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Effect of Additions on Ensiling and Microbial Community of Senesced Wheat Straw

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Abstract

Crop residues collected during or after grain harvest are available once per year and must be stored for extended periods. The combination of air, high moisture, and high microbial loads leads to shrinkage during storage and risk of spontaneous ignition. Ensiling is a wet preservation method that could be used to store these residues stably. To economically adapt ensiling to biomass that is harvested after it has senesced, the need for nutrient, moisture, and microbial additions must be determined. We tested the ensiling of senesced wheat straw in sealed columns for 83 d. The straw was inoculated with *Lactobacillus plantarum* and amended with several levels of water and free sugars. The ability to stabilize the straw polysaccharides was strongly influenced by both moisture and free sugars. Without the addition of sugar, the pH increased from 5.2 to as much as 9.1, depending on moisture level, and losses of 22% of the cellulose and 21% of the hemicellulose were observed. By contrast, when sufficient sugars were added and interstitial water was maintained, a final pH of 4.0 was attainable, with correspondingly low (<5%) losses of cellulose and hemicellulose. The results show that ensiling should be considered a promising method for stable storage of wet biorefinery feedstocks.

Index Entries: Wheat straw; lignocellulose; biorefinery feedstock; silage; ensiling; wet storage.

Introduction

Significant quantities of useable lignocellulosic residues go unused in the United States each year. It has been estimated that more than 600 million t of biomass are available annually in the United States at a cost of up to $40/dry t at the farm gate *(1).* This represents a significant and

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valuable renewable biomass resource that could make a significant contribution to reducing our dependence on foreign oil and thereby have a direct and positive impact on the energy security of the United States *(2)*. Conversion of lignocellulosic biomass into fermentable sugars for production of fuels and chemicals has been widely researched in the last 30 yr, and additional advancements will be necessary to economically process lignocellulose to fuels and chemicals *(3)*. Additional advancements must also be made in the harvest, preprocessing, handling, and storage of biorefinery feedstocks before the complete biorefinery will be economically viable *(4)*. Corn stover and cereal straws are the current focus as feedstock sources for near-term biorefinery commercialization because additional dedicated land use is not required and their production cost is already included in that of the grain *(5)*.

Feedstock harvest and supply issues are critical to the economic success of the biorefinery because of the high costs of existing handling systems and yield losses of fermentable carbohydrates during storage *(6)*. Freshly harvested agricultural residues are available in a short harvest window; can be moist when harvested, depending on climate; and are low in bulk density *(7)*. Bale-handling and storage systems, although well developed, are costly and were developed for smaller-scale forage and bedding operations, rather than large biorefineries *(8)*. Shrinkage during storage typically leads to significant reductions in fermentable carbohydrate yields unless the feedstocks are dried to below 20% moisture *(8)*. Reduction of moisture by field drying is often slow and/or ineffective; mechanical drying is expensive *(7)*; and the application of microbial drying processes, although effective, can lead to significant heat generation and spontaneous combustion *(9)*. Ensiling is a potential solution to these issues because it would allow the feedstock to be handled in bulk, potentially reducing handling costs, and would allow the feedstock to be stored wet, reducing drying costs and fire hazards *(8,10)*.

The production of silage from green (actively growing) plant matter has been practiced in various forms on a variety of scales for more than 3000 yr *(11)*. The literature base on ensiling of green forage crops is extensive, and many excellent books and reviews have been written that offer potentially significant insights into application of ensiling to the storage of crop residues that are harvested after they have senesced *(12–14)*. Improved understanding of the microbial processes that occur during green forage ensiling has led to a fairly complete understanding of the physical and chemical factors that affect the microbial community, the silage pH, and the stability and nutritive value of the silage as feed *(13,15)*. The ability to ensile a green forage material is directly affected by several factors, including the dominant microbial community, the moisture content, and the availability of free sugars *(13,15–18)*. Green forage crops typically contain significant free sugars in the plant sap, as well as sufficient moisture *(17)*. For senesced crop residues, however, during maturation, wilting, and field

drying, the free sugars are consumed, the water evaporates, and the microbial community becomes dominated by aerobic bacteria and fungi (yeasts and molds) *(15,18)*.

In the early stages of ensiling of green forages, lactic acid bacteria grow together with aerobic microbes including bacteria, yeasts, and molds *(15)*. The microbial depletion of O_2 from the interstitial voids promotes the onset of fermentation of available free sugars to lactic and acetic acids and a concomitant decrease in pH. As the pH decreases, yeasts and molds are inhibited by the accumulation of acetic acid, and lactic acid bacteria become dominant and further lower the pH *(16,19)*. In the final stages, usually when the pH has reached about 4.0, lactic acid bacteria predominate as a result of their better tolerance to low pH *(16,19)*. In green forage materials that prove to be difficult to ensile, inoculants comprising primarily homofermentative lactic acid bacteria such as *Lactobacillus plantarum* are often added to stimulate the production of lactic acid so that the pH rapidly drops, which, in turn, allows the indigenous lactic acid bacteria to flourish *(15,20)*. Since these conditions inhibit microbes that decompose plant polysaccharides, ensiling seems to be a natural choice for the stable storage of senesced biorefinery feedstocks.

From an economic perspective, however, the value of biorefinery feedstocks is significantly less than that of green forage materials used for feed. Hence, the costs of constructing, operating, and maintaining the storage systems will be significant factors in determining the potential applicability of ensiling to the storage of senesced crop residues as biorefinery feedstocks. Additionally, the costs of any required additions of sugar, moisture, and inoculant could strongly affect the viability of ensiling systems for storage of senesced biorefinery residues. For the ensiling of green forage materials, a minimum of 2 wt% free sugars is typically required to ensure successful ensiling and production of good-quality feed with low rates of rejection *(15)*. In the nonwoody particleboard and pulp industry, ensiling has been used to store sugarcane bagasse stably *(8)*. In these systems, about 3 wt% residual sugar remains in the bagasse after sucrose extraction, and this sugar is utilized to ensile the bagasse in large storage piles at the mill site *(8)*. If insufficient residual sugar remains, molasses is added to the pile to ensure fermentation *(8)*. Moisture levels of 60–65% on a total weight basis are suggested for good-quality green forage silage *(15)*, whereas 90% moisture has been preferred in the bagasse particleboard and pulp industry *(8)*. The economic bottom line for applying ensiling to senesced residues will include all of these costs.

In the present study, we tested the effects of several levels of moisture and free sugar (molasses) additions on the ensiling of senesced wheat straw inoculated with *L. plantarum*. The overall objective of the study was to determine the potential applicability of ensiling for the storage of senesced crop residues as biorefinery feedstocks. The specific objectives were to determine the levels of moisture and free sugars necessary to

attain a final pH of 4.0 in the presence of an active population of lactic acid bacteria; to determine whether under these conditions the microbial community resembled and/or behaved similarly to that in green forage silage; and to determine the degree of stabilization of the cellulose and hemicellulose compositions of the straw under these conditions, for use as a biorefinery feedstock. Wheat straw produced in Idaho (an arid climate) was used as the test residue because it was readily available at the time the study began. Although this wheat straw was already quite dry and required rewetting, the microbial community structure has been shown to be similar for many crop residues at the various stages of crop maturity *(15)*. Hence, rewetted wheat straw was utilized as an example senesced crop residue for this study. As expected, the ability to ensile the straw was strongly influenced by both moisture and the availability of free sugars. Results indicated that a pH of 4.0 was attainable using bagasse storage parameters, and that cellulose and hemicellulose losses under these conditions were <5% of their initial weights. Future work will be necessary to balance the impacts to the biorefinery in terms of yield losses and overall feedstock costs.

