2950 Niles Road, St. Joseph, MI 49085-9659, USA
269.429.0300 fax 269.429.3852 hq@asabe.org www.asabe.org

An ASABE Meeting Presentation

Paper Number: 084954

INVESTIGATION OF A TWO-STAGE PROCESS FOR INCREASING YIELDS OF ETHANOL FROM SWITCHGRASS

Matthew F. Digman[1,2] Graduate Research Assistant
Kevin J. Shinners[2] Professor of Agricultural Engineering
Bruce S. Dien[3] Research Chemist
Ronald D. Hatfield[1] Plant Physiologist
Richard E. Muck[1,2] Agricultural Engineer
Paul. J. Weimer[1] 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
2008 ASABE Annual International Meeting
Sponsored by ASABE
Rhode Island Convention Center
Providence, Rhode Island
June 29 – July 2, 2008

Abstract.

This research investigated a two-stage process that could be performed on farms to improve the susceptibility of hemicellulose and cellulose present in switchgrass (Panicum virgatum) to enzymatic degradability. The first stage included storage in an anaerobic environment at ambient temperature and pressure in the presence of sulfuric acid given varied chemical loadings (25, 50, 75, 100 and 125 g/kg DM) for 30 days. The second stage was a mild thermal pretreatment (120°C, 1h). Pretreated and untreated samples were fermented to ethanol by Saccharomyces cerevisiae in the presence of several commercial cellulase preparations for 72 h. Xylose yields were also measured following fermentation because it is not metabolized by S. cerevisiae. Potential ethanol yield, lignin and hemicellulose degradation increased with increasing chemical loading; thermal pretreatment and enzyme preparations improved yields significantly, but not substantially. Acetyl bromide lignin was reduced up to 25% during anaerobic storage. Efficiencies of nearly 70% for cellulose conversion to ethanol and 53% for hemicelluloses to xylose were realized, albeit at high chemical loadings.

Keywords. Energy crops, biomass, switchgrass, pretreatment, bioethanol, dilute acid, delignification

Introduction

There is considerable interest in developing agricultural residues and herbaceous perennials as feedstocks for bioethanol production. The logistics of harvesting, storing, and transporting the immense quantities of biomass needed to fuel cost-competitive biorefineries is a great 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; Shinners et al., 2006; Shinners et al., 2007). The advantages over a dry storage system include reduced harvesting costs, lower dry matter (DM) losses during storage, increased product uniformity, improved feedstock susceptibility to enzymatic hydrolysis, and low risk of fire (Shinners et al., 2003; Shinners et al., 2007).

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. In wet storage system, it may be possible to pretreat or remove this recalcitrant nature of the biomass thereby increasing degradability (Ren, 2006; Digman 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 or longer if beneficial. 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 investigates a two-stage process that could be performed on farms to improve the susceptibility of hemicellulose and cellulose present in switchgrass (*Panicum virgatum*) to enzymatic degradability. The first stage included storage in an anaerobic environment at ambient temperature and pressure in the presence of sulfuric acid for varying chemical loadings (25, 50, 75, 100 and 125 g/kg DM) for 30 days. The second stage was a mild thermal pretreatment (120°C, 1h).

Pretreated and untreated samples were fermented to ethanol by *Saccharomyces cerevisiae* in the presence of a multiple commercial cellulase preparations. Enzyme preparations were arranged in a factorial experimental design including two commercial cellulases GC220 and Celluclast 1.5L, with or without Multifect xylanase, and all contained a β-glucosidase, Novozyme 188. Xylose yields were also measured following fermentation because it is not metabolized by *S. cerevisiae*.

Materials and Methods

Substrate

Biomass substrate, switchgrass (*Panicum virgatum*), 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 harvested with a direct-cut forage harvester with a theoretical length of cut of 5 mm on 22 August 2006, and immediately stored at -20°C. The substrate was removed from storage one-day prior to use, transferred to a refrigerator (5°C), and allowed to thaw.

Actual particle-size and moisture content as obtained from direct-cut harvesting was 7.4 mm and 60% (ASAE, 2001; ASAE, 2003). Pretreatment was applied prior to anaerobic storage of grass substrate without adjustment of moisture or size reduction.

Pretreatment

Sulfuric acid was applied as an 18 N solution. Chemical loading was applied in a geometric progression from 25 to 125 g/kg of substrate dry matter (DM). Each loading was administered in duplicate. This range was chosen considering past biomass degradability research. However, pretreatment range was increased for this study as longer duration, but lower substrate temperature and moisture content were considered (Sundstol and Owen, 1984; Kamm et al, 2006). Pretreated material was mixed thoroughly by hand.

