0273-2289/1559–0291 (Online)/07/136–140/221–238/$30.00

The Impact of Enzyme Characteristics on Corn Stover Fiber Degradation and Acid Production During Ensiled Storage

HAIYU REN,[1] TOM L. RICHARD,*,[1] AND KENNETH J. MOORE[2]

[1]Department of Agricultural and Biological Engineering, 249 Agricultural Engineering Building, University Park, Pennsylvania 16802, E-mail: trichard@psu.edu; and [2]Department of Agronomy, 2101 Agronomy Hall, Iowa State University, Ames, Iowa 50011

Abstract

Ensilage can be used to store lignocellulosic biomass before industrial bioprocessing. This study investigated the impacts of seven commercial enzyme mixtures derived from *Aspergillus niger*, *Trichoderma reesei*, and *T. longibrachiatum*. Treatments included three size grades of corn stover, two enzyme levels (1.67 and 5 IU/g dry matter based on hemicellulase), and various ratios of cellulase to hemicellulase (C : H). The highest C : H ratio tested, 2.38, derived from *T. reesei*, resulted in the most effective fermentation, with lactic acid as the dominant product. Enzymatic activity during storage may complement industrial pretreatment; creating synergies that could reduce total bioconversion costs.

Index Entries: Biomass; cellulase; hemicellulase; silage; wet storage; pretreatment.

Introduction

In recent years, corn stover, the above-ground residue of maize plants grown for grain, has attracted intensive interest as lignocellulosic feedstock for bioethanol production because of its considerable availability and biorenewability *(1)*. Around 69 million dry Mg/yr can be sustainably harvested in the United States *(2)*. More than 85% of the available corn stover is concentrated in midwestern states *(3)*, which would reduce industrial-scale harvesting and transportation costs as biomass-based industries develop and mature. The rich content of polysaccharides in corn stover can be hydrolyzed to five- and six-carbon sugars for fermentation and chemical modification to produce value-added products *(4–6)*. Corn stover can also serve as fiber feedstock for particle board manufacturing

*Author to whom all correspondence and reprint requests should be addressed.

(7,8). Considerable efforts have been carried out to develop bioconversion processes and utilization strategies for corn stover *(9–11)*.

Bioconversion of corn stover from its raw form as a plant in the field to final commercial products requires four vital processing phases: harvesting, storage, pretreatment, and bioconversion. Additional steps provide links between these phases, such as transportation of the stover, neutralization of pretreatment chemicals, and removal of toxic byproducts before sugar fermentation. Although technical issues involved in the processes of pretreatment and bioconversion have been extensively investigated, the storage phase has received little attention to date. Because corn stover in the United States can only be harvested once a year, storage is needed to preserve large quantities of stover to provide a continuous supply to future biorefineries. Minimum criteria for storage include: minimizing dry matter (DM) loss, and reducing risk of fire. In addition, it would be preferable if beneficial pretreatment for downstream bioconversion could occur during the preservation period.

Ensilage, a traditional crop preservation method for ruminant feed, has been examined as a preservation method for corn stover and wheat straw *(12–14)*. The high moisture content of silage (up to 60% wet basis) eliminates the risk of accidental fire. A robust lactic acid fermentation initially results in rapidly declining pH, and this low pH then inhibits most microbial activity and DM loss as long as anaerobic conditions are maintained. Ensiled storage can provide stable storage with minimal DM deterioration for as long as 1 yr *(15)*. Acid hydrolysis of the cell wall by lactic and other produced acids can occur during the entire storage period, which may be beneficial for downstream cell wall degradation. However, the low sugar content of corn stover makes it difficult to obtain a robust lactic acid fermentation, resulting in more moderate pH that permits undesirable microbial growth, such as clostridia, whose secondary fermentations reduce stability and degrade the biomass. Amending the corn stover ensilage process with cell wall degrading enzymes has been shown to generate lower pH values, increase fiber hydrolysis to sugars, and conserve water-soluble carbohydrates (WSC), thus providing partial pretreatment for downstream bioconversion into sugar platform chemicals and fuels *(16)*. Enzyme treatment has also been shown to improve downstream manufacturing of stover-based biocomposite materials. Particle board made from ensiled stover had enhanced physical strength and dimensional stability *(12)*.

Enzymes prepared from different aerobic fungi have been observed to have various impacts on improving silage quality, although mechanistic investigations in an ensilage context are lacking. When *Trichoderma reesei* and *Aspergillus niger* were compared as amendments for ryegrass–clover silage, the most active degradation of cellulose and lower pH were found with *T. reesei (17)*. However, the activity of individual enzyme components was not discussed or quantified in their study. Different microorganisms

produce different types and proportions of individual enzymes, and their overall activity is a function of the characteristics and composition of these enzyme components. *T. reesei* excretes a complete set of cellulases with appreciable levels of endoglucanase and cellobiohydrolase *(18)*. However, the level of β-glucosidase is not sufficient to thoroughly hydrolyze cellobiose, thus limiting the complete saccharification of cellulose to glucose *(19)*. *A. niger* is another widely studied and commercially-used enzyme producer. The β-glucosidase productivity was found to be 4.8 times higher that of *T. reesei (20)*. But the activities of endoglucanase and cellobiohydrolase are weaker compared with *T. reesei*, even with genetically improved mutants *(21,22)*. A recent study of enzyme effects on maize silage used enzymes derived from *Flavobacterium xylanivorum*, *T. reesei*, and *Thermoascus acrantiacus*, which are psychrophilic, mesophilic, or thermophilic organisms, respectively *(23)*. Colombatto et al.'s study found that the enzymes derived from *T. reesei* and *T. acrantiacus* reduced pH values and cellulose content significantly more than those of *F. xylanivorum*. These differences may also be caused by the various ratios and activities of individual enzyme components, including xylanase, endoglucanase, exoglucanase, and β-glucosidase, acting in synergistic mixtures.

Similarly, there have been few detailed investigations of the effect of combinations of cellulase and hemicellulase enzymes on silage. The crosslinked spatial orientation of cellulose and hemicellulose in the cell wall is such that complete enzymatic hydrolysis of biomass requires synergistic interactions of cellulases and hemicellulases *(24)*. As most commercial enzyme additives are already mixtures of cellulase and hemicellulase, the effect of these additives on silage chemical composition is actually an integrated effect of these synergistic interactions. As one example of such synergy, hydrolysis of hemicellulose has been reported to increase the effective surface area of cellulose fibrils and, therefore, enhance cellulose hydrolysis *(25)*. But we are not aware of any previous studies that have attempted to optimize the ratios of these enzymes to maximize the synergistic effects.