Materials and Methods

Wheat Straw

Wheat straw (Westbred 936) was obtained from Grant 4-D Farms (Rupert, ID). The straw was produced during the year 2002 cropping season and stored in a field-side stack from the time of harvest in the fall of 2002 until March 2003. One large square bale (approx. 0.5 t) was removed from the stack, transported to a feed mill, and ground to 0.25-in. minus in a tub grinder. To avoid carryover from previously ground materials remaining in the grinder, approximately the first half of the straw to exit the mill was discarded. The remaining straw was placed in a 1-t polypropylene Super Sack (FIBC International, Grand Junction, CO), transported to an indoor storage area, and stored at 21 ± 2°C and 8.43 ± 0.89% moisture until used.

Ash contents of straw samples were determined by ashing at least 1 g of dry 60-mesh straw for 18 h in a muffle furnace at 650°C. Previous testing indicated that 18–24 h was sufficient to completely combust the carbon in the sample without also volatilizing potassium *(21)*. Carbohydrate and lignin compositions were determined by quantitative saccharification *(22)*. Carbohydrates were quantified by high-performance liquid chromatography as previously described *(23)*. The acid-insoluble fraction from the quantitative saccharification was ashed for 18 h at 650°C, and Klason lignin with extractives was calculated by weight difference. The measured components were summed, and the remaining fraction was attributed to unmeasured organic components, such as uronic acids and proteins, as well as recovery errors in the analysis method.

Silage Inoculant

L. plantarum ATCC 39268 (a homofermentative lactic acid bacterium) was obtained from the American Type Culture Collection (Manassas, VA) and maintained at –80°C in MRS medium (Difco, Detroit, MI) containing 15 vol% glycerol. Inoculant for ensiling test columns was prepared by thawing a frozen stock of cells and establishing a seed culture by adding 100 µL of the stock to 10 mL of MRS medium. The seed culture was grown overnight at 30°C with moderate shaking at 150 rpm. An aliquot (0.5 mL) of this culture was then added to 500 mL of fresh MRS medium, and the cells were grown for 24 h as just described. This culture was harvested at 4°C by centrifuging at 6000 *g* for 15 min, and the cell pellet was washed twice in 0.03 *M* phosphate buffer (pH 7.1), resuspended in sterile water, adjusted to an optical density of 0.387 at 540 nm, and held on ice until needed. To determine the cell numbers in the inoculant, five 20-µL aliquots were stained with acridine orange, collected onto a polycarbonate membrane, and direct counts were obtained using an epifluorescent microscope. Cell counts in the inoculant averaged 2.78 ($\pm$0.17) $\times$ 10^8 cells/mL.

Ensiling Tests

Test Columns

Ensiling tests were performed in miniature silos consisting of sealed 5.08 deep $\leftrightarrow$ 15.24 cm high glass columns fabricated from beaded process pipe. End caps were fabricated from Teflon® and were held in place using metal clamps with rubber seals. The top cap of each column was fitted with a 20.7- to 68.9 kPa (3–10 psig) poppet-type pressure relief valve. Because the silos were loaded with initially uncompacted ground wheat straw that settled slightly when wetted with amendment solutions (see Column Preparation and Incubation), each column contained a small amount of headspace; thus, the top of each column was also fitted with a gas sample port for retrieval of gas samples.

Test Variables

A duplicated 2^3 experimental design was used, varying degree of saturation (moisture content) and amount of molasses addition, and is shown in Table 1. The moisture and molasses levels shown are the actual levels achieved in the test columns. The treatments were designated using combinations of L, M, or H according to the levels of the variables being tested, in the order moisture, molasses. Thus, a column receiving the lowest moisture and lowest molasses was designated LL. Similarly, a column receiving the midpoint moisture and the highest molasses was designated MH, and so on. Duplicate columns were designated 1 and 2 for each factorial treatment (see Table 1).

Table 1
Experimental Design Used for Ensiling Tests[a]

Moisture level	Molasses level	Column	Moisture (%)	Molasses (mg/g straw)
Undersaturated (L)	None (L)	LL-1	70.0	0.0
		LL-2	70.0	0.0
Saturated (M)	None (L)	ML-1	80.0	0.0
		ML-2	80.1	0.0
Oversaturated (H)	None (L)	HL-1	90.0	0.0
		HL-2	90.0	0.0
Undersaturated (L)	Midpoint (M)	LM-1	70.0	33.0
		LM-2	70.1	33.8
Saturated (M)	Midpoint (M)	MM-1	80.0	32.6
		MM-2	80.0	31.1
Oversaturated (H)	Midpoint (M)	HM-1	90.0	31.0
		HM-2	90.0	31.6
Undersaturated (L)	Highest (H)	LH-1	70.3	64.1
		LH-2	70.2	62.2
Saturated (M)	Highest (H)	MH-1	79.9	62.1
		MH-2	79.8	61.4
Oversaturated (H)	Highest (H)	HH-1	90.0	62.4
		HH-2	90.0	62.8

[a]L, M, and H refer to the level of the variable being tested in the order moisture molasses, and 1 and 2 refer to designations for the duplicate columns for each treatment.

Moisture levels were chosen to include undersaturated (L), saturated (M), and oversaturated (H) conditions. Preliminary tests indicated uncompacted straw saturation with water at about 80% moisture, which was chosen as the midpoint moisture content. A recent report indicated that a level of 90% moisture is used in oversaturated commercial bagasse ensiling piles *(8)*, providing the upper bound. The lower bound, 70% moisture, was chosen to provide a symmetrical distribution of levels. Note that the degree of saturation, rather than the actual moisture level, was the variable being tested using these moisture levels.

In the wet storage of sugarcane bagasse as silage for the manufacture of nonwoody pulp and paper, when residual sucrose levels in the bagasse are below 3 wt% sucrose, molasses is typically added to the bagasse to restore at least the minimum 3 wt% sucrose content, which equates to 2.72 kg (6 lb) of sucrose/t of bagasse *(8)*. The addition of molasses to the ensiling test columns was thus chosen to encompass no addition of molasses (L) up to the 3 wt% residual sucrose level. Assuming that there were negligible free sugars in the initial straw test material, this gave about 63.3 mg of molasses/g of dry straw (H). A midpoint (M) of 31.6 mg/g was chosen to provide a symmetrical distribution of levels. The actual L and H molasses amendment levels achieved in the test columns averaged 32.2 ± 1.1 and 62.5 ± 0.9 mg of molasses/g of dry straw, respectively.

Column Preparation and Incubation

Approximately 20 g of ground, air-dried 0.25-in. minus wheat straw was added to each column to an average packing density of 0.0883 ± 0.0047 g/cm^3 (5.52 ± 0.29 lb/ft^3) after amendment. Because of settling during and after the addition of amendment solutions (see following paragraph), the columns contained measured headspace volumes averaging 82.1 ± 10.8 mL. Molasses was provided as a product sample by the United States Sugar Corporation (Clewiston, FL). The molasses comprised 21.5% water and 78.5% dry matter (DM). On a total weight basis, the molasses contained 35.9% sucrose, 5.6% fructose, 2.6% glucose, 11.5% reducing substances (as dextrose), 6.30% crude protein, and 16.0% ash *(24)*. Water was added as distilled water, and *L. plantarum* inoculant was prepared as described under Silage Inoculant and added at approx 2.8×10^8 cells/mL.

Aqueous solutions containing the desired additions were prepared separately for each column in clean polypropylene bottles. Concentrated molasses was added by weight, distilled water was weighed into each bottle, and the contents were gently mixed to dissolve the molasses. Shortly before addition to the straw columns, freshly prepared *L. plantarum* inoculant (1 mL) was added to each bottle and the contents were gently mixed, providing 1.35 (±0.02) $\times 10^7$ cells/g of dry straw. For each column, the bottle of amendment solution was poured slowly over the straw into the column. For undersaturated columns, the solution was drawn into a 60-mL syringe and added stepwise to the column at various heights beginning at the bottom to achieve good distribution of the liquid throughout the straw. A small amount of straw tended to float on the excess water in the oversaturated columns; hence, in these columns a 10-g, 1.75-in. perforated Teflon disk was placed on top of the straw to keep it below the liquid level. The columns were then capped, sealed, and incubated without shaking at 35 ± 0.5°C. This temperature was chosen at the midpoint of the 30–40°C temperature range commonly encountered in large-scale bagasse silage piles *(8)*.

