post-storage fermentation product profile after on-farm treatment with calcium hydroxide or sulfuric acid at a rate of 50 g(kg DM)$^{-1}$ followed by anaerobic storage for 60 or 180 days.

	pH	Lactate	Acetate	Butyrate	Ethanol
			g(kg OM)$^{-1}$		
Reed - Acid					
60	2.8*	18	6.2*	1.0*	22
180	3.1	19	8.4	0.62	28
Switch - Acid					
60	3.1	23	9.1*	0.0039	4.7
180	3.2	17	16	0.15	5.9
Reed - Lime					
60	5.6*	34	11	7.4*	31*
180	5.2	32	12	14	50
Switch - Lime					
60	5.6*	20	24*	4.7*	18
180	4.7	21	30	8.7	18

*Indicates significant deviation from control at the $\alpha = 0.05$ level.

APPENDIX B

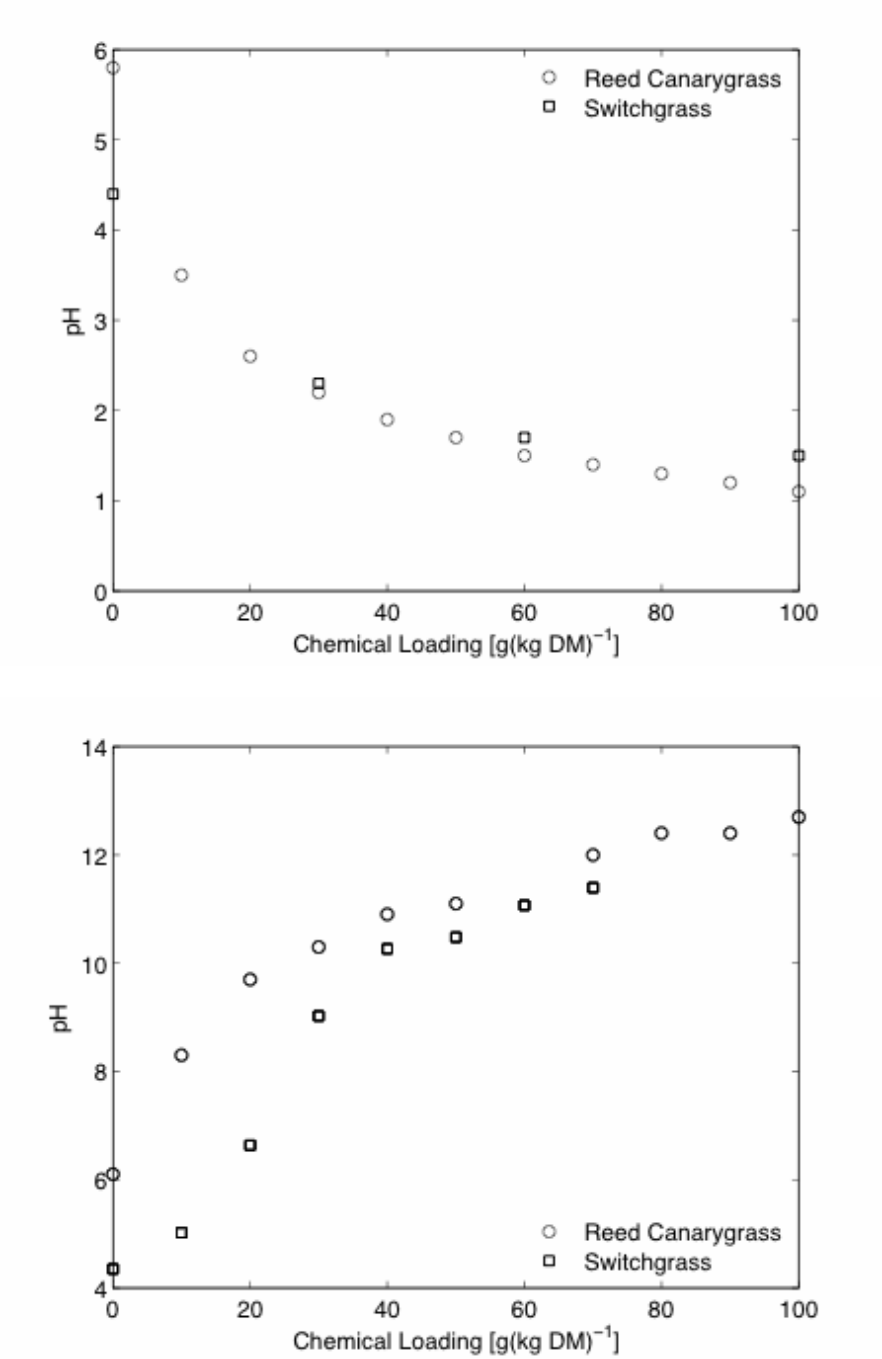

Figure B1. Response of pH in reed canarygrass and switchgrass substrates treated with sulfuric acid (top) and calcium hydroxide (bottom) at varying rates.