Effective and economical application of commercial enzymes for corn stover preservation requires a full characterization of the enzyme additives, including the microbial organisms they are derived from and the ratios of individual enzymes in each mixture. The present study

1. Investigated and characterized cell wall degrading enzymes from three microbial sources: *T. reesei, A. niger,* and *T. longibrachiatum.*
2. Examined the effect of various ratios of cellulase to hemicellulase from these sources on the biochemical transformations of corn stover during ensilage.

Because stover particle size might influence the contact efficiency and hydrolysis efficacy of the enzymes *(26,27)*, particle size was considered as an additional treatment variable in this study.

Methods

Corn Stover and Silage Preparation

Corn stover was harvested by chopping, windrowing, and baling in the fall of 2002. Stover was then milled by using an Art's-Way hammer mill (Art's-Way, Armstrong, IA) 0, 1, and 2 times to obtain three size grades of samples. The original coarse corn stover, once milled (0.5–1.0 cm) and twice milled (0.1–0.5 cm) stover sizes are called course, medium, and fine, respectively. The samples contained 16–20% moisture (wet basis [w.b.]) and were adjusted to 60% (w.b.) by adding water. A moisture level of 60% (w.b.) was previously determined to be the optimum moisture to minimize clostridia and secondary fermentations in a previous study *(28)*.

Six replicates of each treatment (a complete factorial of three size fraction × seven enzyme treatment × enzyme rate) were prepared, with three replicates of each treatment destructively sampled on day 0 and the other three destructively sampled on day 21. For each replicate, 500 g of treated sample was packed tightly into a 20×35 cm^2 polyethylene bag (200 g dry mass mixed with 300 g water), which was immediately placed under 25 in. mercury vacuum and heat sealed. Samples were incubated at 37 ± 1°C for 21 d. At the end of this preservation period samples were taken for DM and pH measurement. The remainder of each sample was stored frozen for later analysis of lactic acid, volatile fatty acids, WSC, and fiber fractions.

Industrial Enzyme Additives

Seven enzymes derived from three different filamentous fungi were added to the three size grades of corn stover at two different levels. These seven enzymes were chosen from an initial pool of 15 commercial enzymes to represent a diversity of microbial sources and a wide ratio of cellulase to hemicellulase. A description of the seven enzyme characteristics is summarized in Table 1.

Endo-1,4-β-glucanase, cellobiohydrolase, and cellobiase were measured according to the methods described by Wood and Bhat *(29)* by using carboxymethylcellulose, avicel, and cellobiose as substrate, respectively. Hemicellulase measurement used 1% birchwood 4-*O*-methyl glucoronoxylan (Roth 7500) as substrates *(30)*. The enzymes were applied in liquid solution with water to adjust moisture content and were mixed evenly with the stover. The amount of enzymes applied was based on two constant hemicellulase activities: 1.67 IU/g dry mass and 5.0 IU/g dry mass. The data in each column is the measured activity for each enzyme, with the ratio of the enzyme to hemicellulase in parenthesis. Every enzyme has the same units as presented for hemicellulase. The last column is the code representing each enzyme, with a letter for microbial source and number for the ratio of endo-1,4-β-glucanase to hemicellulase (C : H). Endo-1,4-β-glucanase was chosen to quantify cellulase based on the facts that:

Table 1
Characteristics of Industrial Enzymes Used to Ensile Corn Stover

Microbial source	Units	Hemi-cellulase	Endo-1, 4-β-glucanase	Cellobio-hydrolase	Cellobioase	ID: source and C : H[a]
A. niger	IU/g	24,805	1904 (0.08)[b]	42.9 (0.002)	87.4 (0.003)	AN0.08
	IU/g	1075	384 (0.36)	34.6 (0.03)	84.3 (0.080)	AN0.36
T. reesei	IU/mL	5712	109 (0.02)	5.31 (0.001)	3.1 (0.0005)	TR0.02
	IU/g	1219	2607 (2.14)	247.1 (0.20)	36.6 (0.03)	TR2.14
	IU/mL	116	278 (2.38)	45.8 (0.39)	5.4 (0.047)	TR2.38
T. longibra-chiatum	IU/g	18,624	5144 (0.28)	50.9 (0.003)	79.5 (0.004)	TL0.28
	IU/mL	390	543 (1.39)	18.2 (0.05)	7 (0.018)	TL1.39

[a]The ratio of combined cellulase activity to hemicellulase activity.
[b]The ratio of individual cellulase activity to hemicellulase activity.

1. The order of the ratios of filter paper unit to hemicellulase of these seven enzymes is the same as that of the ratio of Endo-1,4-glucanase : H.
2. Endo-1,4-β-glucanase initiates the degradation of cellulose by randomly and rapidly shortening the cellulose chain.

Chemical Analysis of Silages

DM was determined by drying 100 g of fresh samples at 60°C in a forced air oven for 48 h, whereas pH was determined using a pH electrode on samples at a 10 : 1 (H_2O : sample) mass dilution. Lactic acid and volatile fatty acids were determined using gas-liquid chromatography with SP-1000/1200-H_3PO_4 columns (Supelco, Inc., Bellefonte, PA) and a flame-ionization detector. The operating temperatures for the oven, injector, and flame detector were 120, 170, and 180°C, respectively. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were determined by the method of Vogel et al. *(31)*. Hemicellulose content was calculated as the difference between NDF and ADF, and cellulose as the difference between ADF and ADL. WSCs were determined by the modified phenol-sulfuric acid method described by Guiragossian et al. *(32)*.

Statistical Analysis

Data were analyzed using generalized linear model procedure of Statistical Analysis System edition 9.1 *(33)*. Differences between treatments

Table 2
Initial pH, Fiber Fraction, and WSC of Each Stover Size Fraction

	Coarse	Medium	Fine
pH	7.32 ± 0.067[a]	7.77 ± 0.078	7.49 ± 0.017
WSC[b] (% d.b.)	1.32 ± 0.05	1.84 ± 0.09	1.88 ± 0.17
NDF[c] (% d.b.)	80.72 ± 0.08	75.61 ± 0.32	72.72 ± 0.75
ADF[d] (% d.b.)	48.32 ± 0.48	43.07 ± 0.45	43.12 ± 1.16
ADL[e] (% d.b.)	5.08 ± 0.13	5.03 ± 0.21	7.56 ± 0.70
Ash (% d.b.)	1.01 ± 0.10	1.62 ± 0.10	4.10 ± 0.94
Cellulose (% d.b.)	43.24 ± 0.57	38.04 ± 0.23	35.56 ± 1.85
Hemicellulose (% d.b.)	32.40 ± 0.41	32.54 ± 0.19	29.59 ± 0.49

[a]Average ± standard error.
[b]WSC, water-soluble carbohydrates.
[c]NDF, neutral detergent fiber.
[d]ADF, acid detergent fiber.
[e]ADL, acid detergent lignin.

were determined by Tukey's test. Significance of all analyses was declared at a 5% probability level.