At approx 33 and 61 d, the columns were removed from the incubator, cooled to room temperature, and the headspaces were sampled and analyzed for oxygen as described under Headspace O_2. The columns were then returned to the incubator until the next sampling. On d 36, the columns were removed from the incubator and photographed to provide an estimate of the headspace volumes and a visual record of visible mycelial growth in the columns.

In addition to the straw columns, three independent samples of straw were separately prepared at 90% moisture without molasses or inoculant amendments and incubated in covered beakers at 35 ± 0.5°C overnight to provide an estimate of the initial pH in the straw, and a sample for enumeration of the initial microbial community. The samples were incubated overnight to allow maximum recovery of the microbes by allowing bacterial

cells time to detach from the surface of the straw, and allowing spores to germinate. Because they were incubated overnight, they are referred to as d 1 samples. The d 1 samples were recovered after the overnight incubation in the same manner as the sealed columns.

Column Harvest

On d 82 and 83, the columns were removed from the incubator and cooled to room temperature. The headspace O_2 in each column was measured and the columns were photographed. The weight of each intact column was noted, and then the cap was removed. The odor of each column was noted, the cap was replaced onto the column, and it was reweighed (this gives the amount of gas and vapor lost on opening the column). Depending on the moisture content, the straw at the top of many columns was initially in contact with oxygen while the straw at the bottom was not. To determine whether this would have a significant effect on straw degradation, the straw in the top and bottom of each column was collected and analyzed separately. It was later observed that the effect was minimal and/or within the standard deviations of the data. Hence, we have combined the data presented herein rather than separately presenting each individual fraction.

During column processing, care was taken to allow detailed mass balance calculations to be performed. Approximately the top half of the wet straw (by visual observation) was quantitatively removed from the column and transferred to a sterile stainless steel blender cup. Thirty-five to 120 mL of distilled water was added to the blender, depending on the initial moisture content of the column being harvested, and the mixture was blended at the highest setting for 1 min. The slurry was quantitatively transferred to a centrifuge bottle and its pH measured using a standard pH meter and probe. A homogeneous 1-mL sample of the slurry was taken for microbial community analysis, and the remaining slurry in the bottle was centrifuged for 10 min at 10,000*g*. The supernatant was decanted and discarded, and the solids remaining in the centrifuge bottle were quantitatively transferred to a tared drying bottle and dried to constant weight at 105°C. After drying, the samples were ground to 60 mesh in a Wiley mill and then stored in capped polyethylene bottles at room temperature until analyzed for composition. The remaining (bottom half) contents in each ensiling column were recovered, homogenized, centrifuged, and dried in an identical manner. After processing, the samples that were collected for microbial analysis from the top and bottom portions of each column were combined and mixed using a vortexer. From this mixture, 1.6 mL was transferred to a clean centrifuge tube and 0.40 mL of 75% glycerol was added. The samples were incubated at room temperature for 10 min and then stored at –80°C until analysis.

Headspace O_2

Headspace O_2 was determined for 100-µL headspace gas samples recovered using a gastight syringe inserted through the sampling port in

the top of each column. Samples were analyzed by direct injection into an HP Model 5890A Series II gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a thermal conductivity detector and a 25-m Molsieve 5Å column (Chrompack Inc., Raritan, NJ) with an id of 0.53 mm. The temperature of the column was maintained at 50°C, and the injector and detector were maintained at 100 and 200°C, respectively. Helium was used as the carrier gas at a flow rate of 10 mL/min. The method was standardized for O_2 measurement by injecting samples of 25, 50, 75, and 100 µL of air at an average temperature and pressure of 21 ± 2°C and 86.1 kPa (0.85 atm). At this temperature and pressure, the average measured concentration of O_2 in the air was 21.07 ± 0.46%. A zero concentration O_2 standard was prepared by flushing a sealed serum bottle with N_2 gas and measured by injecting 100 µL of the nitrogen. All samples and standards were analyzed in duplicate.

Microbial Community

Microbial community analyses were conducted as described by Moon et al. *(25).* Viable bacterial populations were determined for 10-fold serial dilutions of column slurry samples prepared using filter-sterilized distilled water and plating the dilutions onto selective and nonselective agar media *(26,27)*. The agar media included trypticase soy agar (TSA) (Difco, Detroit, MI), for general cultivation of heterotrophic microorganisms; Lactobacillus selective agar (LBSA) (Baltimore Biological Laboratory, Cockeysville, MD), for recovery of Lactobacilli; azide dextrose agar (ADA) (Difco), for growth of lactic acid cocci; violet red bile agar (VRBA) (Difco), for enumeration of coliforms; and rose bengal chlortetracycline agar (RBCA) (Difco), for recovery of yeasts and molds. Plates were incubated at 30°C and colonies were enumerated after 1 wk of incubation. Cell numbers are reported as colony-forming units (CFU) per dry gram of straw.

Results and Discussion

Composition of Wheat Straw

Nine independent grab samples collected from widely spaced locations within the Super Sack of ground straw were analyzed in duplicate for sugar, lignin, and ash compositions. The pooled average composition of the straw in the super sack was 35.9 ± 1.8 wt% glucan, 17.4 ± 1.0 wt% xylan, 0.7 ± 0.4 wt% galactan, 1.7 ± 0.3 wt% arabinan, 0.2 ± 0.4 wt% mannan, 15.9 ± 1.2 wt% Klason lignin with extractives, and 12.3 ± 0.7 wt% ash. The remaining fraction was attributed to uronic acids, proteins, and so on and to recovery errors in carbohydrate analysis technique. This fraction was referred to as "other organics" and was calculated by difference to be 15.9 ± 1.6 wt%. The error range presented for the other organics fraction was estimated by propagating the measurement uncertainties of the other components.

Headspace O_2

The headspace O_2 was measured on d 33 and 61 and at harvest (d 82 to 83, depending on the column) to screen for columns that did not become or remain anaerobic. The headspace O_2 in all but one of the columns (HL-2) was very low by d 33. None of the columns became strictly anaerobic, retaining on average 0.86, 1.3, and 1.7% O_2 in the headspaces on d 33, 61, and 83, respectively. This represents 92–96% reductions of the headspace O_2 from the initial atmospheric concentration of approx 21%. Thus, the columns provided significantly reduced O_2 but did not completely eliminate the O_2. The absence of strict anaerobic conditions suggests that the pressure relief valves may have occasionally opened and reseated slowly enough to allow an unknown amount of O_2 into the columns. Column HL-2 retained 12–19% O_2 over the course of the test (57–90% of atmospheric O_2) and thus was not sealed; this column was therefore excluded from the data analysis.

The initial presence of some amount of O_2 is common in field-scale ensiling methods, which provides O_2 for the undesirable consumption of free sugars via aerobic respiration in the early stages of ensiling. Although this is detrimental to the rapid onset of ensiling, even with compaction of the material it cannot be completely eliminated at the field scale in a practical and cost-effective manner. Thus, we made no effort to remove O_2 from the columns at the beginning of the tests. To ensure that sufficient free sugar remained available for production of organic acids, we estimated the amount of free sugar that could potentially be used for aerobic microbial respiration. At the average local pressure of 86.1 kPa (0.85 atm), and if one assumes that air is an ideal gas, the initial headspace O_2 could have been used to consume an average 97 μmol of *in situ* or exogenous glucose equivalents in each column. Assuming 90% conversion of glucose into lactic acid by a typical mature silage microbial community *(13)*, these free sugars could otherwise have been converted into 174 μmol of lactic acid.

In columns amended with 32.2 and 62.5 mg of molasses/g of straw, there were 1885 ± 54 and 3668 ± 44 μmol of glucose equivalents added, respectively. Thus, the initial O_2 could potentially have been used to consume only 5 and 3% of the total exogenous sugars, respectively. In columns in which no molasses was added, up to 97 μmol of *in situ* glucose equivalents could have been consumed. Because the straw was stored for 6 mo in a field-side stack, it was not expected that it would contain significant *in situ* free sugars. In any event, the fact that the columns amended with 32.2 mg of molasses failed to attain the expected final pH of 3.8–4.0 *(16,19)* indicates that there were not sufficient *in situ* free sugars (see the next section).

