



American Society of  
Agricultural and Biological Engineers

An ASABE Meeting Presentation

Paper Number: 071021

## On-farm Pretreatment Technologies for Improving Enzymatic Degradability of Cellulose and Hemicellulose Present in Perennial Grass

<b>Matthew F. Digman</b> <sup>1,2</sup>	Graduate Research Assistant
<b>Kevin J. Shinnars</b> <sup>2</sup>	Professor of Agricultural Engineering
<b>Bruce S. Dien</b> <sup>3</sup>	Research Chemist
<b>Ronald D. Hatfield</b> <sup>1</sup>	Plant Physiologist
<b>Xin-Liang Li</b> <sup>3</sup>	Molecular Biologist
<b>Richard E. Muck</b> <sup>1,2</sup>	Agricultural Engineer
<b>Paul. J. Weimer</b> <sup>1</sup>	Microbiologist

1 – United States Dairy Forage Research Center (USDA-ARS), Madison, WI

2 – Department of Biological Systems Engineering, University of Wisconsin

3 – National Center for Agricultural Utilization Research (USDA-ARS), Peoria, IL

Written for presentation at the  
2007 ASABE Annual International Meeting  
Sponsored by ASABE  
Minneapolis Convention Center  
Minneapolis, Minnesota  
17 - 20 June 2007

**Abstract.** *This research investigated the ability of on-farm pretreatments with acid, alkali, ozone or novel enzymes to improve enzymatic degradability of cellulose and hemicelluloses in biomass at the biorefinery. Two perennial grasses, switchgrass (*Panicum virgatum*) and reed canarygrass (*Phalaris arundinacea* L.), were direct-cut harvested, pretreated, and stored anaerobically for 30 d. Pretreated and untreated samples were fermented to ethanol by *Saccharomyces cerevisiae* in the presence of commercial cellulase for 72 hr to ethanol. Xylose yields were also measured following fermentation because it is not metabolized by *S. cerevisiae*. Sulfuric acid and lime pretreatment technologies look promising considering the high conversion yields and ease of application. Efficiencies of nearly 80% for cellulose conversion to ethanol and hemicellulose to xylose were realized, albeit at high chemical loadings. Ozonolysis results demonstrated similar success, but integration of this technology into current storage systems will be challenging. Enzyme addition (xylanases or feruloyl esterase) at ensiling only marginally improved conversion efficiency.*

**Keywords.** *Energy crops, biomass, reed canarygrass, switchgrass, pretreatment, bioethanol, feruloyl esterase*

---

The authors are solely responsible for the content of this technical presentation. The technical presentation does not necessarily reflect the official position of the American Society of Agricultural and Biological Engineers (ASABE), and its printing and distribution does not constitute an endorsement of views which may be expressed. Technical presentations are not subject to the formal peer review process by ASABE editorial committees; therefore, they are not to be presented as refereed publications. Citation of this work should state that it is from an ASABE meeting paper. EXAMPLE: Author's Last Name, Initials. 2007. Title of Presentation. ASABE Paper No. 07xxxx. St. Joseph, Mich.: ASABE. For information about securing permission to reprint or reproduce a technical presentation, please contact ASABE at [rutter@asabe.org](mailto:rutter@asabe.org) or 269-429-0300 (2950 Niles Road, St. Joseph, MI 49085-9659 USA).

---

## Introduction

There is considerable interest in developing agricultural residues and herbaceous perennials as feedstocks for bioethanol production as supplements to grains. The logistics of harvesting, storing, and transporting the immense quantities of biomass needed to fuel cost-competitive biorefineries is an immense challenge. Most of the research on biomass conversion assumes the crops will be harvested and stored dry. Recently, wet storage methods have been proposed for feedstock preservation and on-farm storage of perennial grass and corn stover biomass (Richard et al., 2001; Shinnars et al., 2003a; Shinnars and Boettcher, 2006). The advantages over a dry storage system include low risk of fire, reduced harvesting costs, lower dry matter (DM) losses during storage, increased product uniformity, and improved feedstock susceptibility to enzymatic hydrolysis (Shinnars et al., 2003b; Shinnars and Binversie, 2007; Ren, 2006). Also, as the biomass is already wet, it may be possible to pretreat it at the same time for increased degradability. 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 eliminating the need for, pretreatment at the biorefinery, thereby, providing better return for farmers.

This research focuses on optimizing on-farm storage of cellulosic biomass for ethanol production. Here we consider a wet storage system optimized for collection, and preservation of feedstuffs for ruminant animals. This system would not only preserve quality but may also improve enzymatic degradability of the feedstock during storage. This research investigates the ability of on-farm pretreatments with acid, alkali, ozone or novel enzymes to improve enzymatic degradability of cellulose and hemicelluloses in biomass for conversion to ethanol and sugars.