Results

Characterization of Initial Feedstocks

The initial characteristics of each stover size are shown in Table 2. There were some variations in fiber composition among the three sizes, with concentrations of cellulose decreasing and WSC increasing in the ground fractions relative to the coarse, unground size. These losses may result from natural degradation by plant enzymes or microorganisms acting on surfaces exposed by grinding, which could have occurred during dry storage before the stover was used for experiments. This hypothesis is supported by a lower hemicellulose concentration and increases in ADL and ash concentrations in the fine fraction, suggesting that considerable loss of biodegradable constituents may have occurred in these ground fractions.

pH Value of Stover Silage

The pH value of stover silage dropped from 7.32–7.77 to 3.79–4.76 after a 21-d ensilage process. Increasing C : H ratio resulted in lower final pH values within all three microbial sources of enzymes on medium-sized stover (Fig. 1). However, the enzyme treatments with a low C : H ratio, especially at low enzyme concentrations, did not reduce pH significantly compared with the control sample (p = 0.814 for TR0.02; p = 0.114 for AN0.08) (pH value around 4.73–4.8). The *T. reesei* (TR) treatment with the highest C : H ratio of 2.38 had the lowest pH value, 3.79, which should effectively guarantee low levels of microbial activity and high preservation

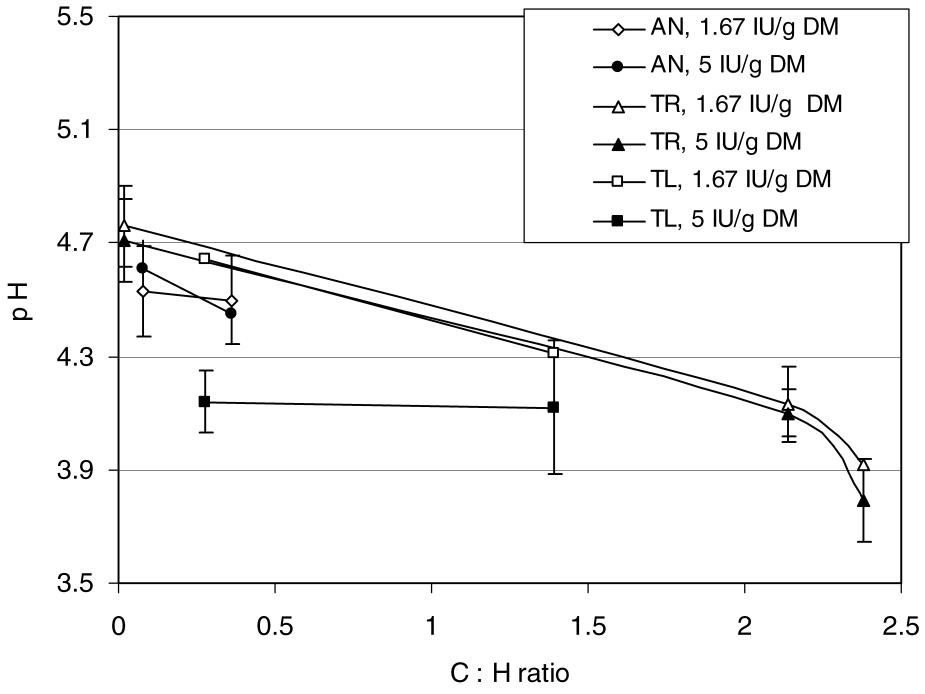

Fig. 1. The pH change of ensiled corn stover vs the C : H ratio of enzyme additives for the medium size stover. (AN represents enzymes from the *A. niger* microbial source; TR represents enzymes from *T. reesei*; and TL represents enzymes from *T. longibrachiatum*. Each enzyme product with a specific C : H ratio is represented by data points, with the white color for low-enzyme concentrations and black for the high concentration).

quality. For ensiled storage of biomass, low pH is desired not only for preservation purposes, but also to create an acidic condition to enhance hydrolysis. These acids partially break down the glycosidic linkages of microfibrils of cell walls, especially hemicellulose, during long-term storage. Dewar et al. *(34)* investigated hemicellulose degradation at various pH levels for 90 d and suggested that considerable hydrolysis of hemicelluloses can be obtained at pH 4.0. This can be presumed to be a beneficial pretreatment for downstream degradation.

Differences among microbial sources are reflected in the observation that higher C : H ratios did not guarantee a lower pH value. For example, at 1.67 IU/g DM, TL0.28 did not resulted in a significantly lower pH value than that of AN0.08 ($p = 0.407$). The enzyme complexes produced by different microbial sources generated different responses in terms of pH. Increasing the level of the TR enzymes did not significantly reduce pH ($p = 0.713$), suggesting that this enzyme system's hemicellulase component was already sufficient at the lower 1.67 IU/g DM rate. Increasing C : H ratio did increase the response for the TR enzymes, demonstrating the importance of synergistic effects. For *T. longibrachiatum* (TL) enzymes, the increase of

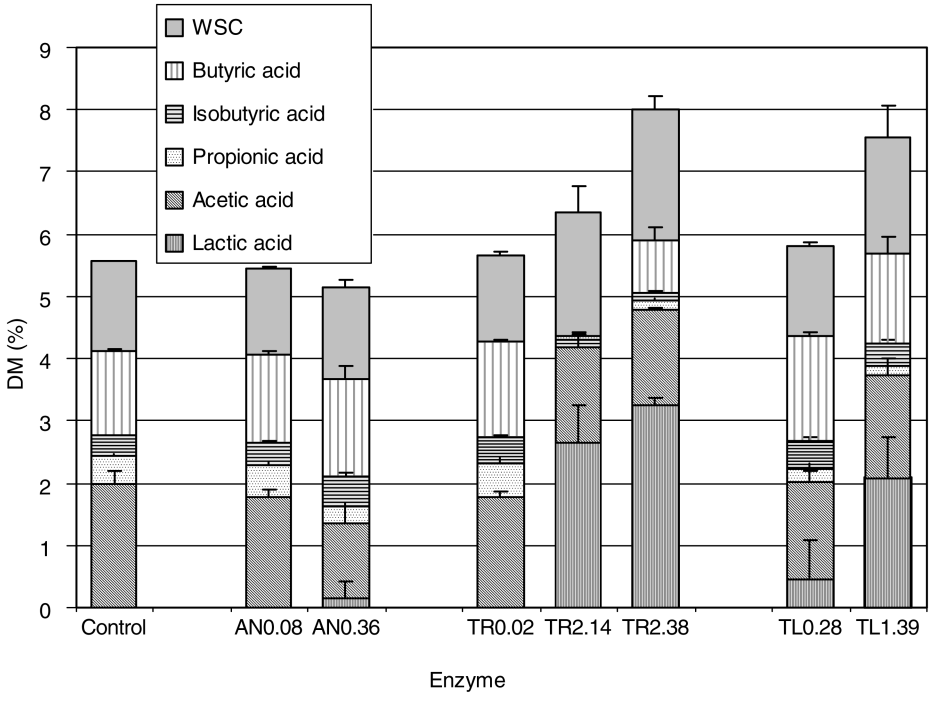

Fig. 2. Concentration of water soluble carbohydrate and organic acids with different enzyme treatments at the level of 1.67 IU/g DM for the fine size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum* respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase [C : H] of the enzyme products).

