Full-scale on-farm pretreatment of perennial grasses with dilute acid for fuel ethanol production

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ABSTRACT

Biorefineries that rely on lignocellulosic feedstocks require dependable and safe methods for storing biomass. Storing biomass wet in the presence of sulfuric acid and the absence of oxygen has been shown to preserve carbohydrates and enhance cellulose conversion but has not been demonstrated at farm-scale. To that end, switchgrass (*Panicum virgatum* L.) and reed canarygrass (*Phalaris arundinacea* L.) were pretreated with sulfuric acid with two methods: during bagging (on-line) and thoroughly mixed in a commercial feed mixer (mixed) and both stored for 90 days. The two methods, applied at rates from 28 to 54 g(kg DM)$^{-1}$ not only helped to preserve biomass substrates under on-farm conditions (anaerobic, ambient temperature and pressure) through inhibition of microbial activity but also enhanced conversion of cellulose to ethanol by SSF using *Saccharomyces cerevisiae*. Acid-pretreated substrate yielded 13 and 5 percentage points higher ethanol conversion efficiencies than fresh reed canarygrass and switchgrass, respectively. The on-line method of pretreatment out-yielded the mixed method both as a preservative and as an agent for enhanced cell wall degradation. This result was thought to be an outcome of more uniform acid application as indicated by the on-line method's more consistent pH profile and decreased fermentation products, as compared to the mixed method. Although significant levels of acetate and lactate were present in the biomass following storage, *concentrations were not sufficient to inhibit S. cerevisiae* in SSFs with a 10% solids loading.

KEYWORDS
pretreatment, biomass storage, energy crop, perennial grass, switchgrass, reed canarygrass, ethanol

INTRODUCTION

There is considerable interest in developing agricultural residues and herbaceous perennials as feedstocks for ethanol production. 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 use wet biomass. These dry harvesting operations may include, but are not limited to, windrowing, merging, baling, collecting and staging, and finally storing either indoors or outdoors (Hess et al. 2007; Shinners et al. 2003; Shinners and Boettcher 2006). Furthermore, delivery to the biorefinery would require loading, transporting, staging, de-twining, size reduction and, finally, rehydration.

Recently, a more cost-effective alternative has been proposed: wet storage. 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 (Chen et al. 2007; Richard et al. 2001; Shinners et al. 2007).

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; Richard et al. 2001). While in-storage pretreatments will be limited to ambient temperature and pressure conditions, reaction times can be on the order of months. Significantly increasing the degradability of the biomass while in storage is expected to add value by either allowing milder, or possibly substituting for, pretreatment at the biorefinery, thereby, providing better return for farmers.

This research investigated two systems for application of sulfuric acid on switchgrass (*Panicum virgatum* L.) and reed canarygrass (*Phalaris arundinacea* L.) before anaerobic storage at ambient temperature and pressure. The experimental design compared material that had been treated during bagging (on-line) to material that had been treated and thoroughly mixed (mixed). The on-line treatment is similar to how producers apply inoculants and forage amendments in the field today. This method would be the most practical and convenient to the farmer, but it may lead to a non-uniform end product, as the only means of homogenization is the action of the bagging rotor. Final product uniformity, including pH, silage acids and fermentability to ethanol after pretreatment and 90 days of storage 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. Reed canarygrass (*Phalaris arundinacea* L.) was harvested on 12 July 2008 from a 2 ha plot established in 2007 at the United States Dairy Forage Research Center Farm in Prairie du Sac, WI. The grass was first cut with a windrower (Model 340HW, CNH; New Holland, PA) followed immediately by precision chopping via the pull-type forage harvester (Model FP240, CNH; New Holland, PA). Theoretical length of cut was set at 4.8 mm.

Switchgrass (*Panicum virgatum* L.) was harvested on the 25 August 2008 from a 4 hectare plot established in 2004 and located at the University of Wisconsin Arlington Agricultural Research Station in Arlington, WI (Shinners and Boettcher 2006). The crop was first cut with a windrower (Model 4995, John Deere; Ottumwa, IA) followed immediately by precision chopping via the self-propelled forage harvester (Model 7800, John Deere; Zweibrücken, Germany) equipped with a windrow pickup. Theoretical length of cut was set at 5 mm.