Biomass needs to be pretreated prior to further processing because the carbohydrates are largely contained in complex cell wall structures that impede their enzymatic conversion into fermentable sugars. *S. cerevisiae* only ferments monosaccharides and sucrose. Biomass recalcitrance also limits digestion in ruminants and as such there is a long history of chemically treating forages for increasing their energy values; only ½ of the carbohydrates are actually utilized when ruminants are fed untreated forage (Hatfield et al, 1999a). In fact, many of the same pretreatments that have been investigated for biofuel applications have been previously evaluated on forages (e.g. lime and AFEX). This literature is a natural place to begin the search. First of all, systems have already been developed for applying some of the milder treatments on the farm. And second, many similarities exist between conversion of forages into volatile fatty acids (VFA) and microbial cell protein in ruminants and enzymatic conversion of biomass into ethanol. Weimer et al. (2004) have correlated in vitro gas production, a widely accepted assay of ruminant forage digestibility, to ethanol yields by simultaneous saccharification and fermentation (SSF). Sundstol and Owen (1984) have reviewed previous research on chemical methods applied to increase forage digestibility. Descriptions of numerous pretreatment strategies and their ability to improve animal digestibility are given. Among the more effective strategies were treating with acids, alkali, and oxidizers.

Alkali chemicals have been the most studied and widely applied for forage treatments (Sundstol and Coxworth, 1984). The more commonly applied chemicals include: sodium hydroxide (NaOH), anhydrous ammonia (NH<sub>3</sub>), aqueous ammonia (NH<sub>4</sub>OH), ammonia freeze-explosion (AFEX), and urea. In the case of NaOH (Wilkinson, 1984), it was found that increasing rate and duration of pretreatment increased organic matter digestibility (OMD). 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 pretreatment success. Feeding trials indicate that NaOH treated

straws were more digestible than ammonia treated, but at the expense of added energy costs (Sunstol and Coxworth, 1984).

Acid pretreatments have received less attention due to their limited success in animal feeding studies (Owen et al., 1984). This has been attributed to low animal intake as pretreatment results in low forage pH. Acid pretreatments were found effective in the laboratory studies however (Owen et al., 1984).

Oxidizing agents applied to forage have included hydrogen peroxide ( $H_2O_2$ ), ozone ( $O_3$ ), and sodium peroxide ( $Na_2O_2$ ) (Owen et al., 1984). Ozone has been found to be as effective as NaOH, but low pH and scale-up difficulties have impeded its widespread application. Ozone's ability to reduce lignin concentration and to improve cell wall degradability has been more recently demonstrated (Neely, 1984; Akin and Morrison, 1988; Morrison and Akin, 1990).

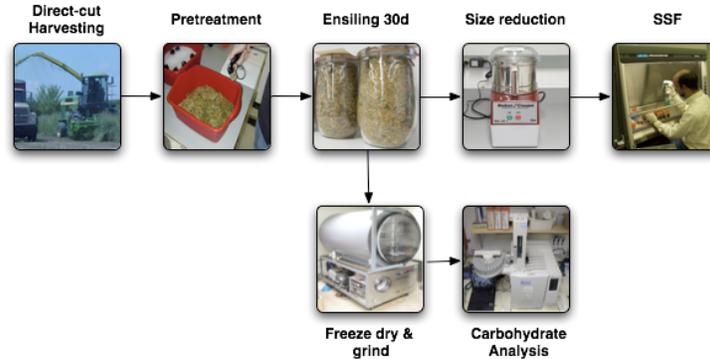
While many enzymes formulations are currently marketed to farmers to improve feeding values, relatively little research has been carried out on applying enzymes during ensiling. Beauchemin et al. (2003) has presented methodology and state of technology for enzyme products for enhancing feed utilization by ruminant animals. Here many enzyme cocktails are presented. Research supporting the use of esterases is limited. A synergistic relationship between esterases and xylanases was suggested.

All of these before mentioned pretreatments have been investigated for biochemical conversion of lignocelluloses to biofuels (for reviews: Grabber et al., 1998; Sun and Cheng, 2002; Mosier et al., 2005; Wyman, 1999; Wyman et al., 2005; Wong, 2005). However, the difference is that the pretreatments were designed to be carried out at the biorefinery, where short-processing times becomes essential. As a consequence, biomass was reacted at high temperatures and/or pressures that would preclude their use on farms.