Storage conditions were anaerobic, 22°C and 200 kg DM/m density, similar to that of on-farm ensiling (Pitt and Muck, 1993). WECK (location needed) 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 typically 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.

At completion of anaerobic storage, representative sub-samples (20g) were taken from the silo with acid loadings of 25 and 100 g/kg DM. These sub-samples were suspended in 200 mL dH_2O and subjected to a second stage thermal pretreatment using a laboratory autoclave; the temperature and duration of treatment were 120°C and 1 hour.

Sample Preparation for Analytical Analysis

After anaerobic storage, a 40 g representative subsample was collected from each silo. Two controls obtained from fresh switchgrass were also processed. Each aliquot was suspended in 150 mL of dH_2O and ground using a BUCHI model B-400 laboratory grinder (BÜCHI Labortechnik AG, Flawil, Switzerland). The samples were then adjusted to neutral pH using 4M NaOH. Each suspension was then 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 to find anaerobic fermentation products.

Dry Matter and Ash

Each analytical method to follow is based on a dry matter and ash free basis. Ash and dry matter for each freeze-dried and ground sample were determined gravimetrically. Dry matter was determined by loss on drying in a forced air oven; the temperature and drying time were 103°C and 24 hours per ASABE S358.2. Ash content was determined as residue remaining after combusting at 500°C for 4 hours.

Composition Analysis

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

Sub-samples for each silo and control 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 in a 55°C oven for X h prior to weighing for analysis. Approximately 50 mg of sample was weighed into 15 mL polypropylene conical tubes. These samples were hydrolyzed using the Saemen 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_2SO_4, 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_2O (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 3 h. 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. 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_2O 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).

Isolated cell walls were weighed out (~ 25 mg) for lignin determination by the acetyl bromide method using the modified procedure of Hatfield et al. (1999). The extinction coefficient used to determine lignin concentration was based on a purified HCl-dioxane lignin isolated from corn stems (Fukushima and Hatfield, 2001).

Anaerobic Fermentation Products

Post ensiling, a portion of wet sample (20 g) was diluted 10:1 with distilled water and blended with a B-400 BÜCHI laboratory mixer (BÜCHI Labortechnik AG, Switzerland). The water extract was then poured through four layers of cheesecloth, and pH was immediately measured on the filtrate. A 20-mL aliquot of the filtrate was placed into a 50 mL polypropylene tube and centrifuged at 25,000 × g for 25 min. Liquid decanted from the centrifuge tube was frozen at −20°C and used for measuring fermentation products. Fermentation products (lactate, acetate, propionate, butyrate, and ethanol) were determined by HPLC with a refractive index detector (Muck and Dickerson, 1988).

Fermentation to Ethanol

The dilute acid experiment was of a factorial experimental design. The goal was to determine the influence that chemical loading, a second stage thermal pretreatment (120°C, 1h) and various enzyme preparations had on cellulose and hemicellulose degradation (Table 1).

Table 1: Factorial design for two-stage dilute acid process.

Factor	1	2	3	4
Chemical Loading (% DM)	2.5	10	-	-
Thermal Pretreatment	120°C, 1h	None	-	-
Enzyme Preparation	GC220	Celluclast 1.5L	GC220	Celluclase 1.5L
" "	Novo188	Novo188	Novo188	Novo188
" "			Xylanase	Xylanase

Ethanol and pentose sugar yields were determined using a modified simultaneous saccharification and fermentation (SSF) method of Dowe and McMillan (2001). First, a 1.5 g DM aliquot of freeze-dried substrate was added to a 25 mL Pyrex media bottle (Corning Glass, Corning, NY) along with 8 mL of distilled water. The fermentation was buffered using 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.

Next, GC220 (5 FPU/g DM, Genencor-Danisco, Rochester (NY or MN?), USA) or Celluclast 1.5L (15U/g DM, Novozymes, Bagsvaerd, Denmark), Novo188 β-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. When called upon by the experimental design, an equivalent volume as cellulase of Multifect xylanase (Genencor-Danisco, Rochester (NY or MN), USA) was added. Enzymes and YP stock were sterilized by filter sterilizing or autoclaving, respectively.

Each bottle was inoculated with *Sacchromyces cerevisiae* D5A to an O.D. at 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 µL) 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_2SO_4 at 0.6 mL/min and 65°C.