Approximately 2 Mg of dry matter was harvested for each experimental replicate. In total, approximately 20 Mg DM was harvested and treated during the experiment.

Pretreatment and storage. At both locations, forage was immediately transported via a forage wagon (Model 4518, Meyer; Dorchester, WI) to the storage site. To compare on-line and TMR mixed methods all loads delivered from the field were divided in half to distribute any field variation across both treatments. This was accomplished by first unloading half the wagon directly into a 2.7 m diameter bagger (Model G6000, Ag-Bag; St. Nazianz, WI). Acid was applied as material was being unloaded via a low-pressure nozzle array. The second half of the material was unloaded on the ground, scraped up with a loader tractor, mixed in a TMR mixer (Model 3575, Knight; Brodhead, WI) while acid was applied with a second low-pressure nozzle array.

In both cases the low-pressure nozzle array consisted of six quarter-circle irrigation nozzles (Model 54011, Orbit Irrigation; Bountiful, UT) connected to a polyvinyl chloride pipe manifold. Acid was supplied to the array via a four-roller pump (Model 4101 A,

Hypro; New Brighton, MN) powered by a 1.1 kW electric motor equipped with a variable frequency drive (Model AF-300 Mini, GE Fuji; Saddle Brook, NJ). The system was operated using an empirical relationship that was developed between 18 *N* sulfuric acid mass-flow rate and drive frequency. This allowed adjustment of acid application rate based on wagon unloading speed and biomass dry matter content during bagging. Acid was supplied to the application system from a 250 L polyethylene drum situated on a platform scale (Model HV-200-KGL, AND; Milpitas, CA). The scale was used to verify application rate after the load of biomass was treated.

Safety of those involved in applying the acid pretreatment was of great concern. The low pH and strong affinity for water of the 18 *N* solution make contact with skin, eyes and respiratory tract extremely dangerous. All those working on this project not only wore safety glasses but also were fitted with respirators (Model 7500, 3M; St. Paul, MN) equipped with acid gas cartridges with P100 filters. The technician working directly with the pumping system wore a full-face respirator (Model 6000, 3M; St. Paul, MN) equipped with the same cartridges as well as a high-density polyethylene smock and nitrile-coated gloves.

The following field procedure was followed for each experimental replicate. First, enough biomass to complete one experimental replicate was direct-cut harvested from the field. Two moisture samples were taken from the beginning of the load to estimate moisture content using a microwave oven per ASABE S358.2 (ASABE 2008). The pump frequency was then selected to treat at a level of 50 g(kg DM)$^{-1}$ based on projected mass-flow of biomass from the wagon given total harvested mass, dry matter content and expected unloading time. The weight of the wagon was monitored throughout unloading by load cells in the running gear and the wagon operator was charged with unloading the wagon in set time period of 8 minutes. The nozzle array was primed and supplied acid immediately at the beginning of the load. Beginning and ending acid barrel weights were recorded to verify acid application rate.

After half the load had been bagged, the wagon was pulled forward and unloaded on the ground. At this time, three samples were taken to represent the initial moisture and carbohydrate contents. Initial carbohydrate samples were sealed in 950 ml plastic bags and stored at -20°C until they could be assayed. The remainder of the material was then loaded into the TMR mixer with a loader tractor. Calculations were made to determine the total acid applied (DM basis) during the previous on-line application so that it could be matched during mixer application. After acid application, material was allowed to mix for 10 minutes and then was unloaded into the bagger. This entire process was replicated three times with two additional loads. An untreated load of biomass was bagged at each end to serve as a buffer against any oxygen that might enter the end of the bag. Bags were marked with paint to define treatment location.

Both reed canarygrass and switchgrass substrates were stored for 90 days before destructive sampling. Each replicate and treatment was sampled using a 0.3 x 0.3 m grid. Approximately sixty 200 g samples were taken per replicate (720 total) and frozen for later analysis. Additionally, each section of the bag was measured to estimate density.

The samples were used to generate pH distributions for each treatment and replicate and five were randomly selected from each treatment (60 total) to be assayed for fermentation (ensilage) products, cell wall carbohydrates and fermentation to ethanol.

Sample preparation for analysis. Representative subsamples (40 g) from the before-mentioned 60 samples were 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, 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 (ensilage) 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 103°C 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 60°C 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 103°C and 24 hours. Ash content was determined as residue remaining after combustion at 500°C for 4 hours. These data were used to convert all data to 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 500°C for 4 hours. Cellulose was estimated by subtracting ADL from ADF, and hemicelluloses by the difference of NDF and ADF.