In this work switchgrass (*Panicum virgatum*) and reed canarygrass (*Phalaris arundinacea L.*) were pretreated with calcium hydroxide, dilute-sulfuric acid, ozone, or ferouyl esterase. While all experiments were at laboratory scale, pretreatment conditions were carefully selected for application on the farm during wet storage. Therefore, in each case the wet-harvested biomass was treated "as is" under ambient temperature and pressure for 30 days. Following the pretreatment, the biomass was fermented to ethanol using the yeast *Saccharomyces cerevisiae* D5A in the presence of commercial cellulase. Pretreatments were compared for increases in ethanol and xylose yields over untreated biomass samples.

## Materials and Methods

This research was conducted as a screening to assess the success of calcium hydroxide, sulfuric acid, gaseous ozone, and feroyl esterase considering on-farm storage technology typically used in preservation of forages for ruminant nutrition (figure 1). This system employs an anaerobic environment promoting activity of microorganisms that will ensure the stability and nutritive value of the forage (Collins and Owens, 2003).



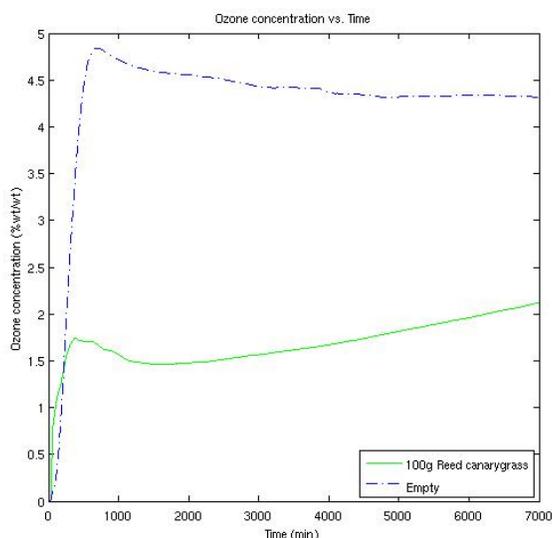
**Figure 1:** Screening method used to identify successful on-farm pretreatments.

Biomass substrates included switchgrass (*Panicum virgatum*), and reed canarygrass (*Phalaris arundinacea L.*). Crop was obtained from two 4 ha plots established in the spring of 2004 at the Arlington Agricultural Research Station located in Arlington, WI (Shinners and Boettcher, 2006). Substrate studied was direct harvested on 22 August 2006, and stored at  $-20^{\circ}\text{C}$ . The substrate was removed from storage one-day prior to use, transferred to a refrigerator ( $5^{\circ}\text{C}$ ), and allowed to thaw.

Particle size and moisture content as obtained from direct-cut harvesting was 9.9 mm, 50% and 7.4 mm and 60% for reed canarygrass and switchgrass respectively (ASAE, 2001; ASAE, 2003). Pretreatment was applied to prior to storage of grass substrate without adjustment of moisture or size reduction.

Method of pretreatment application varied for each scheme. Sulfuric acid was applied as a 6 N solution at a rate of 30 or 90 g  $\text{H}_2\text{SO}_4/\text{kg}$  substrate dry matter (DM). This range was chosen considering past animal digestibility research (Owen et al., 1984). Pretreated material was mixed thoroughly with a 475-Watt household mixer. Calcium hydroxide was applied in the same manner but as dry powder (1.2% moisture content wet basis) at a rate of 5 or 15% of substrate DM (Sundstol and Coxworth, 1984). After considering past biomass degradability research, pretreatment range was increased for this study as longer duration, but lower substrate temperature and moisture content were considered (Kaar and Holtzapple, 2000; Chang et al., 2001; Kim and Holtzapple, 2005, 2006a and 2006b).

A Pacific Ozone model L21 (Pacific Ozone Technology Inc., Benicia, CA) cathode discharge ozone generator was used to generate ozone for reaction with substrate. An AirSep Onxy+ (AirSep Corp., Buffalo, NY) oxygen concentrator provided oxygen feed gas at a purity of no less than 90%. Ozone flow rate and concentration were 2.4 L/min and 5%wt/wt respectively. One hundred grams of substrate loosely filled a 1.2 L mesh cylinder suspended in a 2.2 L reaction chamber. The design was an effort to ensure uniformity of gas diffusion and resulting reaction. Reaction chamber ozone concentration was destructively sampled at a rate of 0.5 L/min by means of a Teledyne 450H UV (Advanced Pollution Instrumentation Inc., San Diego, CA) ozone monitor. Utilization was not quantitatively studied for this exploratory research, but was observed to vary inversely with reaction time (figure 2).