Effects of chemical loading, thermal treatment and enzyme preparation 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

Anaerobic Storage

Preservation of herbaceous crops via anaerobic storage consists of four phases including: aerobic, fermentation, stable, and feed out (Barnes et al., 2003). During the first phase, trapped air in the forage pile, bag, or bunker (clamp) is utilized in plant biochemical processes related to respiration and proteolysis. The anaerobic phase or fermentation phase is dominated by homo and heterofermentive lactic acid bacteria, which ferment soluble sugars to organic acids, thereby lowering the pH of the plant material. At this lower pH, typically between 3.8 and 5.0 depending on the plant species, undesirable microorganisms such as enterobacteria, fungi and clostridia bacteria are inhibited (McDonald, 1981).

The dilute acid pretreatment lowered the pH well below the range expected for anaerobic microorganism activity (figure 1). The pH ranged from 3.4 to 1.5 and was observed to be inversely proportional to chemical loading. Increasing chemical loading also resulted in suppression of lactic acid production while increasing levels of acetate. We suspect that increased acetate is the result of hemicellulose degradation. This phenomenon was observed in the conventional (temperature and pressure catalyzed) dilute acid process (Kamm et al., 2007).

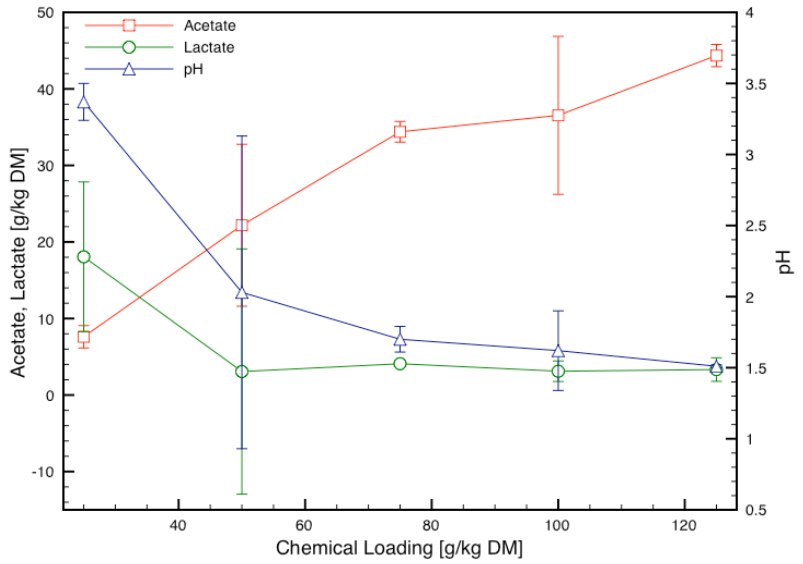

Figure 1: Means and 95% CI bars for acetate, lactate and post fermentation pH after acid treatment and anaerobic storage for 30 days.

Post anaerobic storage compositional analysis revealed changes in switchgrass substrate that occurred during storage (figure 2). Glucose and xylose were observed to increase with chemical loading. This would suggest that pretreatment improves recovery of these two sugars during the Uppsala dietary fiber method. Arabinose yield decreased with chemical loading providing further evidence that hemicellulose was degraded with increasing chemical loading.

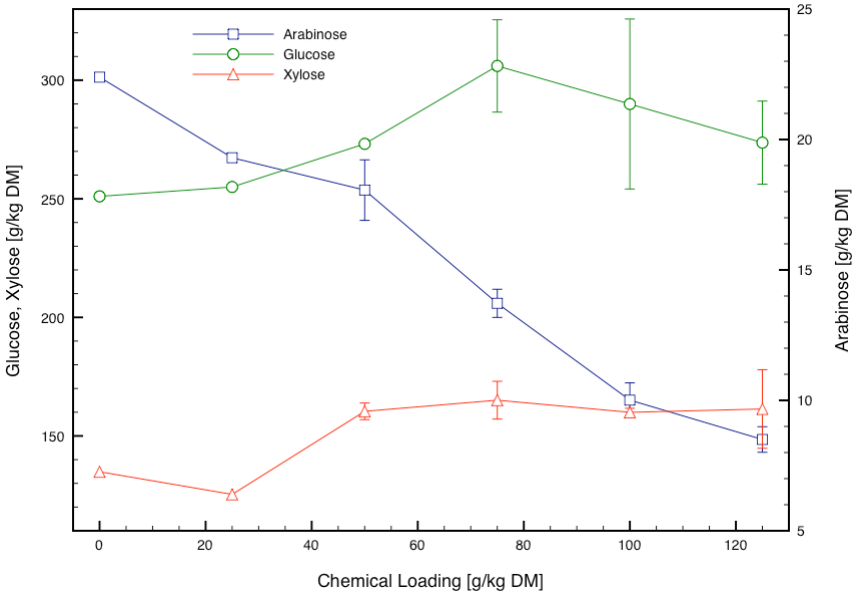

Figure 2: Means and 95% CI bars for arabinose, glucose and xylose after acid treatement and anaerobic storage for 30 days.