Column pH

The pH of the d 1 samples averaged 5.23 ± 0.23. The observation of a pH significantly below neutral could have been influenced by the overnight

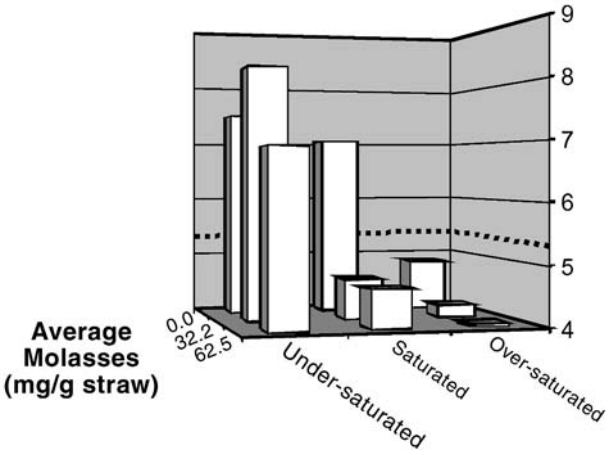

Fig. 1. Variation of final pH in columns with moisture and molasses addition. The undersaturated, saturated, and oversaturated moisture levels correspond to 70, 80, and 90% moisture in the straw, respectively. The dashed line at pH 5.23 indicates the initial pH in the straw.

incubation at 35°C. The incubation was performed to obtain good recovery of indigenous microbes and allow any indigenous spore formers to germinate.

The variation of final pH (averaged for each set of duplicates) with moisture and molasses added in the ensiling columns is shown in Fig. 1; The dashed line indicates the initial pH of 5.23. Ensiling, defined here as a drop in pH, did not occur in any of the undersaturated (70% moisture) test columns regardless of whether molasses was added, with the pH increasing to >7.0 and as high as 8.60. In these columns, a strong "barnyard" odor emanated from the straw, indicating that ammonification and butyric acid production may have occurred. Ensiling also did not occur in the saturated (80% moisture) columns without the addition of molasses. A slight drop in pH to 4.90 was observed in the oversaturated (90% moisture) columns without the addition of molasses. When molasses was added to saturated and oversaturated columns, some degree of ensiling occurred, with the best ensiling (lowest pH) occurring in oversaturated columns having the highest level of addition of molasses with an average pH of 4.05. In general, ensiling improved with both increased moisture and increased addition of molasses. Moderate drops in pH to approx. 4.6 led to faint "vinegar" odors in the columns, suggesting some acetic acid production. Columns exhibiting final pH values approaching 4.0 had strong "vinegar" odors, suggesting the presence of significant amounts of acetic acid. Clearly, maintaining interstitial water and adding free sugars to the straw (molasses) was necessary for significant ensiling to occur in the wheat straw.

Microbial Communities in Test Columns

The suite of organic acids produced in green forage silage depends on the microbial community and has a significant effect on the nutritive value of the forage, the final pH attained, and the susceptibility of the silage to spoilage once exposed to air *(15)*. For example, the presence of significant butyrate indicates poorly fermented silage and also the activity of Clostridia, which negatively impacts intake by ruminants *(28)*. Acetic acid inhibits aerobic bacteria as well as yeast and molds, thereby protecting the ensiled biomass from exposure to air *(15,29)*. Lactic acid provides both a quick drop in pH and a lower pH than the other organic acids typically produced *(16)*. Homofermentative lactic acid bacteria are often used as inoculants in silage making for forage applications to improve the organic acid profile in the silage by increasing the amount of lactic acid produced *(15,16)*. Because another potential market for crop residues harvested after senescence and ensiled as biorefinery feedstocks could be as forage, similar constraints could be required for these residues. In the present study, we inoculated with the homofermentative lactic acid bacterium *L. plantarum*.

Table 2 presents the results of the microbial community analysis. Selective and nonselective media were used to recover specific groups of microbes. TSA plate counts represent the total population of culturable aerobic and aerotolerant anaerobic bacteria. LBSA medium selected for *Lactobacillus*, and ADA medium selected for lactic acid cocci, including *Streptococcus*, *Pediococcus*, *Enterococcus*, and *Leuconostocs (16)*, which produce acetic, lactic, and propionic acids. VRBA medium selected for coliforms, and RBCA medium selected for fungi.

Initial Microbial Populations

Culturable microbial numbers in the d 1 samples averaged 3.57 (±2.79) $\times$ 10^8 CFU/dry gram of straw, which is slightly lower than the approx 6×10^8 CFU/g reported for fully ripe corn *(15)*. The distribution of microbes in fully ripe corn was reported to be dominated by aerobic bacteria (approx 98%), followed by yeasts and molds (approx 2%), lactic cocci (approx 0.01%), and lactic rods (<0.0005%) *(15)*. It was expected that microbial numbers in the straw would be lower than at harvest because it was stored for 6 mo in a field-side stack before being utilized for the ensiling tests. For d 1 samples A and B (Table 2), comparison of the microbial numbers enumerated on TSA medium with those enumerated on the selective media indicated that nearly 98% of the culturable aerobic bacteria were not lactobacilli or lactic acid cocci, which is identical to that reported for fully ripe corn. Day 1 sample C was significantly different at 83% but was not outside the 95% confidence limits. The selective media indicated that the initial straw harbored a significantly larger population of lactic acid bacteria than that reported for fully ripe corn, as well as a much smaller fungal population. Day 1 samples A and B

Table 2
Culturable Microbes Recovered on Selective Plates from Initial Straw and d 83 Columns

		Microbial numbers recovered using each medium (CFU/g dry straw)[b]				
Column	pH[a]	TSA	LBSA	ADA	VRBA	RBCA
Day 1 A	5.50	6.0×10^8	6.8×10^6	5.2×10^6	2.4×10^7	2.9×10^5
Day 1 B	5.10	4.2×10^8	3.9×10^6	5.7×10^6	1.3×10^7	3.8×10^5
Day 1 C	5.10	5.2×10^7	3.6×10^6	4.6×10^6	1.5×10^7	4.9×10^5
LL-1	8.66	4.8×10^7	1.9×10^5	1.7×10^5		
LL-2	6.24	1.7×10^8	1.3×10^6	1.2×10^6	6.7×10^3	
ML-1	8.57	4.5×10^9	3.2×10^6	9.8×10^4	1.4×10^5	7.0×10^3
ML-2	5.46	2.7×10^7	9.0×10^6	6.2×10^5		
HL-1	4.85	1.0×10^8	1.1×10^7	1.1×10^6		
HL-2[c]	4.95	1.4×10^9	1.6×10^3	2.4×10^4		
LM-1	7.84	1.0×10^8	3.8×10^6	5.7×10^6	9.1×10^3	
LM-2	8.60	1.8×10^8	2.0×10^6	1.7×10^7	2.8×10^3	
MM-1	4.89	6.7×10^5	2.2×10^5	1.5×10^5		
MM-2	4.44	7.3×10^5	1.0×10^5	4.5×10^5		
HM-1	4.25	9.5×10^6	4.1×10^6	3.0×10^6		
HM-2	4.16	1.1×10^6	5.1×10^5	3.5×10^5		
LH-1	5.18	6.8×10^5	1.4×10^5	1.6×10^5		
LH-2	8.59	1.5×10^7	2.3×10^6	6.9×10^4	2.6×10^3	
MH-1	4.63	2.4×10^6	1.3×10^6	2.0×10^5		
MH-2	4.65	3.5×10^6	9.0×10^5	6.8×10^5		
HH-1	4.12	7.9×10^5	2.6×10^5	2.5×10^5		
HH-2	3.97	9.2×10^5	1.5×10^5	5.3×10^5		

[a]pH on d 83 in the bottom of the columns, except for the time zero samples, for which it is the pH after incubation for 1 d.