Post-storage fermentation 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 H2SO4 in 0.0034 M EDTA free acid at 0.7 ml min^{-1} and 45°C.

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 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 1.5L (Novozymes, Bagsvaerd, Denmark) was added at 5 FPU(g DM)$^{-1}$ substrate, Novo188 ß-glucosidase (Novozymes, Bagsvaerd, Denmark) was added at 15 FPU(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) 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 5 mM H_2SO_4 at 0.6 ml min^{-1} and 65°C.

Analysis. Effects of the two treatment application methods 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 AND DISCUSSION

Composition. Composition and physical properties of fresh reed canarygrass and switchgrass are summarized in Table 1. Mean particle size was lower than theoretical length of cut: 4 mm for both reed canarygrass and switchgrass. This may be attributed to substrate homogenization and handling. The moisture content measured after harvest was 57 and 49% w.b. for reed canarygrass and switchgrass, respectively. Table 1 also presents cell wall carbohydrates as measured by detergent fiber methods.

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

	MC	Ash	MPS	Cellulose	Hemicelluloses	Lignin
	%w.b.	g(kg DM)$^{-1}$	mm	g(kg OM)$^{-1}$		
Reed Canarygrass	57	82	4	320	220	102
Switchgrass	49	65	4	350	350	90

Moisture Content, MC; Mean Particle Size, MPS; Organic Matter, OM

Table 2 summarizes the density and application rates for each load of substrate. The application rates varied due to a lack of control of the mass-flow of the substrate from the wagon. Densities in the bag were similar between loads and crops but were higher than that reported in literature (Muck and Holmes 2000, 2006; Pitt and Muck 1993). This may be a result of the pretreatment but could also be the result of low mass-flow rates, which may have enhanced the baggers ability to densify the substrate.

Table 2. Storage density and sulfuric acid application rates for each load of substrate.

	Load*	Density (kg DM)m^{-3}	Application Rate g(kg DM)$^{-1}$
Reed Canarygrass	1	230	32
	2	230	53
	3	220	30
Switchgrass	1	190	28
	2	240	31
	3	230	45

*Each load consisted of mixed and on-line treatments.

Detergent fibers were assayed to better understand the preservative or detrimental effects of pretreatment and storage (Table 3). With both substrates, hemicelluloses were significantly lower for the on-line method of pretreatment compared to mixed. Lower levels of hemicelluloses for the on-line method of pretreatment may indicate that it was more effective than was the mixed, in that there was partial hydrolysis and solublization

of the hemicellulose fraction of the cell wall. On-farm animal digestibility work has documented release of acetyl groups from hemicellulose constituents resulting from pretreatment (Chesson 1981). Acid pretreatment did not significantly effect changes in other major cell wall carbohydrates regardless of application method.

Table 3. Post storage cell-wall composition where sulfuric acid pretreatment was applied at a target rate of 50 g(kg DM)$^{-1}$ via on-line or mixed methods.

	Cellulose	Hemicelluloses	Lignin
		g(kg OM)$^{-1}$	
Reed Canarygrass			
On-line	296	161*	90
Mixed	282	195	95
Switchgrass			
On-line	333	173*	106
Mixed	346	210	105

*Indicates significance between treatments at the $\alpha = 0.05$ level.

Pretreatment with acid resulted in a low level of fermentation products normally associated with anaerobic fermentation, suggesting the acid pretreatment limited microbial activity (Table 4) (McDonald 1981a). Specifically, the on-line method of pretreatment significantly decreased pH and lactate while significantly increasing acetate, compared to the mixed method of pretreatment for reed canarygrass. Similar trends for lactate and pH were observed in switchgrass, but acetate levels were lower. For both methods of pretreatment, butyrate levels were similar for reed canarygrass, but the on-line method produced slightly higher levels of butyrate in switchgrass. However, the values in both cases were close to the detection limit. The fermentation product profile is not surprising, as pH levels in acid-pretreated substrates were extremely low which would greatly limit activity of microorganisms known to populate silages (McDonald 1981b; Muck 1988).