enzyme levels had a more significant effect in reduced pH value at the C : H ratio of 0.28 than at a C : H ratio of 1.39, suggesting that hemicellulase sufficiency for this enzyme complex occurred between these two rates, and that cellulase was sufficient for both C : H ratios at the higher 1.67 IU/g DM hemicellulase rate. Only two relatively low C : H ratios were tested for the *A. niger* (AN) enzymes. For this enzyme source there was not a significant effect of hemicellulase level, suggesting that enzyme concentration was sufficient at the lower rate. Although not significant, there was a slight trend with increasing C : H, which might have been significant if enzyme mixtures had been available over a larger C : H range.

Chemical Composition of Corn Stover Silage

The nature of the mixed fermentation process and the quality of ensiled stover are reflected in the final chemical composition. Combinations of high concentrations of lactic acid and low concentrations of butyric acid in silage indicate a more active lactic acid fermentation and more dormant secondary fermentations *(12,35)*. The chemical composition of stover samples treated

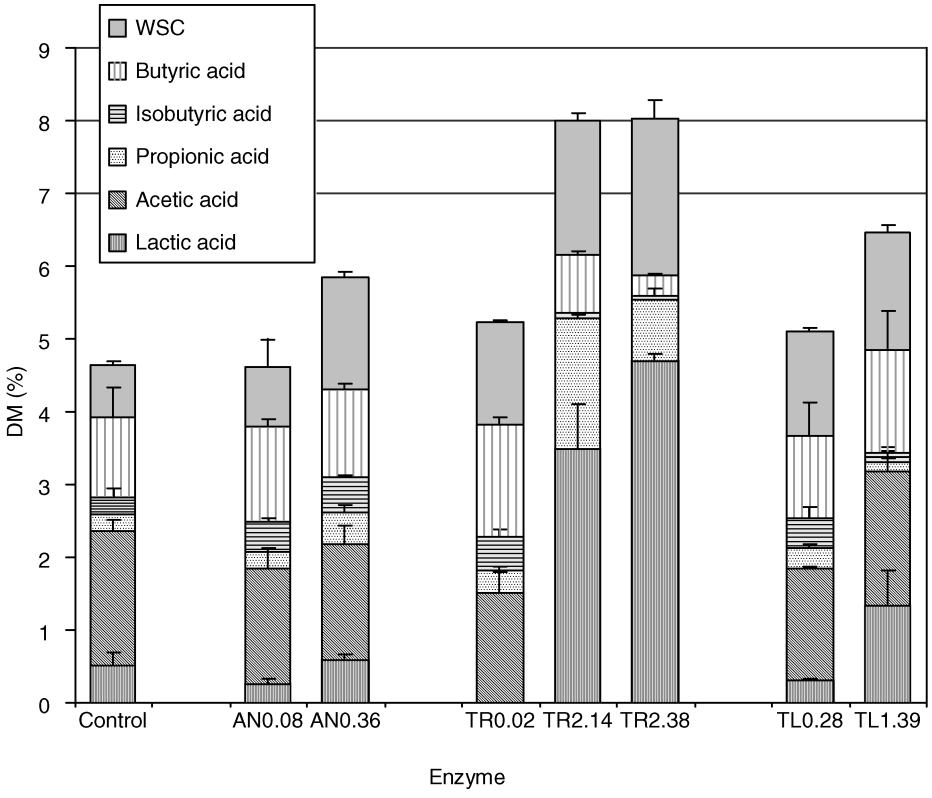

Fig. 3. Concentration of water soluble carbohydrate and organic acids with different enzyme treatments at the level of 1.67 IU/g DM for the medium size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum*, respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase [C : H] of the enzyme products).

and stored for 21 d are presented in Figs. 2–4. Each figure is for one stover size, and includes results for all seven enzyme types at 1.67 IU/g DM hemicellulase as well as the control. For each microbial source, increases of C : H ratio generally resulted in higher concentrations of lactic acid and lower concentrations of butyric acid. This trend was most significant for all three sizes of stover treated with enzymes from the TR microbial source ($p < 0.001$ for lactic acid and $p = 0.013$ for butyric acid). The fraction of lactic acid in the total acid products was highest for the TR 2.38 treatment in the medium size, where it was 80%. This represented the most efficient treatment for silage preservation, as fermented WSCs were primarily metabolized into lactic acid to lower the pH. For the enzymes derived from AN and TL microbial sources, increasing the C : H ratio only increased the lactic acid concentration, without consistently decreasing the amount of acetic acid and butyric acid. The fraction of lactic acid was not improved by the increased C : H ratio because of similar or greater increases in total acid products.

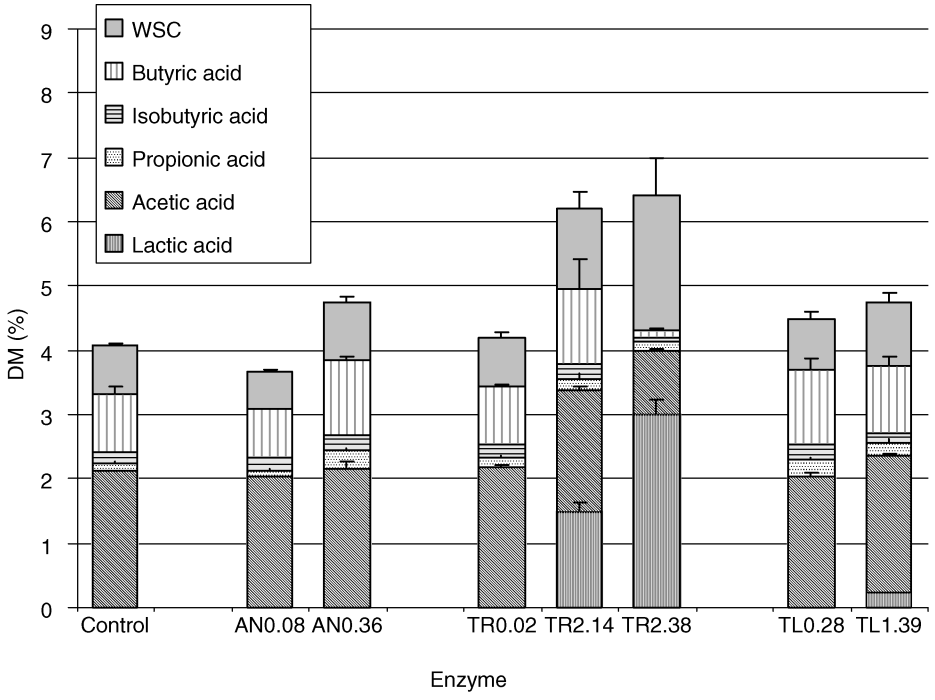

Fig. 4. Concentration of water soluble carbohydrate and organic acids with different enzyme treatments at the level of 1.67 IU/g DM for the coarse size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum*, respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase [C : H] of the enzyme products).