[b]Where no data are listed, no colonies grew at any dilution.

[c]Although enumerated and included here, air leaked into column HL-2 during the course of the incubation; thus, this column did not remain at low O_2 and was dropped from the overall analysis.

averaged 1.1% lactic acid cocci, 1.0% lactobacilli, and 0.07% fungi, whereas d 1 sample C yielded 8.9% lactic acid cocci, 6.9% lactobacilli, and 0.9% fungi. Although the fungal numbers were smaller than the bacterial numbers, in the literature, fungi have been shown to metabolically dominate the microbial community in mature forage materials *(15)*. The data indicate that the d 1 samples contained a much higher number of lactic acid bacteria than expected. This lends support to the hypothesis that the lower-than-expected initial pH of 5.23 was owing to fermentative activity either while the straw was field drying (before baling) or during the overnight incubation. It is plausible that rapid field drying in the arid Idaho climate could have evaporated sufficient water quickly enough to limit consumption of the free sugars via aerobic microbial respiration and allow some free sugars to remain.

Day 83 Microbial Communities and Correlation with Ensiling

The microbes recovered from the d 83 test columns are shown in Table 2. Lactic acid bacteria are the most beneficial microbes involved in ensiling *(16)*. This group consists of both homofermentative and heterofermentative Gram-positive non-spore-forming bacteria. Homofermentative strains ferment sugars almost exclusively to lactate, whereas heterofermentative strains also produce acetate and ethanol *(16)*. The plate counts demonstrate that both groups of bacteria are established in the columns that ensiled the best and attained the lowest pH values. Hence, the low pH is owing to production of both lactic and acetic acids. The "vinegar" odor emitted by these columns suggested accumulation of acetic acid, which inhibits growth of yeasts and mold *(29)*. In support of this, no fungal growth was observed either visually (not shown) or by plate counts in these columns. In the columns that ensiled significantly (final pH of <4.5), coliform growth and Clostridia activity were not observed. This is consistent with the literature; a combination of both low pH and low oxidation-reduction potential has been shown to be detrimental to Enterobacteria (coliforms) and to inhibit growth of spore-forming bacteria (Bacilli and Clostridia) *(29)*.

Ensiling did not occur in undersaturated and saturated columns lacking molasses, with the average final pH in these columns ranging from 5.46 to 8.66. The total number of culturable bacteria in these columns was similar to that observed in the d 1 samples. Growth of both coliforms and fungi was observed; although fungi were not cultured on RBCA medium in all of these samples, visible fungal mycelia were present as well as more dense filamentous actinomycetes (not shown). In addition, all of the columns emitted the aforementioned "barnyard" odor, which suggests Clostridial activity—Clostridia ferment sugars and lactic acid to butyric acid and generate NH_3 from deamination and decarboxylation of amino acids *(30)*. In some of the undersaturated and saturated no-molasses columns, coliform growth was not observed. Because low pH is detrimental to coliform growth *(16)*, the presence of coliforms in a column indicates that low pH was not established during the incubation. In columns that did not ensile and exhibited Clostridial activity but no coliform growth, the pH most likely dropped at some point during the incubation but did not stabilize at the lower value. This would occur because the lowered pH would largely eliminate coliforms but spore-forming bacteria such as Clostridia would survive and begin to thrive as the pH increased *(16)*.

A slight amount of ensiling occurred in oversaturated columns lacking molasses, with the pH dropping from 5.23 to about 4.9. This indicates that there was at least a small amount of free sugars available either as *in situ* free sugars or as free sugars released during degradation of the plant polysaccharides. The total number of culturable bacteria in these columns was again similar to that observed in the d 1 samples. Neither coliforms nor fungi were

cultured and no visible fungal mycelia were present in the columns (not shown), which is consistent with the lowered pH. However, these columns also emitted the "barnyard" odor, suggesting Clostridial activity; hence, the final pH of 4.9 was not sufficient to suppress the activity of detrimental microbes. The final pH of 4.9 was an improvement over the lower-moisture columns lacking molasses, but it was not a significant gain with respect to ensiling. This result may reflect better distribution of initial *in situ* organic acids and nutrients in the column owing to interstitial water.

The lowest final pH was observed in columns amended with molasses and oversaturated with water. Increasing the addition of molasses correspondingly decreased the final pH to 4.0, which is near the lower end of the 3.8–5.0 range commonly observed in green forages *(13)*. The total number of cells in the oversaturated columns was lower and the population was heavily dominated by lactic acid bacteria (Lactobacillus and lactic acid cocci). Because this did not occur in the oversaturated columns that were not amended with molasses, the addition of a readily available energy source was necessary, indicating that there were insufficient free sugars in the straw to support significant ensiling.

The results indicated that the more rapid the consumption of O_2 and onset of fermentation, the lower the final pH that will be attained. We inoculated the straw in each test column with approx 1.35×10^7 metabolically active *L. plantarum* cells/gram of dry straw. In Table 2, the d 83 populations of these inoculated cells would be enumerated (included) on the TSA and LBSA media for samples from the ensiling test columns, but not for the d 1 samples (because they were not inoculated). The inoculated *L. plantarum* would be expected to have a competitive advantage over the dormant epiphytic bacteria. It is likely that the addition of the inoculant assisted significantly in rapidly consuming the initial O_2 and lowering the pH, because *L. plantarum* is homofermentative and thus would convert the sugars in the molasses solely into lactic acid, thereby causing a rapid drop in pH. The low pH would, in turn, inhibit the growth of detrimental bacteria and enhance the growth of the epiphytic lactic acid bacteria. As the acids continued to accumulate, the activity of the lactic acid bacteria would decrease, resulting in a lower total number of cells (as was observed).

Overall Mass Balances

Table 3 presents the mass balances for the column harvests. The total mass recoveries (solid and liquid) averaged $98.0 \pm 2.0\%$ of the initial inputs. In these calculations, it was assumed that the net amount of water in the columns, which can be produced from aerobic microbial respiration and consumed in the microbial hydrolysis of plant polysaccharides, did not increase or decrease. There were significant decreases in the DM in all columns, regardless of whether the columns ensiled. There were corresponding increases in the dissolved solids, which would be expected both

Table 3
Overall Mass Balances for Test Columns

	Weights into columns at time zero				Weights harvested on d 83				
Column	DM (g)[a]	Water (g)[b]	Dissolved solids (g)[c]	Total (g)	DM (g)	Water (g)[d]	Dissolved solids (g)[d]	Total (g)	Mass balance (%)[e]
LL-1	20.04	46.74	0	66.78	12.95	46.74	4.02	63.71	95.4
LL-2	19.97	46.70	0	66.67	15.40	46.70	2.45	64.55	96.8
ML-1	20.03	80.30	0	100.33	13.60	80.30	3.16	97.07	96.8
ML-2	19.99	80.22	0	100.21	16.96	80.22	2.12	99.30	99.1
HL-1	19.25	173.50	0	192.75	13.54	173.50	4.44	191.48	99.3
HL-2[f]	20.06	180.94	0	201.00	13.91	180.94	3.55	198.40	98.7
LM-1	20.11	46.42	0.41	66.95	13.39	46.42	3.48	63.29	94.5
LM-2	19.98	46.23	0.42	66.63	13.48	46.23	2.83	62.54	93.9
MM-1	20.19	80.23	0.41	100.83	17.39	80.23	2.15	99.77	98.9
MM-2	20.52	81.55	0.40	102.47	17.27	81.55	2.99	101.81	99.4
HM-1	20.32	182.05	0.39	202.76	17.33	182.05	3.55	202.93	100.1
HM-2	20.53	183.21	0.40	204.15	17.06	183.21	3.55	203.82	99.8
LH-1	20.27	46.86	0.80	67.93	17.83	46.86	2.23	66.91	98.5
LH-2	20.30	46.70	0.78	67.78	14.95	46.70	2.88	64.53	95.2
MH-1	19.99	77.96	0.77	98.72	16.88	77.96	3.02	97.85	99.1
MH-2	20.50	79.71	0.78	100.99	17.33	79.71	3.35	100.39	99.4
HH-1	20.47	180.78	0.79	202.04	17.22	180.78	3.55	201.55	99.8
HH-2	20.08	178.12	0.78	198.98	17.13	178.12	2.85	198.10	99.6

[a]Includes dry straw and ash from the added molasses.
[b]Includes water added in the wheat straw.
[c]Includes the added yeast extract and dissolved solids in the added molasses.
[d]Final water and dissolved solids weights calculated by difference from the weight of the recovered liquid phase, assuming no net change in water content owing to microbial respiration and metabolic activity.
[e]Does not include the weight lost on opening the column, which was calculated by weight difference from the initial weights and final weights in the opened columns.
[f]Although recovered and included here, air leaked into column HL-2 during the course of the incubation; thus, this column did not remain at low O_2 and was dropped from the overall analysis.

from dissolution of water-soluble ash and extractables and from microbial solubilization of initially insoluble plant matter.