Acetyl bromide lignin was significantly reduced with increased acid loading (figure 3). Lignin reduction was extremely repeatable when comparing replicate silos.

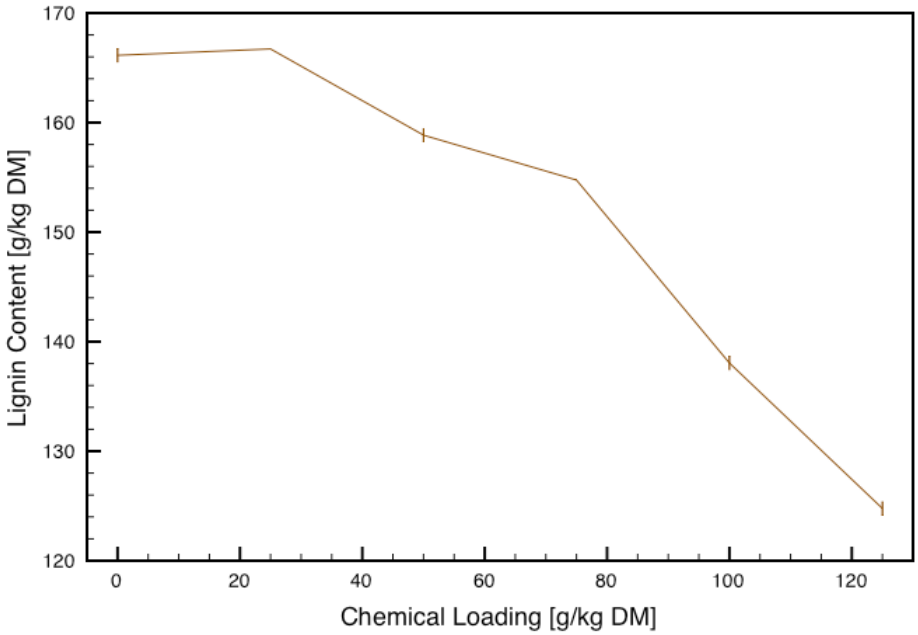

Figure 3: Means and 95% CI bars for lignin after acid treatment and anaerobic storage for 30 days.

Fermentation to Ethanol

Conversion efficiency is reported as the amount of cellulose or xylose recovered after pretreatment, anaerobic storage and simultaneous saccharification and fermentation compared to cellulose available before pretreatment and storage. Cellulose yield was measured by ethanol obtained from the SSF assay whereas available cellulose was measured from untreated substrate by the Uppsala total dietary fiber method. Chemical loading, second stage thermal pretreatment, and enzyme preparation were investigated by way of factorial experimental design.

Cellulose and hemicellulose (xylose) recovery were significantly enhanced by second stage thermal pretreatment (table 2). Xylose recovery was enhanced more than cellulose.

Table 2: Second stage thermal treatment factor's main effect means for cellulose and xylose recovery after simultaneous saccharification and fermentation.

Thermal Treatment	Cellulose Recovery† (% of available)	Xylose Recovery† (% of available)
0	48.7_a	19.3_a
1h, 120°C	56.2_b	46.2_b

†LSD = 1.7% and 3.7% at $\alpha = 0.05$ for cellulose and xylose, respectively.

Increasing the level of acid had the most significant effect on both cellulose and hemicellulose (xylose) recovery (table 3). Increasing the chemical loading from 25 to 100 g H_2SO_4/kg DM resulted in recoveries of 67.8 and 53.1% of available cellulose and xylose, respectively.

Table 3: Chemical loading factor's main effect means for cellulose and xylose recovery after simultaneous saccharification and fermentation.

Chemical Loading (g/kg DM)	Cellulose Recovery† (% of available)	Xylose Recovery† (% of available)
25	37.2_a	12.5_a
100	67.8_b	53.1_b

†LSD = 1.7% and 3.7% at $\alpha = 0.05$ for cellulose and xylose, respectively.