Ideally, for silage preservation purposes, the enzyme treatments would exclusively encourage lactic acid fermentation with little or no contribution to secondary fermentation. If the enzyme treatment is not sufficient to encourage lactic acid production and reduce the pH low enough to inhibit clostridia, enzymatically hydrolyzed sugars will be mostly converted to an undesirable acid mixture *(36)*. For example, Mandebvu et al. *(37)* found that the treatment of bermudagrass silage with enzymes increased the butyric acid concentration without improving lactic acid or WSC production. In contrast, Jakhmola et al. *(38)* reported that the addition of cellulase had no significant effect on the forage quality of perennial ryegrass, nor on a mixture of perennial ryegrass and white clover mixed with shredded barley straw. Inadequate enzyme levels may also encourage yeast growth and ethanol production *(36)*. Enzyme treatments will not be beneficial for preservation if degraded sugars are not sufficient to initiate a dominate lactic acid fermentation. If hydrolyzed sugars are instead allowed to assimilate by undesirable microorganisms, these treatments could lead to substantial DM loss. Environmental and chemical conditions in the first hours and days of a silage process are crucial for establishing and maintaining a lactic-acid-dominated system and an appropriate microbial ecosystem.

For all three stover sizes, some enzyme treatments with a low ratio of C : H (AN0.08, AN0.36, TR0.02, and TL0.28) did not produce much lactic acid. It is interesting that for the medium size stover, the lactic acid produced by AN0.08, TR0.02, and TL0.28 treatments was even lower than that of the control samples, although some of these differences were not statistically significant ($p = 0.074$ for AN0.08; $p = 0.007$ for TR0.02; and $p = 0.21$ for TL0.28). Only TR2.14, TR2.38, and TL1.39, which had higher C : H ratios, enhanced lactic acid concentration significantly ($p < 0.001$ for TR2.14; $p < 0.001$ for TR2.38; and $p = 0.033$ for TL1.39). Cellulases play a much more important role in silage preservation than hemicellulase, because most of the hydrolyzed sugars come from the cellulose fraction *(15)*. Ren et al. *(12)* observed that cellulose degradation is more sensitive to enzyme addition than hemicellulose. This difference was attributed to the heterogeneous structure of the hemicellulose and hemicellulase specificity. Thus, for the purpose of encouraging lactic acid fermentation, a certain amount of cellulase is required in the enzyme mixture. A higher ratio of C : H can result in an elevated lactic acid content in the final silage composition.

It is difficult to examine the effects of microbial enzyme sources on stover silage using the present enzyme selection. These enzymes were produced and separated by different processes, and therefore cannot be assumed to have the same composition for individual enzyme components even when produced by the same microbial source. However, the results seem to provide useful insights. When choosing enzymes available in the market for enzyme screening work, it was noticed that enzymes from AN always had a lower ratio of C : H compared with those of TR. Our results indicated that cellulases produced by AN always have lower activity of endo-1,4-β-glucanase per gram enzyme than those of TR when the comparison is based on the same hemicellulase activity , which is consistent with the work of Kang et al. *(21)* and Kim et al. *(22)*.

An expected effect of enzyme addition is increased hydrolysis of the cell wall, releasing sugars that increase WSC content *(39,40)*. However, in this study AN and TL enzymes did not enhance WSC content significantly in fine and coarse size material when compared with control samples ($p = 0.66$ for AN in fine size; $p = 0.93$ for AN in coarse size; $p = 0.227$ for TL in fine size; $p = 0.402$ for TL in coarse size). Only in the medium size, AN0.36, TL0.28, and TL1.39 treatments resulted in significant increase of WSC compared with control samples ($p < 0.001$). TR enzymes did significantly increase WSC content in each of the three sizes with the increase of C : H ratio ($p = 0.006$ for fine size; $p = 0.002$ for medium size; and $p = 0.03$ for coarse size). The different effects of enzyme addition on WSC content depend on the fate of degraded WSC during the ensilage process *(15)*. The WSC content is the difference between the amount of initial WSC and hydrolyzed WSC, and the amount of the WSC metabolized by microorganisms *(17,41)*. The amount of both hydrolyzed WSC and metabolized WSC is influenced by the activity of microbial communities, especially

lactic acid bacteria, throughout the ensilage process. This microbial consumption of hydrolyzed glucose can reduce or eliminate the glucose inhibition effects on cellobioase, resulting in an accelerated and more complete hydrolysis of the cell wall *(42)*.

The net result of these inputs and outputs to the WSC pool is determined by the initial biomass characteristics including its WSC content, the amounts of cellulose and hemicellulose hydrolyzed to WSC by native and introduced enzymes, the population dynamics of vegetative microorganisms, and the metabolic pathways used by the microorganisms. For enzymes with a low ratio of C : H, such as AN0.08, TR0.02, and TR0.28, the relatively constant WSC can be attributed to a low level of cellulase activity that is not sufficient to generate large amounts of WSCs. Low levels of WSCs early in the ensilage process limited lactic acid production and the resulting pH decline, allowing secondary fermentations by clostridia to dominate in these treatments. For TR2.14 and TR2.38, a significant amount of lactic acid was fermented from the available WSCs in silage, and although this lactic acid consumed WSCs, low pH and reduced secondary fermentation resulted in a final WSC that was still significantly higher than the control samples ($p < 0.001$). Previous long-term trials have demonstrated that cell wall hydrolysis continues throughout the ensilage process *(12)*. The conversion of some of the initial sugars to lactic acid encourages more sugar production, resulting in higher final WSC concentrations in these treatments.