Although the initial mass of gas in the columns was negligible, all of the columns were slightly pressurized after cooling to room temperature, and a loss of mass from the columns was measured on opening them to the laboratory atmosphere. This indicates the release of gases and potentially water, ammonia, or organic vapors, which are included in the mass lost from the solid and liquid phases in Table 3. Any additional (unmeasured) loss of gases through opening of the pressure relief valves is accounted for in the 2% of the initial weight that was not recovered. Gases that could

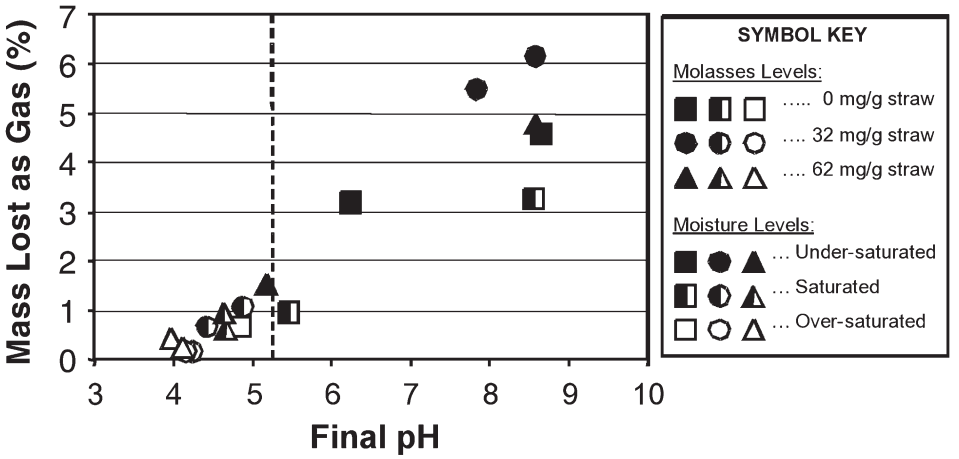

Fig. 2. Mass lost as gas from columns on opening, presented as percentage of initial column contents, as function of pH in bottom of columns. The dashed vertical line indicates the initial pH of 5.23.

have been generated include CO_2, H_2, and NH_3, and at higher pH values, small amounts of CH_4 *(19)*. Although the straw did not contain nitrates, NO, N_2O, and N_2 could also be generated in forages containing nitrates. The "vinegar" odors observed indicate accumulation of acetic acid, which has a significant vapor pressure and thus could also have been released from the columns. The weight lost on opening the columns was defined as a the mass lost as gas and is presented as a percentage of initial column contents vs final pH for all columns in Fig. 2; the dashed line indicates the initial pH of 5.23. Weight losses as gas generally increased as moisture decreased and as molasses decreased. Although there was some scatter in the data at higher pH, losses increased with increasing pH, indicating lower conversion of the DM and/or dissolved solids into gases when ensiling occurred. An average 4.7 ± 1.2% loss of matter as gas was observed above a final pH of 8.0, whereas an average 0.27 ± 0.12% loss as gas was observed as the final pH approached 4.0 (pH 3.97–4.25).

DM Balances

The 83-d losses of DM (as percentage of initial straw) are plotted vs final pH in Fig. 3; the dashed line indicates the initial pH of 5.23. DM losses generally increased as moisture decreased and as molasses decreased. The trend observed was to increasing DM losses as final pH increased, indicating a potential correlation among moisture, molasses, and pH attained. There was again scatter in the data, especially at higher pH. The increased scatter in the data at higher pH may be a reflection of continued aerobic activity owing to the approx 1% headspace O_2 levels. At lower pH values, the aerobic microbes would have been inhibited by both the low pH and the accumulation of acetic acid (observed as a "vinegar" odor) *(29)*.

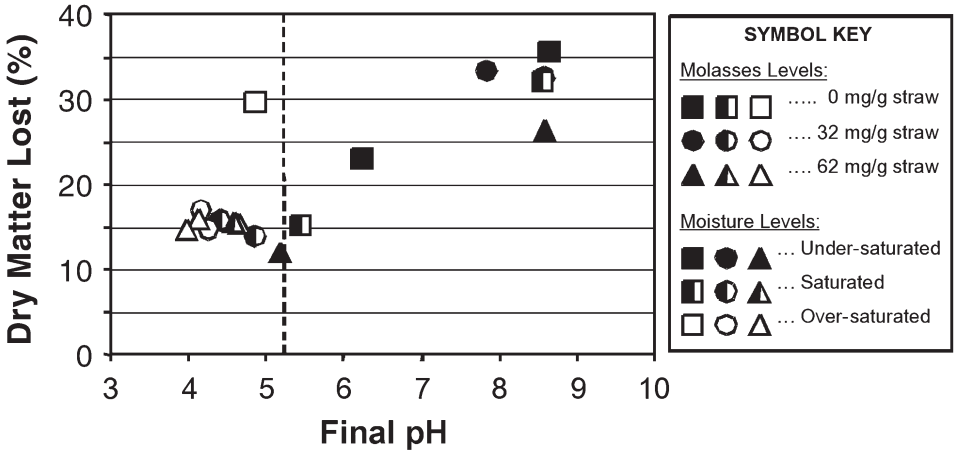

Fig. 3. DM lost from columns, presented as percentage of initial straw, as function of pH in bottom of columns. The dashed vertical line indicates the initial pH of 5.23.

Assuming that the pressure relief valves did not open and reseat identically in all of the columns, there would be different levels of headspace O_2 in the columns, leading to increased scatter in the DM loss data.

Average DM losses of 31.6 ± 3.8% were seen above pH 8.0. When the final pH of the columns reached the range of 4.0 to 5.0, the DM losses were all about 15% (15.3 ± 1.0%) save one data point at 30% lost (the duplicate column for this outlier, HL-2, significantly leaked air and was dropped from the data analysis). These data suggest that the 15% loss of DM observed in the columns that reached pH 4.0 to 5.0 may have derived primarily from dissolved ash and extractables, and at higher pH the increased losses may have included significant mass derived from additional plant components. The average increase in dissolved solids in columns that attained a pH <5.0 was 13.6 ± 4.2% of the initial DM, which supports this hypothesis.

Wheat Straw Component Balances

Table 4 gives the component mass recoveries on the DM in the columns. The DM component inputs included the wheat straw components, which includes plant polysaccharides, lignin, ash, and "other" organics (uronic acids, proteins, and so on), as well as ash from the molasses. Ash introduced in the molasses comprised 4.03 ± 0.14 and 7.53 ± 0.10% of the total ash in columns amended with 32.2 and 62.5 mg of molasses/g of straw, respectively. This amounted to 0.52 ± 0.02 and 1.00 ± 0.01% of the total straw in these columns, respectively. In most columns, seemingly significant amounts of plant polysaccharides, "other" organics, and ash were removed from the solid phase. Columns LM-1 and LM-2 showed 13.7 and 1.2% increases in ash content, respectively, which most likely indicates incomplete combustion of the organic fractions of the recovered material during ash determination.