Table 4. Post storage fermentation product profile where sulfuric acid pretreatment was applied at a target rate of 50 g(kg OM)$^{-1}$ via on-line or mixed methods.

	pH	Lactate	Acetate	Butyrate	Ethanol
			g(kg OM)$^{-1}$		
Reed Canarygrass					
On-line	2.4*	0.33*	1.1*	0.00	0.35*
Mixed	2.6	1.6	0.60	0.00	0.68
Switchgrass					
On-line	2.4*	2.1*	1.1*	0.11*	0.46*
Mixed	2.9	2.9	1.4	0.04	0.40

*Indicates significance between treatments at the $\alpha = 0.05$ level.

Cellulose Conversion. Cellulose conversion to ethanol was not significantly different between on-line and mixed application methods in reed canarygrass (Table 5). In switchgrass, however, cellulose conversion was observed to be 6 percentage points greater for the on-line method of application compared to the mixed method. Acid-pretreated substrate out-yielded fresh by 5 and 13 percentage points for switchgrass and reed canarygrass, respectively. These numbers were low primarily because of low chemical loadings 28 to 53 g(kg DM)$^{-1}$; previous work had explored ranges from 0 to 100 g(kg DM)$^{-1}$ (Digman et al. 2008; Digman et al. 2007). Data at the high chemical loadings were significantly better than at the lower loadings. In that research, sulfuric acid pretreatment and anaerobic storage yielded conversion of cell wall glucose to ethanol as high as 46 to 83% for switchgrass and reed canarygrass, respectively.

Glucose was not found in hydrolysate leading us to conclude that organic acids produced by pretreatment and storage did not inhibit fermentation to ethanol by *Saccharomyces cerevisiae* in a SSF at 10% solids loading. Finally, xylose measured in the hydrolysate was low and was not measured at levels greater than 10% of the total hemicelluloses measured in fresh substrate by the detergent fiber method.

Table 5. Cellulose converted to ethanol by simultaneous saccharification and fermentation (SSF) where sulfuric acid pretreatment was applied at a target rate of 50 g(kg DM)$^{-1}$ via on-line or mixed methods. Subscripts indicate significance at the $\alpha = 0.05$ level.

	Cellulose Conversion (% of Total)
Reed Canarygrass	
On-line	45_a
Mixed	44_a
Fresh	32_b
Switchgrass	
On-line	30_a
Mixed	24_b
Fresh	25_b

Subscripts indicate significance at the $\alpha = 0.05$ level.

pH Profiles. The final comparisons made in this study were among the resulting pH profiles after either mixed or on-line application of acid. Each profile is representative of a replicate (i.e. half-load) for both mixed and on-line application methods. Figures 1 and 2 compare on-line and mixed pretreatment methods for reed canarygrass and switchgrass, respectively. Additional comparisons were made but with similar trends. For both substrates, it is evident that the mixed method of application resulted in a less uniform pH distribution than that of the on-line method. However, spatial distribution of pHs within treatments did not yield any clear patterns. This data is consistent with previously presented pH, storage associated fermentation products, hemicellulose and cellulose conversion data because it is expected that a more uniform pretreatment would result in lower pH, levels of fermentation products and hemicelluloses as well as increased acetate concentration and cellulose conversion yields.

The less uniform pH distribution with the mixed method was the opposite of what was expected; however, qualitative observations made during application of the acid pretreatment may explain these findings. During the on-line method of pretreatment, crop layer thickness in the feeding conveyor was thin (low mass-flow), resulting in application of chemical over a larger surface area (better coverage), compared to the mixed method. This effect should have been mitigated by the mixing process, unless the acid was not "mobile" after application and, therefore, its effect cannot be homogenized simply by substrate interaction.