Fiber Degradation of Corn Stover Silage

Degradation of cellulose and hemicellulose at the two levels of enzyme addition are presented in Figs. 5 and 6. Degradation is reported as the difference between initial and final concentrations divided by the initial concentration (all on a dry matter basis [d.b.]), and thus assumes negligible DM loss. The average DM loss in previous experiments was 2.7% d.b. with the highest DM loss of 6.1% d.b. Note that these DM losses are considerably less than the fiber degradation indicated in Figs. 5 and 6, as much of the fiber is converted to organic acids and WSC (Figs. 2–4) that are largely conserved at the drying temperature of 60°C. Fine and coarse size material gave similar results of cellulose degradation with medium size, whereas resulting in no significant increase of hemicellulose degradation. In the control sample, only hemicellulose degradation was observed. This is consistent with ensilage results for orchardgrass and lucerne *(43)*, Italian ryegrass *(44)*, and perennial ryegrass *(45)*.

All these studies reported an overwhelming preferential degradation of hemicellulose, relative to cellulose. The degradation of hemicellulose during the ensilage process is catalyzed by indigenous plant hemicellulases, bacterial enzymes produced during ensilage, and resulted from acid hydrolysis by produced acids *(46)*. Although plant enzymes have been credited as contributors to sugar production *(47–49)*, experimental results have been inconsistent. Bousset et al. *(50)* did not observe hemicellulase

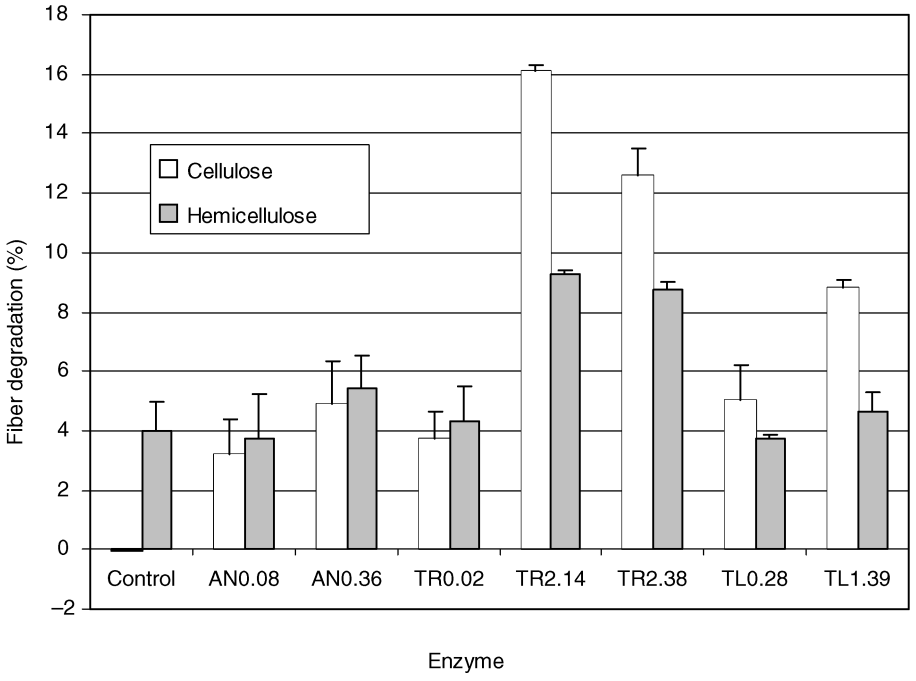

Fig. 5. Degradation of cellulose and hemicellulose with different enzyme treatments at the level of 1.67 IU/g DM for the medium size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum*, respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase [C : H] of the enzyme products).

activity in sterilized silage, and argued that if such activity did exist, the activity should be low because of compartmentalization, plasmolysis, and the short life of these enzymes. Dewar et al. *(34)* found that plant enzymes lost most of their activities after 3 d of ensiling. Direct acid hydrolysis has been suggested by Dewar et al. *(34)* and Morrison *(45)* to be mainly responsible for the degradation of hemicellulose. High levels of hemicellulose degradation have been reported to occur at a pH level of 4 *(34)*. Cellulose degradation at this pH is much lower, as cellulose has more resistant microfibrils with extensive crystalline regions, and relatively moderate acid hydrolysis cannot break down the β 1–4 glycosidic links buried in this three-dimensional structure.

It is not surprising that enzyme additions increased cellulose degradation significantly. For treatments with enzymes from the same microbial source, cellulose degradation increased with the increase of C : H ratio, except for TR2.38. This anomalous result may be an artifact of higher DM loss in the TR 2.38 treatment. Increased cell wall degradation would have reduced the DM basis for the measured concentrations, resulting in a higher percentage of cellulose in the silage, and a corresponding smaller

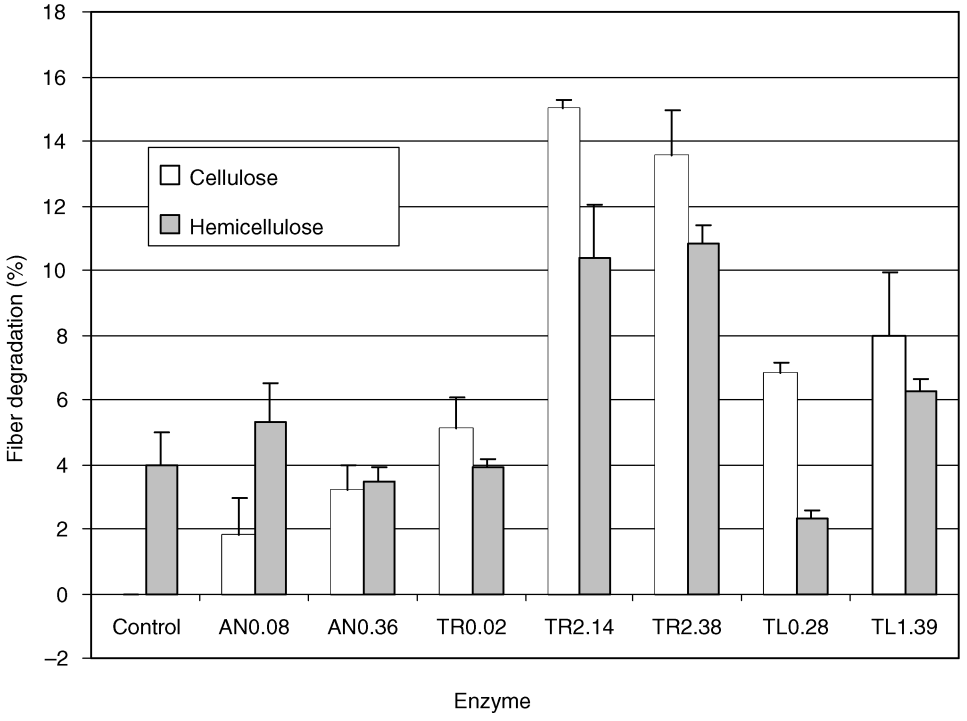

Fig. 6. Degradation of cellulose and hemicellulose with different enzyme treatments at the level of 5 IU/g DM for the medium size stover (AN, TR, and TL represent the microbial sources of various enzyme products, standing for *A. niger*, *T. reesei*, and *T. longibrachiatum*, respectively. The numbers following AN, TR, and TL are the ratios of endo-1,4-β-glucanase to hemicellulase [C : H] of the enzyme products).