Table 4
Wheat Straw Component Percentage Mass Recoveries for Test Columns

Column	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Mannan (%)[a]	Lignin (%)[b]	Ash (%)[c]	Other (%)[d]
LL-1	81.9	88.0	78.6	74.3	100.0	130.2	90.7	39.0
LL-2	92.5	87.9	50.0	77.1	100.0	139.4	94.3	33.4
ML-1	83.6	91.7	57.1	71.4	25.0	117.0	85.4	43.7
ML-2	101.7	105.7	92.9	91.4	50.0	131.1	76.3	49.8
HL-1	93.2	103.9	128.6	105.9	200.0	118.0	92.8	53.8
HL-2[e]	45.0	52.3	28.6	42.9	ND	63.3	42.7	43.1
LM-1	67.1	69.1	35.7	71.4	75.0	113.2	113.7	72.2
LM-2	67.9	71.1	14.3	42.9	25.0	111.7	101.2	69.5
MM-1	94.5	93.7	64.3	114.3	125.0	112.5	96.9	72.1
MM-2	97.7	94.4	93.3	69.4	25.0	108.0	88.1	93.5
HM-1	98.1	98.9	142.9	94.3	ND	108.4	89.5	92.8
HM-2	99.5	100.3	173.3	94.4	75.0	115.1	85.8	81.2
LH-1	91.4	95.4	42.9	77.1	50.0	111.3	96.6	83.0
LH-2	79.9	77.1	71.4	80.0	25.0	108.2	94.4	63.6
MH-1	94.8	99.4	107.1	82.9	ND	112.1	87.5	79.6
MH-2	94.8	98.0	135.7	108.6	75.0	114.6	84.0	86.0
HH-1	96.6	97.5	150.0	125.7	175.0	115.2	86.2	80.1
HH-2	91.3	94.2	42.9	91.4	ND	112.0	80.7	100.6

[a]ND, none detected (below the detection limit).
[b]Lignin represents Klason lignin and ethanol-benzene extractives.
[c]Ash includes the ash components of both the wheat straw and the molasses.
[d]Other organics, attributed to contents of uronic acid, protein, and so on and to recovery errors in carbohydrate analysis technique.
[e]Although recovered and included here, air leaked into column HL-2 during the course of the incubation; thus, this column did not remain at low O_2 and was dropped from the overall analysis.

Inspection of the data in Table 4 indicates possible increases in the measured Klason lignin with extractives component in nearly all columns. However, statistical analysis of the data indicates that these putative increases were within the 95% confidence intervals for the amounts of lignin input to the columns. Thus, statistically there were no changes in the lignin contents in the columns, which would be expected under conditions of low oxygen. The lone exception was column HL-2, which remained at high O_2 and showed a statistically significant 37% decrease in Klason lignin with extractives.

Cellulose

Cellulose contents were estimated as the total measured glucan. This estimate thus includes the relatively small fraction of glucose that originates from the hemicellulose. Figure 4 presents the cellulose losses from the columns (as percentage of initial cellulose); the dashed line indicates the initial pH of 5.23. There was significant scatter in the data at

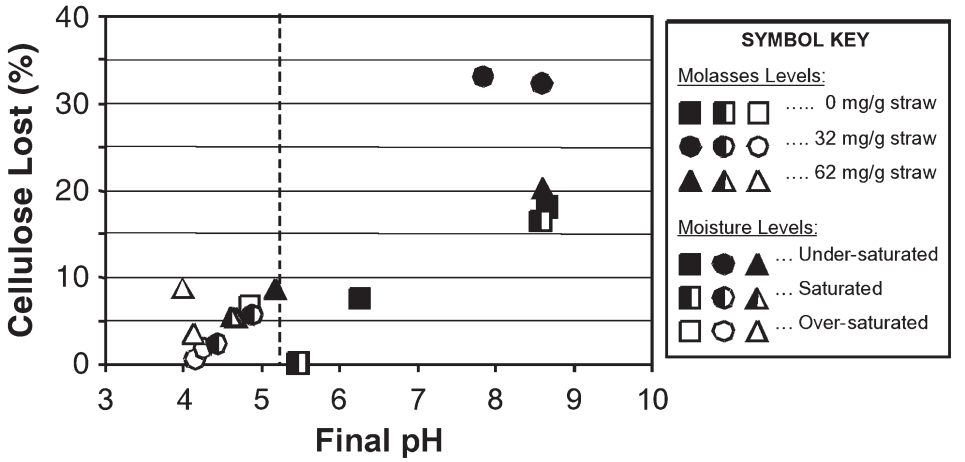

Fig. 4. Cellulose lost from columns, presented as percentage of initial cellulose, as function of pH in bottom of columns. The dashed vertical line indicates the initial pH of 5.23.

the highest pH values. As hypothesized for the DM losses, the increased scatter in the cellulose loss data at higher pH may have been owing to variations in the continued aerobic activity in these columns because of the approx 1% headspace O_2. The cellulose loss data below pH 5.0 show considerably less scatter, which suggests that in the presence of the low levels of headspace O_2 the low pH combined with the accumulation of acetic acid was sufficient to greatly reduce microbial degradation of the cellulose fraction of the straw. The reduced data scatter at lower pH can probably also be at least partially attributed to the presence of interstitial water because it would provide a diffusional barrier to O_2 penetration. However, the saturated and oversaturated columns lacking molasses also showed significant scatter in the data, which indicates that more than one parameter contributed to the variability.

As observed for the data on mass lost as gas and for the data on DM, cellulose losses generally increased as moisture decreased and as molasses decreased. In the undersaturated columns, in which only column LH-1 had a slight drop in pH, to 5.18, cellulose losses did not correlate with the addition of molasses, with columns receiving either level of molasses showing significant losses of cellulose. However, in both saturated and oversaturated columns, the addition of molasses directly resulted in lower cellulose losses. An average 21.7 ± 7.2% loss of cellulose was observed in columns attaining a final pH above 8.0. This is consistent with the DM balance and indicates higher degradation of the cellulose component of the wheat straw when ensiling did not occur. An average 3.4 ± 3.2% loss of cellulose was observed in the final pH range of 4.0–4.5.

Column ML-2 showed a 0% loss of cellulose at a final pH of 5.46, but its duplicate (column ML-1) showed a 16.4% loss of cellulose at a final pH

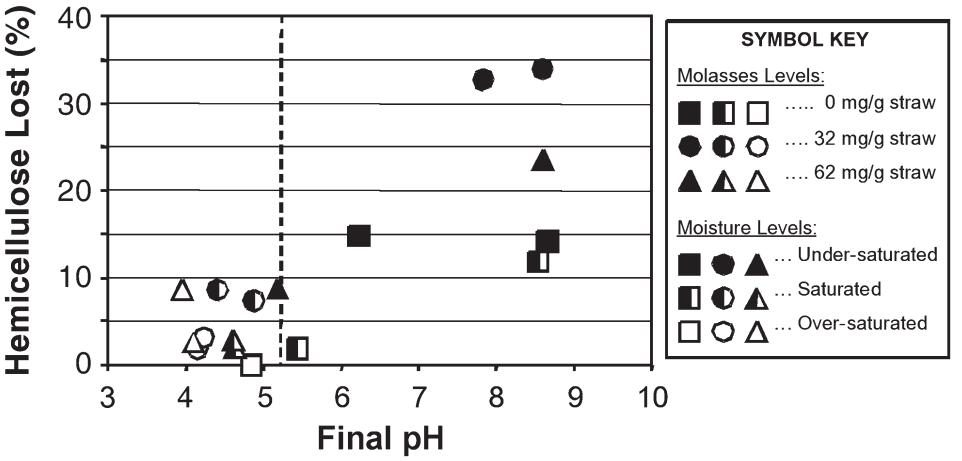

Fig. 5. Hemicellulose lost from columns, presented as percentage of initial hemicellulose, as function of pH in bottom of columns. The dashed vertical line indicates the initial pH of 5.23.

of 8.57. Because no molasses was added to these columns, *in situ* free glucose or sucrose remaining from added molasses could not have contributed to the excess measured glucose. In addition, any free glucose initially in the straw would have been accounted for in the initial cellulose content. The widely different final pH values in these columns clearly indicate that each took a different path regarding the biochemistry that occurred during their incubations. However, whether the lack of cellulose loss in column ML-2 was owing to a measurement error or some other factor cannot be determined from the available information. Taken as a whole, the overall trend of cellulose loss with pH indicates that this result can probably be attributed to a measurement error.