The effect of enzyme preparation was statistically significant for preparations containing the Multifect xylanase (table 4; rows 3 & 4), but yields were only slightly higher. Hemicellulose (xylose) yields were unaffected by enzyme preparation except in one instance.

Table 4: Enzyme preparation factor's main effect means for cellulose and xylose recovery after simultaneous saccharification and fermentation.

Enzyme Preparation (see table 1)	Cellulose Recovery† (% of available)	Xylose Recovery† (% of available)
1	51.1_b	28.5_b
2	49.0_b	31.0_a
3	55.1_a	35.9_a
4	54.8_a	35.6_a

†LSD = 2.4% and 5.2% at $\alpha = 0.05$ for cellulose and xylose, respectively.

Summary

The following compositional changes were observed after pretreatment with dilute acid and anaerobic storage of switchgrass:

- Increased chemical loading suppressed of lactic acid production while increasing levels of acetate.
- Glucose and xylose were observed to increase with chemical loading.
- Acetyl bromide lignin was significantly reduced with increased acid loading.

The compositional changes were observed to influence conversion efficiency of cellulose and xylose measured after simultaneous saccharification and fermentation in the following ways:

- Increasing the chemical loading from 25 to 100 g H_2SO_4/kg DM resulted in recoveries of 67.8 and 53.1% of available cellulose and xylose, respectively.
- The second stage thermal pretreatment only slightly increased cellulose recovery, but had a more pronounced impact on hemicellulose degradation.
- Enzyme preparations that contained Multifect xylanase significantly improved cellulose recovery.

Future Work

- Pretreatments will be investigated at lower concentrations but longer durations.
- 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.

Acknowledgements

Many thanks are due to Michael Boettcher, Steve Esch, Patricia J. O'Bryan, Jimmy Henderson, Jane Marita, Paul Schildgen and Rachel Wepner for their technical assistance and advice throughout this study.

References

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.

Barnes, R., C. Nelson, M. Collins, and K. Moore. 2003. Forages: An Introduction to Grassland Agriculture. 6th Edition. USA: Iowa State Press, Ames, IA.

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.*

Blumenkrantz, N., and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. *Anal. Biochem. 54:484-489.*

Collins, M. and V.N. Owens. 2003. Preservation of Forage as Hay and Silage. In *Forages:* An Introduction to Grassland Agriculture, 443-471. USA: Iowa State Press, Ames, IA.

Digman, M.F., K.J. Shinners, B.S. Dien, R.D. Hatfield, X. Li, R.E. Muck and P.J. Weimer. 2007. On-farm pretreatment technologies for improving enzymatic degradability of cellulose and hemicellulose present in perennial grass. ASAE Paper No. 071021. St. Joseph, Mich.: ASABE.

Dowe, N. and J. McMillan. 2001. SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation. LAP-008. Golden, CO.: NREL.

Fukushima, R.S., and R.D. Hatfield. 2001. Extraction and isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *J. Agric. FoodChem. 49:3133–3139.*

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.R. Wilson, and D.R. Mertens. 1999. Composition of cell walls isolated from cell types of grain sorghum stems. *Journal of the Science of Food and Agriculture. 79:891-899.*

Iiyama, K., Lam, T. B. T., and B. A. Stone.1990. Phenolic bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry. 29:733–737.*

Kamm, B., P. Gruber, and M. Kamm. 2006. Biorefineries - Industrial Processes and Products Volume 2. Wiley-VCH.

McDonald, P. 1981. The Biochemistry of Silage. New York, NY: John Wiley & Sons, Ltd.

Muck, R. E., and J. T. Dickerson. 1988. Storage temperature eon proteolysis in alfalfa silage. *Trans. ASAE 31:1005–1009.*

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 pre-treatment 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. 2003. Whole-plant corn harvesting for biomass: comparison of single -pass and multiple-pass harvest systems. ASAE Paper No. 036089. St. Joseph, Mich.: ASABE.

Shinners, K.J., G.C. Boettcher, R.E. Muck, M. D. Casler and P.J. Weimer. 2006. Drying, harvest and storage characteristics of perennial grasses as biomass feedstocks. ASABE Paper No. 061012 St. Joseph, Mich.: ASABE.

Shinners, K.J., B.N. Binversie, R.E. Muck and P.J. Wiemer. 2007. Comparison of wet and dry corn stover harvest and storage. *Biomass & Bioenergy.* 31(4): 211-221.

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 Fiberous By-products as Feed*. 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*.