apparent cellulose loss. Comparing across microbial sources, the cellulose degradation in the TL0.28 treatment was higher than that with AN0.36 at the 5 IU/g DM hemicellulase level, even though the latter had a higher C : H ratio. A similar but smaller difference between these treatments was observed at the 1.67 IU/g DM level. This was also the case when comparing TR0.02 and AN0.08 treatments, with the lower C : H ratio from the *T. reesei* source resulting in higher cellulose degradation. However, we need to be cautious before definitively concluding that enzymes from *T. longibrachiatum* and *T. reesei* are more effective at cellulose degradation than those of *A. niger* at the same C : H ratio. Cellulose degradation is a complicated synergistic process that includes contributions by at least six individual enzyme components *(42)*. Complete elucidation of the effects of the microbial source of enzymes on cell wall degradation requires enzyme characterization on the molecular level.

Significant increases in the degradation of hemicellulose were observed only for the treatments with TR2.14 and TR2.38 in medium size. Other enzyme treatments did not significantly enhance hemicellulose

degradation. Similar results have been reported by Ren et al. *(12)* and Van Vurren et al. *(51)*. The increased hemicellulose degradation observed with TR2.14 and TR2.38 in medium size is probably because the higher lactic acid concentration and lower pH value (<4.0), enhance acid hydrolysis as previously discussed. The higher degradation of cellulose observed with these treatments may also have contributed, by partially removing the structural hindrance around hemicellulose and increasing access of hemicellulase to the substrate *(25)*.

Increasing the hemicellulase enzyme level did not change fiber degradation significantly. The chemical composition of stover silage for enzyme treatments at the higher hemicellulase level were also similar to previously reported data at the lower (1.67 IU/g) level *(12)*. To decrease the cost of enzyme additives in full-scale industrial applications, the lower level of enzyme amendment is preferable.

Discussion

Mixtures of different ratios of cellulase and hemicellulase enzymes from different microbial sources had varying effects on fiber hydrolysis during ensiled storage of corn stover biomass feedstock. To facilitate comparisons among treatments, mixtures were normalized on the basis of hemicellulase activity. For each enzyme mixture and C : H ratio, two hemicellulase levels were tested, 1.67 and 5 IU/g, and results indicated the lower level of hemicellulase was sufficient to achieve the most beneficial effects. At each of these hemicellulase levels, the ratio of cellulase to hemicellulase was important for improving the quality of stover silage. Increasing ratios of C : H reduced pH, increased lactic acid concentration, and decreased butyric acid concentration.

Successful development of ensilage as a biomass storage strategy will require minimizing DM loss. This loss often results from secondary fermentations, which can be suppressed by high concentrations of lactic acid and the resulting reduced pH. In order to increase the concentration of lactic acid and suppress these secondary fermentations, a critical C : H ratio is required. Mixtures at or more than this critical C : H ratio will have sufficient cellulase to hydrolyze glucose for fermentation into lactic acid. Although this study examined seven different ratios, they were derived from three fungal sources and were not evenly distributed over the entire range. To determine the critical C : H ratio for a particular fungal organism's enzyme suite, additonal C : H ratios derived from that single source should be investigated.

One of the potential benefits of an ensiled storage process would be *in situ* pretreatment and hydrolysis of polymers during storage to produce WSC for downstream bioconversion. This benefit was observed in many of our ensilage treatments. The WSC content of stover silage depended on the ratio of C : H in the applied enzymes as well as the size of stover

material. For each of the stover sizes tested, enzymes from *T. reesei* increased WSC content at increasing rates with increasing C : H ratios. Results for other stover sizes and microbial sources were less consistent, but similar trends were observed. The effect of the microbial source of enzyme mixtures cannot be completely elucidated based on our current results. But enzyme mixtures derived from *T. reesei* and *T. longibrachiatum* appear to hydrolyze more cellulose than those derived from *A. niger*, even when the ratio of C : H in the former mixtures is less than that of the latter. These differences suggest that optimized enzyme mixtures can provide significant pretreatment benefits during ensiled biomass storage.

Hemicellulose is more easily hydrolyzed than cellulose by the acid conditions that prevail during the normal ensilage process. Therefore, it was not surprising that the addition of enzymes improved cellulose degradation more significantly than that of hemicellulose. However, this improved cellulose degradation also contributed to lower pH and presumably increased access of hemicellulase to substrate, resulting in considerably improved hydrolysis of hemicellulose for the high C : H treatments. Development of microbial strains that can convert both five- and six-carbon sugars to ethanol and other value-added chemicals makes increased hemicellulose hydrolysis important for maximizing product yields.

Because the hemicellulase enzyme concentration did not have a significant influence on pH, chemical composition, or fiber degradation of the final stover silage, minimizing the enzyme treatment level should maximize economic returns. For the high C : H ratios of 2.14 and 2.38 in mixtures derived from *T. reesei*, the low 1.67 IU/g hemicellulase level appears more than sufficient to achieve positive results. Further research to optimize these levels and the increase in synergies among enzyme mixture components appears likely to result in attractive ensilage strategies for industrial storage of large volumes of biomass feedstocks.

Acknowledgments

The authors acknowledge the assistance of Patricia Patrick with laboratory analysis. This research was supported by the Iowa Energy Center, Iowa State University, Penn State University, and the United States Department of Agriculture.

References

1. Kadam, K. L. and McMillan, J. D. (2003), *Bioresour. Technol.* **88,** 17–25.
2. Perlack, R. D., Wright, L. L., Turhollow, A. F., Graham, R. L., Stokes, B. J., and Erbach, D. C. (2005), *Biomass as Feedstock for Bioenergy and Bioproducts Industry: The Technical Feasibility of a Billion Ton Annual Supply*. Oak Ridge National Laboratory, Oak Ridge, TN.
3. Walsh, M. E., Perlack,R. L., Turhoolow, A., et al. (1999), *Biomass Feedstock Availability in the United States: 1999 State Level Analysis*. Oak Ridge National Laboratory, Oak Ridge, TN.
4. Sudha Rani, K., Swamy, M. V., and Seenayya, G. (1998), *Process Biochem.* **33,** 435–440.
5. Riera, F. A., Alvarez, R., and Coca, J. (1991), *J. Chem. Technol. Biotechnol.* **50,** 149–155.
6. Buhner, J. and Agblevor, F. A. (2004) *Appl. Biochem. Biotechnol.* **119,** 13–30.