REFERENCES

Farrell, A. E., R. J. Plevin, B. T. Turner, A. D. Jones, M. O'Hare, and D. M. Kammen. 2006. Ethanol can contribute to energy and environmental goals. *Science* 311(5760):506-508.

Hess, J. R., C. T. Wright, and K. L. Kenney. 2007. Cellulosic biomass feedstocks and logistics for ethanol production. *Biofuels Bioproducts & Biorefining-Biofpr* 1(3):181-190.

Shinners, K. J., B. N. Binversie, and P. Savoie. 2003. Whole-plant corn harvesting for biomass: Comparison of single-pass and multiple-pass harvest systems. In *ASAE Annual International Meeting*. Las Vegas, NV.

Shinners, K. J., and G. C. Boettcher. 2006. Drying, harvesting and storage characteristics of perennial grasses as biomass feedstocks. In *ASABE Annual International Meeting*. Minneapolis, MN.

Richard, T. L., S. Proulx, K. J. Moore, and S. Shouse. 2001. Ensilage technology for biomass pre-treatment and storage. In *ASAE Annual International Meeting*. Sacramento, CA.

Digman, M. F., K. J. Shinners, B. S. Dien, R. E. Muck, L. Xin-Liang, R. D. Hatfield, and P. J. Weimer. 2007. On-farm Pretreatment Technologies for Improving Enzymatic Degradability of Cellulose and Hemicellulose Present in Perennial Grass. In *ASABE Annual International Meeting*. Minneapolis, MN.

Ren, H., T. L. Richard, K. J. Moore, and P. Patrick. 2004. Long-term kinetics of corn stover bioconversion in an enzyme enhanced mixed culture fermentation. In *ASAE/CSAE Annual International Meeting*. Ottawa, ON.

Ren, H. Y., T. L. Richard, Z. L. Chen, M. L. Kuo, Y. L. Bian, K. J. Moore, and P. Patrick. 2006. Ensiling corn stover: Effect of feedstock preservation on particleboard performance. *Biotechnology Progress* 22(1):78-85.

Ren, H. Y., T. L. Richard, and K. J. Moore. 2007. The impact of enzyme characteristics on corn stover fiber degradation and acid production during ensiled storage. *Applied Biochemistry and Biotechnology* 137:221-238.

Hatfield, R. D., J. Ralph, and J. H. Grabber. 1999. Cell wall structural foundations: Molecular basis for improving forage digestibilities. *Crop Science* 39(1):27-37.

Weimer, P. J., B. S. Dien, T. L. Springer, and K. P. Vogel. 2005. In vitro gas production as a surrogate measure of the fermentability of cellulosic biomass to ethanol. *Applied Microbiology and Biotechnology* 67(1):52-58.

Sundstol, F., and E. Owen. 1984. *Straw and other fiberous by-products as feed*. Elsevier Science Publishers B.V., Amsterdam, The Netherlands.

McDonald, P. 1981a. *The Biochemistry of Silage*. John Wiley and Sons, Inc., New York, NY.

Muck, R. E. 1988. Factors Influencing Silage Quality and Their Implications for Management. *Journal of Dairy Science* 71(11):2992-3002.

Owen, E., T. Klopfenstien, and N. Urio. 1984. Treatment with other chemicals. In *Straw and other fiberous by-products as feed*, 248-275. F. Sundstol, and E. Owen, eds. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Sundstol, F., and E. Coxworth. 1984. Ammonia Treatment. In *Straw and other fiberous by-products as feed*, 196-247. F. S. a. E. Owen, ed. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Jackson, M. 1977. Review article: The alkali treatment of straws. *Animal Feed Science and Technology* 2:105.

Wilkinson, J. 1984. Ensiling with soduim hydroxide. In *Straw and other fiberous by-products as feed*, 181-195. F. Sundstol, and E. Owen, eds. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.

Hadjipanayiotou, M. 1984. Effect of Level and Type of Alkali on the Digestibility Invitro of Ensiled, Chopped Barley Straw. *Agricultural Wastes* 10(3):187-194.

490 Chandra, R. P., R. Bura, W. E. Mabee, A. Berlin, X. Pan, and J. N. Saddler. 2007. Substrate pretreatment: The key
491 to effective enzymatic hydrolysis of lignocellulosics? *Biofuels* 108:67-93.