**Figure 2:** Plot comparing ozone concentration vs. time with reactor vessel empty and 100g reed canarygrass.

Feruloyl esterase (FAE) and its synergistic effect with Family 10 and 11 xylanases were investigated. The FAE and the family 10 xylanase are the FAE and xylanase domains of *Clostridium thermacellum* XynZ separately produced by *Escherichia coli* BL21(DE3) (Blum et al., 2000). The family 11 xylanase is the xylanase A (XynA) of *Orpinomyces* PC-2, an anaerobic fungus (Li et al., 1997). Activities of feruloyl esterase, family 10 xylanase, and family 11 xylanase were 338, 1250, and 13.1 U/mL respectively. One unit of enzyme activity for xylanase and feruloyl esterase was defined as the amount of activity required to release one micromole xylose and ferulic acid from 0.5% oat spelt xylan and 1.0 mM methyl ferulate, respectively at pH 6.0 and 50°C. This experiment was arranged in a 2<sup>3</sup> full factorial design (table 1).

**Table 1:** Pretreatment rates for feruloyl esterase study (U/kg DM).

<b>Factor</b>	<b>Level (U/kg SDM)</b>	
	<b>High</b>	<b>Low</b>
Feruloyl esterase	100	10
Family 10 xylanase	1000	0
Family 11 xylanase	5000	0

Storage conditions for all treatments were anaerobic, 22°C, and 200 kg DM/m<sup>3</sup> density, similar to that of on-farm ensiling (Pitt and Muck, 1993; Muck and Holmes, 2000, 2001, 2004, and 2006; Bernier-Roy et al., 2001; D'Amours and Savoie, 2004 and 2005). WECK 1 L canning jars were used to provide an anaerobic storage environment for each treatment (Pflaum et al., 1996). Under normal ensiling conditions active fermentation is complete within twenty-one days (Collins and Owens, 2003). A more conservative thirty days was chosen as the storage duration for this study even though typical fermentation was not expected.

A modified version of the Uppsala Total Dietary Fiber Method was used to determine fermentable carbohydrates of the controls in each experiment (Theander et al., 1995). These values would be used to assess a pretreatment's success in increasing cellulose and hemicellulose susceptibility to enzymatic degradation.

Controls for each experiment were freeze-dried and ground in a vortex mill (Udy Corporation, Fort Collins, CO) through a 1 mm screen. Sub-samples of material were weighed into 50 mL conical centrifuge tubes (0.5 g). Tris buffer (50 mM, pH 6.7) was added to each sample and placed in a 90°C water bath for 2 h to gelatinize the starch. Samples were transferred to a 55°C water bath and incubated for 2 h after adding amylase (Sigma A3403 10 U/tube) and amyloglucosidase (Fluka 10115 10 U/tube) for starch removal. Ethyl alcohol (EtOH, 95%) was added to each tube to produce a final EtOH concentration 80%. Samples were stirred with a spatula before centrifuging at 3200 x g for 15 min. Insoluble residues/pellets recovered from the starch extraction procedure were washed extensively (1 mL solvent/g fresh tissue) with a series of solvents. The solvent series included: 80% EtOH (4X), and acetone (3X) to remove cytoplasmic contaminants (Hatfield, 1992; Hatfield et al., 1999b). Each wash included 15 minutes sonication, centrifugation (3200 x g for 15 min) and solvent removal. The retained insoluble residues, mainly cell walls, were allowed to air dry under a hood and used for structural analysis.

Cell wall residues were dried overnight in a 55°C oven prior to weighing for analysis. Approximately 50 mg of sample was weighed into 15 mL polypropylene conical tubes. These samples were hydrolyzed using the Saeman method (Saeman et al., 1963) as modified by Hatfield (Hatfield, 1992). Dried samples were suspended in 1.5 mL of cold (4°C) 12 M H<sub>2</sub>SO<sub>4</sub>, incubated at room temperature (23-24°C) for 2 h, and stirred occasionally using a stainless steel spatula. At the completion of the first stage hydrolysis, the acid was diluted with dH<sub>2</sub>O (10 mL), washing off the spatulas as water was added. All samples were capped tightly and placed in a 100°C forced air oven for 3h. Samples were mixed twice during this secondary hydrolysis step by carefully inverting 2-3 times. After the secondary hydrolysis, samples were cooled in an ice waterbath; a 200 mL aliquot was removed from each sample for total uronosyls analysis. Inositol was added (10 mg, 200 mL of 50 mg/mL solution in water) as internal standard. Tubes were recapped and thoroughly mixed before a 0.75 mL sub-sample was diluted to 10 mL with dH<sub>2</sub>O in a 50 mL centrifuge tube. Sub-samples were neutralized with barium carbonate, centrifuged (3200 x g 15 min) and the supernatant filtered through a glass fiber filter (0.2 micron, Acrodisc). Four mL of the filtered sub-samples were dried and sugars converted to alditol acetate derivatives using the procedure of Blakeney et al. (1983) and analyzed by FID-GLC (Supelco SPB-225 column 30m X 0.25mm with 0.25 micron film thickness). Samples removed for total uronosyls were diluted 10 fold with dH<sub>2</sub>O and analyzed using the method of Blumekrantz and Asboe-Hansen (1973).