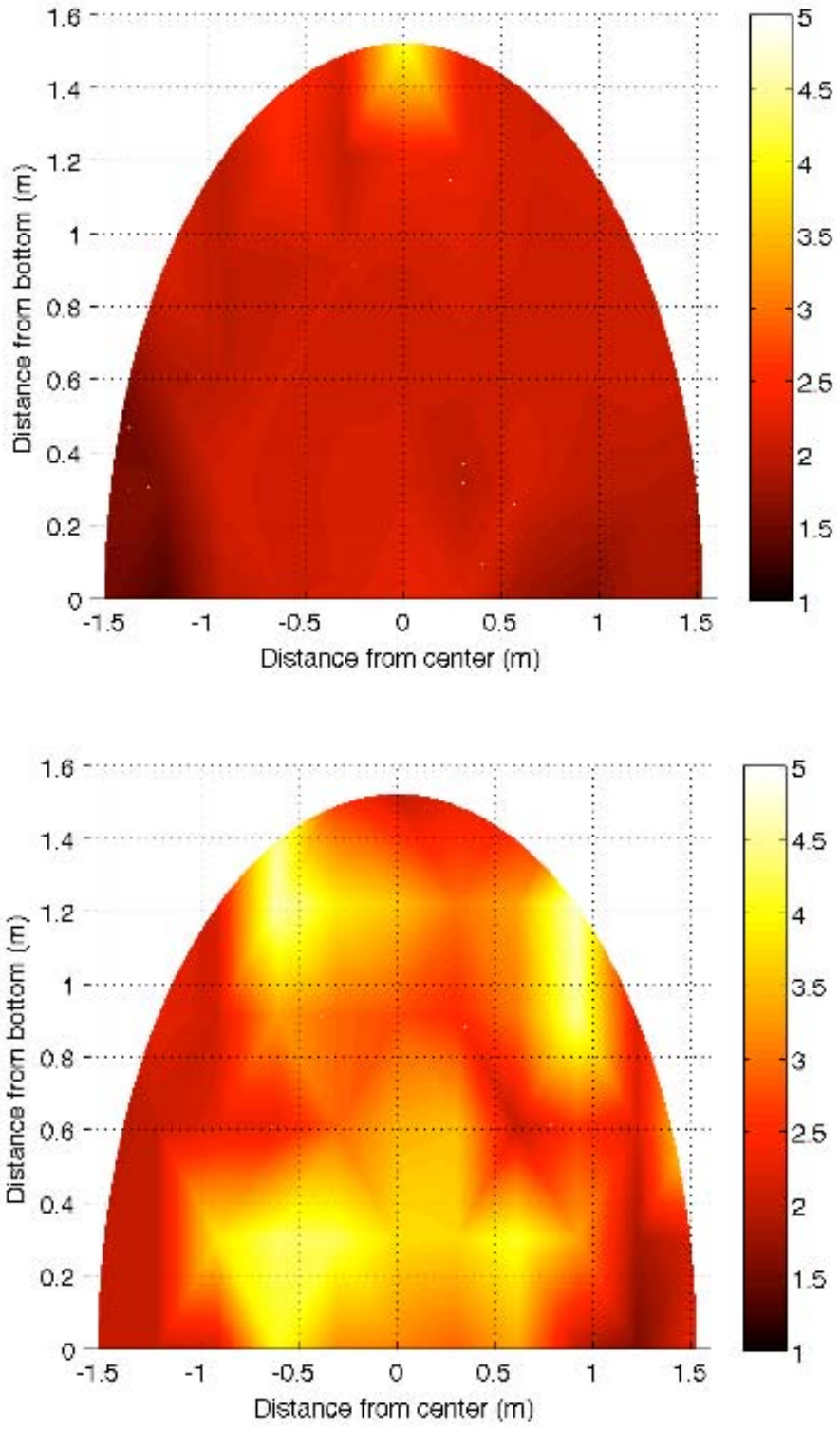

Figure 1. Post-storage pH distribution for on-line (top) versus mixed (bottom) treatment of reed canarygrass with sulfuric acid applied at a rate of 53 g(kg DM)$^{-1}$.