492 McMillan, J. 1993. Enzymatic Conversion of Biomass for Fuels Production. In *ACS Symposium Series 566*.
493 Washington, DC: American Chemical Society.

494 Mosier, N., C. Wyman, B. Dale, R. Elander, Y. Y. Lee, M. Holtzapple, and M. Ladisch. 2005. Features of promising
495 technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology* 96(6):673-686.

496 Sun, Y., and J. Y. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review.
497 *Bioresource Technology* 83(1):1-11.

498 Wyman, C. E., B. E. Dale, R. T. Elander, M. Holtzapple, M. R. Ladisch, and Y. Y. Lee. 2005. Comparative sugar
499 recovery data from laboratory scale application of leading pretreatment technologies to corn stover.
500 *Bioresource Technology* 96(18):2026-2032.

501 ASABE.2008.S358.2: Moisture Measurment - Forages. St. Joseph, MI.

502 ASABE.2007.S424.1: Method of determining and expressing particle size of chopped forage materials by screening.
503 St. Joseph, MI.

504 Vogel, K. P., J. F. Pedersen, S. D. Masterson, and J. J. Toy. 1999. Evaluation of a filter bag system for NDF, ADF,
505 and IVDMD forage analysis. *Crop Science* 39(1):276-279.

506 Muck, R. E., and J. T. Dickerson. 1988. Storage-Temperature Effects on Proteolysis in Alfalfa Silage. *Transactions*
507 *of the ASAE* 31(4):1005-1009.

508 Dowe, N., and J. McMillan. 2001. Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation.
509 National Renewable Energy Center. Golden, CO.

510 Muck, R. E., and B. J. Holmes. 2000. Factors affecting bunker silo densities. *Applied Engineering in Agriculture*
511 16(6):613-619.

512 Muck, R. E., and B. J. Holmes. 2006. Bag silo densities and losses. *Transactions of the ASABE* 49(5):1277-1284.

513 Pitt, R. E., and R. E. Muck. 1993. A Diffusion-Model of Aerobic Deterioration at the Exposed Face of Bunker Silos.
514 *Journal of Agricultural Engineering Research* 55(1):11-26.

515 McDonald, P. 1981b. Clostridia. In *The Biochemistry of Silage*, 77-90. New York, NY: John Wiley & Sons, Ltd.

516 McDonald, P. 1981c. Other Micro-organisms. In *The Biochemistry of Silage*, 91-102. New York, NY: John Wiley &
517 Sons, Ltd.

518 McDonald, P. 1981d. Lactic Acid Bacteria. In *The Biochemistry of Silage*, 62-76. New York, NY: John Wiley &
519 Sons, Ltd.

520 Tetlow, R. M., V. C. Mason, and G. Deschard. 1987. Treatment of Whole-Crop Cereals with Alkali .2. Voluntary
521 Intake and Digestibility by Sheep of Rye, Barley and Wheat Crops Ensiled with Sodium-Hydroxide.
522 *Animal Feed Science and Technology* 18(4):271-281.

523 Klopfenstein, T. J. 1978. Chemical Treatment of Crop Residues. *Journal of Animal Science* 46(3):841-848.

524 Chesson, A. 1981. Effects of Sodium-Hydroxide on Cereal Straws in Relation to the Enhanced Degradation of
525 Structural Polysaccharides by Rumen Microorganisms. *Journal of the Science of Food and Agriculture*
526 32(8):745-758.

527 Tarkow, H., and W. Feist. 1969. Celluloses and their application. Washington, DC: American Chemical Society.

528 Petersen, M. K., V. M. Thomas, and R. E. Roffler. 1981. Reconstituted Kentucky Bluegrass Straw .2. Ensiled with
529 Hydroxide and Soybean-Meal, Cull Beans or Peas. *Journal of Animal Science* 52(2):406-412.

530 Wyman, C. E. 1999. Biomass ethanol: Technical progress, opportunities, and commercial challenges. *Annual*
531 *Review of Energy and the Environment* 24:189-226.

532 ICIS. 2006. Chemical Market Reporter. Surrey, United Kingdom: Reed Business Information Limited.

533 Miller, M. 2002. Lime in the United States 1950 to 2001. U.S. Geological Survey.

534 Digman, M. F., K. J. Shinners, B. S. Dien, R. D. Hatfield, R. E. Muck, and P. J. Weimer. 2008. Investigation of a
535 two-stage process for increasing yields of ethanol from switchgrass. In *ASABE Annual International*
536 *Meeting*. Providence, RI.

537
538