Ethanol and pentose sugar yields were determined using a modified method of Dowe and McMillan (2001) for simultaneous saccharification and fermentation (SSF). Samples were chopped in a Robot Coupe RSI 2Y-1 industrial processor (Robot Coupe USA, Inc., Jackson, MS) to a particle size of approximately 4 mm. A 1.5 g DM aliquot of wet substrate was added to a 25 mL Pyrex media bottle (Corning Glass, Corning, NY) along with 8 mL of distilled water. The pH of the substrate was adjusted to 4.5-5.0 by adding either 2 N sulfuric acid or calcium hydroxide, depending on post-storage pH, and buffered to pH 4.8 by adding 0.5 mL of sodium citrate buffer (1 M, pH 4.8). Additional distilled water was added to give a final solids loading to 7.5%, which took into account differences in moisture content and additions. The bottles were sealed and autoclaved for 15 min at 121°C. Next, GC220 (5 FPU/g DM, Genencor-Danisco, Rochester, USA), Novo188  $\beta$ -glucosidase (15U/g DM, Novozymes, Bagsvaerd, Denmark) and 10x YP stock (2 mL, stock: 100 g/l yeast extract and 200 g/l peptone) were added to each bottle. Enzymes and YP stock were sterilized by filter sterilizing or autoclaving, respectively. Each bottle was inoculated with *Sacchromyces cerevisiae* D5A to an O.D. @600 nm of 1.0. Fermentation was cultured for 48 hr at 35°C with gentle shaking (100 RPM) and sampled for sugars and ethanol. The inoculum was prepared by growing the yeast overnight in YP5D (10g/l yeast extract, 20 g/l peptone, and 50 g/l dextrose) at 35°C and 200 rpm. The cells were harvested by centrifugation and resuspended in a dilute peptone solution (1 g/l peptone).

Monosaccharide and ethanol concentrations were measured by HPLC (SpectraSYSTEM liquid chromatography system, Thermo Electron Corporation, CA) equipped with an automatic sampler, column heater, isocratic pump, refractive index detector, and computer based integrator running Chromquest ver. 2.5 (Thermo Electron Corporation, CA). Samples were injected (20  $\mu$ L) onto an organic acid column (Aminex HPX-87H Column, 300 x 7.8 mm, Bio Rad Laboratories, Inc., Hercules, CA) and eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at 0.6 mL/min and 65°C.

## Results

Laboratory silo DM densities averaged 180 kg DM/m<sup>3</sup>, which is within the range observed in on-farm silos (Pitt and Muck, 1993; Muck and Holmes, 2000, 2001, 2004, and 2006; Bernier-Roy et al., 2001; D'Amours and Savoie, 2004 and 2005). Dry matter losses averaged 0.4%, lower than on-farm silos, but typical of laboratory silos (Buckmaster et al., 1993; Pflaum et al., 1996). The pretreatment conditions did not alter dry matter loss.

In the first study, acid (H<sub>2</sub>SO<sub>4</sub>) and hydrated lime (Ca(OH)<sub>2</sub>) were applied at two levels. All pretreatments resulted in a significant increase in cellulose availability for fermentation when compared to their respective control (Table 2). Higher chemical loadings gave higher product yields independent of substrate or chemical. Treatment with Ca(OH)<sub>2</sub> outperformed sulfuric acid at high treatment levels, but was comparable at lower treatment levels. RCG gave higher yields than SWG for both acid and base pretreatments.

Xylose yields were significantly higher than the control for all pretreatments (Table 2). Alkaline pretreatment not only resulted in higher xylose yields than acid when comparing high and low levels, but it was also found that high acid was not significantly better than low alkali, a result independent of substrate. Xylose yields for comparable pretreatment levels were similar for RCG and SWG samples.

**Table 2:** Cellulose and xylose recovery relative to control for switchgrass (SWG) and reed canarygrass (RCG) after pretreatment with calcium hydroxide or sulfuric acid, and anaerobic storage at 22°C for 30 days.