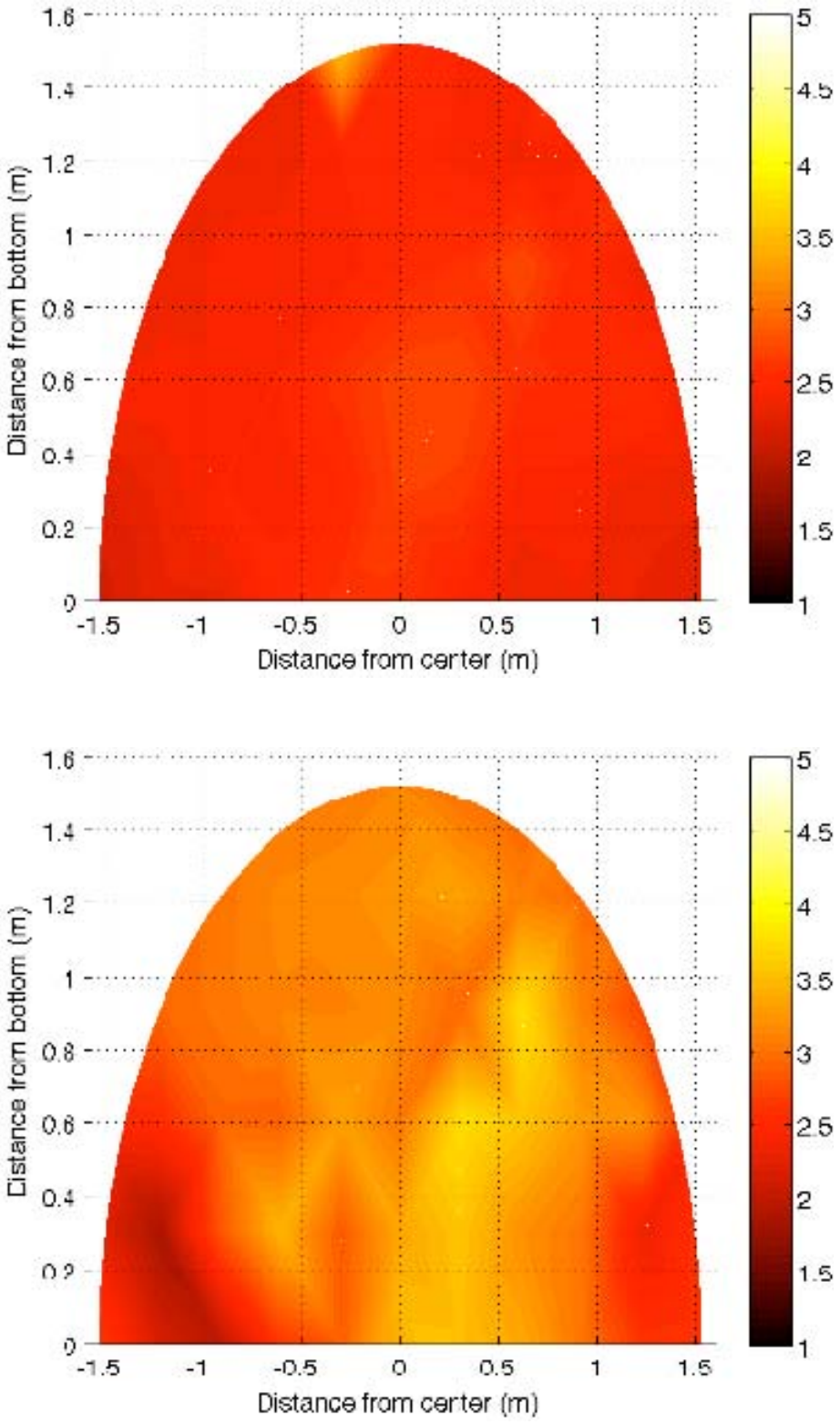

Figure 2. Post-storage pH distribution for on-line (top) versus mixed (bottom) treatment of switchgrass with sulfuric acid applied at a rate of 31 g(kg DM)$^{-1}$.

We believe these results show promise for the on-farm pretreatment system for acid pretreated substrates. On-farm pretreatments 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 be compatible with fermentation to ethanol by *Saccharomyces cerevisiae* at a relatively high solids loading. On-farm pretreatment at the rates explored in this study did not yield complete conversion of available cellulose, but the residual sulfuric acid could be used to further degrade the cell wall matrix at the biorefinery through an additional thermal process (Digman et al. 2008).

CONCLUSIONS

The two methods of application of sulfuric acid studied not only preserved biomass substrates through inhibition of microbial activity under anaerobic storage but also enhanced conversion of cellulose to ethanol in switchgrass and reed canarygrass substrates. Acid-pretreated substrate produced 13 and 5 percentage units more ethanol after SSF than untreated for reed canarygrass and switchgrass, respectively. The on-line method of pretreatment was a better preservative and agent for enhanced cell wall degradation than the mixed method. This result was thought to be an outcome of more uniform acid application as indicated by the on-line method's more consistent pH profile and the reduction of fermentation products, compared to the mixed method.

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