Hemicellulose

Hemicellulose contents were estimated as the sums of the nonglucan anhydro-sugars in the straw, including the xylan, galactan, arabinan, and mannan, measured in the compositional analyses. This estimate neglects glucose originating from the hemicellulose fraction (included in the cellulose fraction), as well as the unmeasured uronic acids. Figure 5 presents the hemicellulose losses from the columns (as a percentage of initial hemicellulose); the dashed line indicates the initial pH of 5.23. There was again significant scatter in the data at higher final pH values. The data on hemicellulose loss were completely consistent with both the data on DM and cellulose loss; as observed for the other component losses, hemicellulose losses generally increased as moisture decreased and molasses decreased. An average 20.8 ± 10.1% hemicellulose loss was observed in columns with final pH values >8.0, whereas losses in the pH range of 4.0–4.5 averaged 5.0 ± 3.4%. The trends of cellulose and hemicellulose losses with pH clearly

indicate that failure to attain a final pH near 4.0 in the feedstock would result in significant losses of fermentable sugar yield from a wet biorefinery feedstock.

Ash

The ash contents were measured by ashing the straw samples to constant weight at 650ºC. It was expected that contacting the straw with moisture would lower the ash content of the ensiled straw through dissolution of inorganic salts, with potentially higher removals as the moisture content increased. Generally, higher ash removals were observed at lower pH values, although there was significant scatter in the data (not shown).

Other Organics

The "other organics" component was calculated as the difference between the measured components and 100% in the compositional analysis; these values also contain the recovery errors in the compositional analysis method. It was expected that contacting the straw with increasingly higher moisture would dissolve increasingly more uronic acids, proteins, and other water-soluble organic components from the straw. However, the highest removals of the other organics component (>50%) occurred in columns at undersaturated or saturated conditions (columns LL-1, LL-2, ML-1, and ML-2). These columns had significant visible fungi, although few of the fungi must have been culturable because few were cultured in the microbial analysis (Table 2). It is also possible that the low fungal counts could reflect the technique used to process the column contents (the fungi may have stayed associated with the solid phase or may not have survived the homogenization for some reason). These columns seemed to ammonify (based on an observed "barnyard" odor), which suggests that there could have been increased microbial degradation of the other organics component (which would include proteins). A trend that was generally similar to those seen with DM, cellulose, and hemicellulose was seen for the other organics component, namely that of increased losses with increased pH (not shown). However, the data were significantly scattered.

Implications for the Use of Ensiling for Storage of Biorefinery Feedstocks

The objectives of these tests were to estimate levels of moisture and free sugars necessary to attain a final pH of 4.0 in the presence of a population of active lactic acid bacteria, to determine whether under these conditions the microbial community resembled and/or behaved similarly to that in green forage silage, and to determine the degree of stabilization of the cellulose and hemicellulose compositions of the straw under these conditions. Losses of DM, cellulose, and hemicellulose were the lowest when the final pH approached 4.0, with losses of both cellulose and hemicellulose observed to

be <5%. Although the columns did not attain strict anaerobic conditions, there was a pattern of decreasing losses of fermentable sugars with decreasing pH. Thus, the fermentable sugar losses probably more closely approximate the expected yield losses nearer to the surface of a silage pile at the boundary between oxic and anoxic conditions. Hence, the results indicate that ensiling should be considered a promising alternate method for wet storage of biorefinery feedstocks that are harvested after they have senesced.

In the tests, we varied the addition of free sugar from 0 to 3 wt% (as sucrose equivalents) and moisture content from undersaturated to oversaturated levels. Addition of free sugars was found to be absolutely critical for the successful ensiling of the wheat straw tested, primarily because the *in situ* free sugar content was too low to provide sufficient carbon for rapid organic acid production. The 3 wt% sucrose equivalent level used in the bagasse industry was sufficient to reach a pH of 4.0 in the wheat straw, but addition at the 1.5 wt% level was not. Moisture was also found to be critically important to achieving ensiling and stabilization of the polysaccharides, with interstitial water being necessary for the best ensiling (lowest pH) and thus the best stabilization of polysaccharides. Many of the water-saturated columns ensiled nearly as well as the oversaturated columns but none of the undersaturated columns ensiled. This indicates that the critical parameter is not moisture content but degree of saturation.

The results indicated that the ensiling process that occurred in the senesced wheat straw was similar to that which occurs in silage made from green forages, but only if the straw was maintained at water saturation or above and if sufficient free sugars were added to allow a rapid drop in pH to 4.0. When these conditions were met, the microbial community behaved in a manner similar to that described for green forage silage *(12–14)*. The primary differences between ensiling of senesced residues and green forage ensiling are, then as follows:

- The metabolically dominant microbial population in the material at the time of ensiling is fungi, and the indigenous lactic acid bacteria are dormant (not metabolically active).
- Sufficient *in situ* free sugars may not be available to stimulate the indigenous lactic acid bacteria and provide sufficient carbon for rapid and sustained lactic and acetic acid production.
- The initial water content is too low.

Compared to green forages, senesced residues have lower microbial numbers and are dominated by microbes that are detrimental to the stable long-term storage of the biomass *(15)*. Although present and perhaps previously active for a time, the necessary Lactobacilli and lactic acid cocci were not dominant and were not in an environment that was conducive to their activity and, hence, were in a state of low metabolic activity. In addition, the level of free sugars, required to stimulate their activity, was too low owing primarily to microbial consumption soon after senescence.

Although not specifically tested in this study, it is also likely that the addition of the *L. plantarum* inoculant assisted in rapidly consuming the initial O_2 and lowering the pH, because this bacterium is homofermentative and was metabolically active when introduced into the straw. Under these circumstances, the inoculant would be expected to rapidly convert the sugars in the molasses almost exclusively into lactic acid, thereby causing a rapid drop in pH. Having established the necessary environment, the epiphytic lactic acid bacteria then would grow and produce acetic and lactic acids, inhibiting the detrimental bacteria and fungi more quickly than would likely be seen in an uninoculated system. Although no uninoculated columns were prepared as part of this study, we have seen significant improvement in both the lowest attainable pH and the stabilization of the plant polysaccharides in senesced wheat straw with the addition of the *L. plantarum* inoculant.

Conclusion

Ensiling should be considered a promising alternate method for storage of wet biorefinery feedstocks that have senesced before they are harvested. There was a pattern of decreasing losses of fermentable sugars with decreasing pH. Losses of cellulose and hemicellulose <5% were achieved when the final pH was near 4.0. The ability to attain this final pH was strongly influenced by the maintenance of interstitial water in the straw and by the addition of sufficient free sugars. When these conditions were provided, the ensiling process that occurred was similar to that which occurs in green forage silage, with three primary differences. In senesced straw, the microbial community was metabolically dominated by fungi, and the necessary lactic acid bacteria were metabolically dormant. This, combined with insufficient *in situ* free sugars and a lack of interstitial water, led to significant losses of DM, cellulose, and hemicellulose when too little molasses or water was added. The results were also likely improved by the addition of a metabolically active homofermentative lactobacillus. Thus, amendment of senesced biomass at the time of ensiling with saturating or oversaturating amounts of water, sufficient free sugars, and an inoculant of a homofermentative lactic acid bacterium would significantly reduce shrinkage of the yield of fermentable sugars.

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