	<b>Crop Treatment (% DM)</b>	<b>Recovered (% Available)</b>	
		<b>Cellulose<sup>†</sup></b>	<b>Xylose<sup>†</sup></b>
	Control	33c	6a
<b>RCG</b>	Acid Low (3%)	54e	13b
	Acid High (9%)	67f	26c
	Alkali Low (5%)	54e	23c
	Alkali High (15%)	79g	67d
	Control	15a	9a
<b>SWG</b>	Acid Low (3%)	25b	16b
	Acid High (9%)	29b,c	23c
	Alkali Low (5%)	24b	23c
	Alkali High (15%)	42d	77e

<sup>†</sup>LSD = 4% and 3% at  $\alpha = .05$  for cellulose and xylose respectively.

In this study, gaseous ozone was applied at 1, 2, and 5% of substrate dry matter (table 3). Rate of application was controlled by duration of pretreatment, as ozone concentration and flow rate were held constant. All pretreatment levels resulted in a significant increase in cellulose availability when compared to their respective control. Pretreatment of reed canarygrass showed an increase in cellulose availability as ozone increased from 0 to 2%. A decline in ethanol yield was observed at the 5% level. This result may indicate the formation of fermentation inhibitors at this pretreatment level. Previous ozonolysis work has attributed lower digestibility to production of phenolic aldehydes including vanillin and p-hydroxybenzaldehyde, chemicals known to be toxic to microorganisms (Morrison and Akin, 1990). Xylose yield increased from the 1 to 2% level but not for the 5% level. These results suggest the optimal treatment level for RCG is between 2 and 5%. For SWG, cellulose and xylose availability continued to increase with higher amounts of ozone, suggesting that the optimal level of ozone had not yet been reached.

**Table 3:** Cellulose and xylose recovery relative to control for switchgrass (SWG) and reed canarygrass (RCG) after pretreatment with gaseous ozone, and anaerobic storage at 22°C for 30 days.

	<u>Crop</u>	<u>Treatment (%DM)</u>	<u>Recovery (% Available)</u>	
			<u>Cellulose</u> <sup>†</sup>	<u>Xylose</u> <sup>†</sup>
<u>RCG</u>		Control	42d	10a
		1	67f	30c
		2	71g	41d
		5	59e	41d
<u>SWG</u>		Control	16a	11a
		1	27b	21b
		2	32c	33c
		5	41d	53e

<sup>†</sup>LSD = 3% at  $\alpha = .05$  for both cellulose and xylose.

In this final study, feruloyl esterase was applied with either family 11 or family 10 xylanase in a full factorial experimental design. Only statistically significant results are presented (table 4). Pretreatment with feruloyl esterase only marginally improved cellulose degradation. Family 10 xylanase significantly improved yields over family 11.

**Table 4:** Statistically significant cellulose recoveries for switchgrass (SWG) and reed canarygrass (RCG) after pretreatment with feruloyl esterase (FAE), and family 10 or 11 xylanase and anaerobic storage at 22°C for 30 days.

<u>Crop</u>	<u>FAE</u> (U/kg DM)	<u>Family 10</u> (U/kg DM)	<u>Cellulose</u> <u>Recovery</u> <sup>†</sup>
<u>RCG</u>	100	0	38%
	100	1000	40%
	10	0	40%
	10	1000	37%
<u>SWG</u>	100	0	19%
	100	1000	19%
	10	0	19%
	10	1000	19%

†LSD = 3% at a = .05

## **Conclusions**

- Conversion efficiencies of nearly 80% for cellulose and hemicellulose were realized, albeit at high chemical loadings.
- Sulfuric acid and lime pretreatment technologies look promising based upon conversion yields and ease of application.
- Ozonolysis demonstrated similar yields as the above, but integration into current storage systems will be challenging.
- Adding enzymes (xylanases or feruloyl esterase) during ensiling only marginally improved conversion efficiency.
- In general, alkali pretreatments were similar to or more effective than acid in increasing the degradability of cellulose and hemicellulose.
- Switchgrass was found to be more recalcitrant than reed canarygrass for all the pretreatments.
- Extended duration of ozonolysis inhibited fermentation of reed canarygrass.

## **Future Work**

- Response surfaces will be constructed to determine optimal pretreatment conditions for given substrate properties.
- Pretreatments will be performed on-farm in parallel to laboratory experiments to identify scale-up challenges.
- Ozonolysis will be applied at lower concentration and longer durations with attention to uniformity of treatment and gas diffusion characteristics.

## ***Acknowledgements***

Many thanks are due to Michael Boettcher, Garritt Boettcher, Patricia J. O'Bryan , and Jane Marita for their technical assistance and advice during this study.