7. Wang, D. and Sun, X. S. (2002), *Ind. Crops Prods.* **15,** 43–50.
8. Chow, P., Bowers, T. C., Bajwa, D. S., et al. (1999), *Proceeding of 5th International Conference on Woodfiber-plastic Composites*, Madison, WS, 26–27 May 1999, pp. 312–313.
9. Green, A. E. S. and Feng, J. (2005), *J. Anal. Appl. Pyrolysis* **76,** 60–69.
10. Hu, Z. and Yu, H. (2005), *Process Biochem.* **40,** 2371–2377.
11. Teymouri, F., Laureano-Perez, L., Alizadeh, H., and Dale, B. E. (2005), *Bioresour. Technol.* **98,** 2014–2018.
12. Ren, H., Richard, T. L., Chen, Z., et al. (2006), *Biotechnol. Prog*. **22,** 78–85.
13. Richard, T. L., Moore, K. J., Tobía, C., and Patrick, P. (2002), *Proceedings Institute of Biological Engineering*, Baton Rouge, LA, 18–21 January, vol. 3, pp. 45–53.
14. Thompson, D. N., Barnes, J. M., and Houghton, T. P. (2005), *Appl. Biochem. Biotechnol.* **121–124,** 21–46.
15. McDonald, P., Henderson, A. R., and Heron, S. J. E. (1991), *The Biochemistry of Silage*, 2nd ed. Chalcobe Publications, Marlow, Bucks, UK.
16. Ren, H., Richard, T. L., Moore, K. J., and Patrick, P. (2004), ASAE paper No. 047066. ASAE, Ottawa, Canada.
17. Henderson, A. R. and McDonald, P. (1977), *J. Sci. Food Agric*. **28,** 486–490.
18. Allen, A. L. and Roche, C. D. (1989), *Biotechnol. Bioeng*. **33,** 650–656.
19. Sternberg, D., Vijayakumar, P., and Reese, E. T. (1977), *Can. J. Microbiol* **23,** 139.
20. Flachner, B., Brumbauer, A., and Reczey, K. (1999), *Enzyme Microb. Technol*. **24,** 362–367.
21. Kang, S. W., Park,Y. S., Lee, J. S., Hong, S. I., and Kim, S. W. (2004), *Bioresour. Technol.* **91,** 153–156.
22. Kim, S. W., Kang, S. W., and Lee, J. S. (1997), *Bioresour. Technol.* **59,** 63–67.
23. Colombatto, D., Mould, F. L., Bhat, M. K., Phipps, R. H., and Owen, E. (2004), *Anim. Feed Sci. Technol.* **111,** 145–159.
24. Mielenz, J. R. (2001), *Curr. Opin. Microb.* **4,** 324–329.
25. White, B. A., Mackie, R. I., and Doerner, K. C. (1993), In: *Forage Cell Wall Structure and Digestibility*, American society of Agronomy, Madison, Inc., pp. 715–767
26. Mooney, C. A., Mansfield, S. D., Beatson, R. P., and Saddler, J. N. (1999), *Enzyme Microb. Technol*. **25,** 644–650.
27. Allan, G. G., Ko, Y. C., and Ritzenthaler, P. (1991), *Tappi J.* **74,** 205–212.
28. Richard, T. L., Proulx, S., Moore, K. J., and Shouse, S. (2001). ASAE paper No. 016019. ASAE, St. Joseph, Mich.
29. Wood, T. M. and Bhat, K. M. (1988), *Methods Enzymol*. **160,** 97–112.
30. Bailey, M. J., Biely, P., and Poutanen, K. (1992), *J. Biotechnol*. **23,** 257–270.
31. Vogel, K. P., Pedersen, J. F., Masterson, S. D., and Toy, J. J. (1999), *Crop Sci*. **39,** 276–279.
32. Guiragossian, V. Y., Scoyoc, S. W. V., and Axtell, J. D. (1977), In: *Chemical and Biological Methods for Grain and Forage Sorghum*, Purdue University, W. Lafayette, IN: pp. 174–178.
33. SAS, SAS/STAT User's Guide, Version 9.1. (2004), Statistical Analysis System Institute Inc., Cary, NC. Available at http://support.sas.com/91doc/docMainpage.jsp.
34. Dewar, W. A., McDonald, P., and Whittenbury, R. (1963), *J. Sci. Food Agric*. **14,** 411–417.
35. Leibensperger, R. Y. and Pitt, R. E. (1987), *Grass Forage Sci.* **42,** 297–313.
36. Rauramaa, A., Setala, J., Moisio, T., and Sivela, S. (1987), *J. Agric. Sci. Finland* **59,** 371–377.
37. Mandebvu, P., West, J. W., Froetschel, M. A., Hatfield, R. D., Gates, R. N., and Hill, G. M. (1999), *Anim. Feed Sci. Technol.* **77,** 317–329.
38. Jakhmola, R. C., Weddell, J. R., and Greenhalgh, J. F. D. (1990), *Anim. Feed Sci. Technol.* **28,** 39–50.
39. Nadeau, E. M. G., Buxton, D. R., Russell, J. R., Allison, M. J., and Young, J. W. (2000), *J. Dairy Sci.* **83,** 1487–1502.
40. Adogla-Bessa, T., Owen, E., and Adesogan, A. T. (1999), *Anim. Food Sci. Technol.* **82,** 51–61.
41. Morrison, I. M. (1988), *J. Agric. Sci.* **111,** 35–39.
42. Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S. (2002), *Microbiol. Mol. Biol. Rev.* **66,** 506–577.
43. Yahaya, M. S., Kimura, A., Harai, J., et al. (2001), *Anim. Feed Sci. Technol.* **92,** 141–148.

44. Kawamura, O., Fukuyama, K., and Niimi, M. (2001), *Anim. Sci. J.* **72,** 134–138.
45. Morrison, I. M. (1979), *J. Agric. Sci.* **93,** 581–586.
46. McDonald, P., Stirling, A. C., Henderson, A. R., et al. (1960), *Edinburgh School of Agriculture Technical Bulletin* **24,** 70–77.
47. Heron, S. J. E., Edwards, R. A., and McDonald, P. (1986), *J. Sci. Food Agric.* **37,** 979–985.
48. Ohyama, Y. and Masaki, S. (1977), *J. Sci. Food Agric.* **28,** 78–84.
49. Pitt, R. E., Muck, R. E., and Leibensperger, R. Y. (1985), *Grass Forage Sci.* **40,** 279–303.
50. Bousset, J., Bousset-Fatianoff, N., Gouet, Ph., et al. (1972), *Ann. Biol. Anim. Biochim. Biophys.* **12,** 453–477.
51. Van Vuuren, A. M., Bergsma, K., Krol-Kramer, F., and Van Beers, J. A. C. (1989), *Grass Forage Sci.* **44,** 223–230.