## References

- Akin, D.E. and W.H. Morrison. 1988. Ozone Pretreatment of Forage: Structure and Digestibility of Different Types of Lignified Cell Walls. *Crop Sci.* 28:337-342.
- ASAE Standards. 2001. S424.1: Method of determining and expressing particle size of chopped forage materials by screening. St. Joseph, Mich.: ASABE.
- ASAE Standards. 2003. S358.2: Moisture measurement – Forages. St. Joseph, Mich.: ASABE.
- Beauchemin, K.A., D. Colombatto, and D.P. Morgavi. 2003. A rationale for the development of feed enzyme products for ruminants. Contribution No. (387) 02102. Lethbridge, Alberta Canada.: Canadian Journal of Animal Science
- Bernier-Roy, M., Y. Tremblay, P. Pomerleau, and P. Savoie. 2001. Compaction and Density of Forage in Bunker Silos. ASAE Paper No. 011089. St. Joseph, Mich.: ASABE
- Blakeney, A.B., P.J. Harris, R.J. Henry, and B.A. Stone. 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydrate Research* 113:291-299.
- Blum, D.L., I.A. Kataeva, X.L. Li, and L.G. Ljungdahl. 2000. Feruloyl Esterase Activity of the *Clostridium thermocellum* Cellulosome Can Be Attributed to Previously Unknown Domains of XynY and XynZ. *J Bacteriol.* 182:1346-1351.
- Blumenkrantz, N., and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem.* 54:484-489.
- Buckmaster, D. R., C.A. Rotz. and R.E Muck. 1989. A comprehensive model of forage changes in the silo. *Transactions of the ASABE.* 32(4):1143-1152. St. Joseph, Mich.: ASABE
- Chang, V.S., W.E. Kaar, B. Burr and M.T. Holtzaple. 2001. Simultaneous saccharification and fermentation of lime-treated biomass. *Biotechnology Letters.* 23:1327-1333.
- Collins, M. and V.N. Owens. 2003. Preservation of Forage as Hay and Silage. In *FORAGES: An Introduction to Grassland Agriculture*, 443-471. Ames, IA: Iowa State University Press
- D'Amours, and P. Savoie. 2004. Density profile of Corn Silage in Bunker Silos. ASAE Paper No. 041136. St. Joseph, Mich.: ASABE
- D'Amours, and P. Savoie. 2005. Density Profile of Herbage Silage in Bunker Silos. ASAE Paper No. 051051. St. Joseph, Mich.: ASABE

- Dowe, N. And J. McMillan. 2001. SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation. LAP-008. Golden, CO.: NREL.
- Grabber, J.H., R. D. Hatfield, and J. Ralph. 1998. Diferulate Cross-Links Impede the Enzymatic Degradation of Non-Lignified Maize Walls. *J Sci Food Agric.* 77, 193-200.
- Hatfield, R.D. 1992. Carbohydrate composition of alfalfa cell walls isolated from stem sections differing maturity. *Journal of Agricultural and Food Chemistry* 40:424-430.
- Hatfield, R.D., J. Ralph, and J.H. Grabber. 1999a. Sorghum: Cell wall structural foundations: Molecular basis for improving forage digestibilities. *Crop Sci.* 39:27-37.
- Hatfield, R.D., J.R. Wilson, and D.R. Mertens. 1999b. Composition of cell walls isolated from cell types of grain sorghum stems. *Journal of the Science of Food and Agriculture.* 79:891-899.
- Kaar, W.E., and M.T. Holtzaple. 2000. Using lime pretreatment to facilitate the enzymic hydrolysis of corn stover. *Biomass and Bioenergy.* 18:189-199.
- Kim, S. and M.T. Holtzaple. 2005. Lime pretreatment and enzymatic hydrolysis. *Bioresource Technology.* 96:1994-2006.
- Kim, S. and M.T. Holtzaple. 2006a. Effect of structural features on enzyme digestibility of corn stover. *Bioresource Technology.* 97:583-591.
- Kim, S. and M.T. Holtzaple. 2006b. Delignification kinetics of corn stover in lime pretreatment. *Bioresource Technology.* 97:778-785.
- Li, XL. and L.G. Ljungdahl. 1997. Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. *Appl. Environ. Microbiol.* 63:628-634.
- Morrison, W.H. and D.E. Akin. 1990. Water-Soluble Reaction Products from Ozonolysis of Grasses. *J. Agric. Food Chem.* 38:676-681.
- Mosier, N., C. Wyman, B. Dale, R. Elander, Y.Y. Lee, M. Holtzaple, and M. Ladisch. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource Technology.* 96:673-686.
- Muck, R.E. and B.J. Holmes. 2000. Factors Affecting Bunker Silo Densities. *Applied Engineering in Agriculture* 16:613-619.
- Muck, R.E. and B.J. Holmes. 2001. Density and Losses in Pressed Bag Silos. ASAE Paper No. 011091. St. Joseph, Mich.: ASABE

- Muck, R.E. and B.J. Holmes. 2004. Bag Silo Densities and Losses. ASAE Paper No. 041141. St. Joseph, Mich.: ASABE
- Muck, R.E. and B.J. Holmes. 2006. Bag Silo Densities and Losses. *Transactions of the ASABE*. 49(5):1277-1284. St. Joseph, Mich.: ASABE
- Neely, W.C. 1984. Factors Affecting The Pretreatment of Biomass with Gaseous Ozone. *Biotechnology and Bioengineering XXVI:059-065*.
- Owen, E. Klopfenstien, T. and Urio, N. 1984. Treatment with other chemicals. In *Straw and other fibrous by-products as feed*, 196-247. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.
- Pflaum, J., L. Gartner, C. Demarquilly, J. P. Andrieu, H. Honig, -H. W. Staudacher, and U. Wyss. 1996. Silage additive testing: Comparison of the German DLG and the French INRA schemes. *Wirtschaftseigene-Futter*. 42(3):217-248
- Pitt, R.E. and R.E. Muck. 1993. A Diffusion Model of Aerobic Deterioration at the Exposed Face of Bunker Silos. *J. agric Engng Res*. 55, 11-26.
- Ren, H. 2006. Effect of Cell Wall Degrading Enzymes and Chemicals on Corn Stover Preservation and Pretreatment during Ensilage Process. Ph.D. thesis. University Park, Pennsylvania: Pennsylvania State University, Department of Agricultural and Biological Engineering.
- Richard, T.L., S. Proulx, K.J. Moore, and S. Shouse. 2001. Ensilage Technology for Biomass Pretreatment and Storage. ASAE Paper No. 01-6019. St. Joseph, Mich.: ASABE.
- Saeman, J.F., W.E. Moore, and M.A. Millett. 1963. Sugar units present. Hydrolysis and quantitative paper chromatography. In R. L. Whistler, ed. *Cellulose, Vol. 3*. 54-69. Academic Press, New York.
- Shinners, K.J., B.N. Binversie, and P. Savoie. 2003a. Harvest and Storage of Wet and Dry Corn Stover as a Biomass Feedstock. ASAE Paper No. 036088. St. Joseph, Mich.: ASABE.
- Shinners, K.J., B.N. Binversie, and P. Savoie. 2003b. Whole-Plant Corn Harvesting for Biomass: Comparison of Singe-pass and Multiple-pass Harvest Systems. ASAE Paper No. 036089. St. Joseph, Mich.: ASABE.
- Shinners, K.J. and B.N. Binversie. 2007. Comparison of wet and dry corn stover harvest and storage. *Biomass and Bioenergy*. 31(4):211-221.
- Shinners, K.J. and G.C. Boettcher. 2006. Drying, Harvesting, and Storage Characteristics of Perennial Grasses as Biomass Feedstocks. ASAE Paper No. 061012. St. Joseph, Mich.: ASABE.

- Sun, Y. and J. Cheng. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresource Technology*. 83:1-11.
- Sundstol, F., and E. Owen 1984. *Straw and other fibrous by-products as feed*. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.
- Sunstol, F. and E.M. Coxworth. 1984. Ammonia Treatment. In *Straw and other fibrous by-products as feed*, 196-247. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.
- Theander, O., P. Aman, E. Westerlund, R. Andersson, and D. Petterson. 1995. Total dietary fiber determined as neutral sugar residues, uronic acid residues, and Klason lignin (The Uppsala Method): collaborative study. *Journal of AOAC International*. 78:1030-44.
- Weimer, P.J. 2005. In vitro gas production as a surrogate measure of the fermentability of cellulosic biomass to ethanol. *Appl Microbiol Biotechnol*. 67:52-58.
- Wilkinson, J. 1984. Ensiling with Sodium Hydroxide. In *Straw and other fibrous by-products as feed*, 196-247. Amsterdam, The Netherlands: Elsevier Science Publishers B.V.
- Wong, C.E. 2005. Feruloyl Esterase: A Key Enzyme in Biomass Degradation. *Applied Biochemistry and Biotechnology* 133:87-112.
- Wyman, C.E. 1999. Biomass Ethanol: Technical Progress, Opportunities, and Commercial Challenges. *Annu. Rev Energy Environ*. 24:189-226.
- Wyman, C.E., B.E. Dale, R.T. Elander, M. Holtzapple, M.R. Ladische, and Y.Y. Lee. 2005. Coordinated development of leading biomass pretreatment technologies. *Bioresource Technology*. 96:1959-1966