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Ensiling Agricultural Residues for Bioethanol Production

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Abstract The potential of using ensiling, with and without supplemental enzymes, as a cost-effective pretreatment for bioethanol production from agricultural residues was investigated. Ensiling did not significantly affect the lignin content of barley straw, cotton stalk, and triticale hay ensiled without enzyme, but slightly increased the lignin content in triticale straw, wheat straw, and triticale hay ensiled with enzyme. The holocellulose (cellulose plus hemicellulose) losses in the feedstocks, as a result of ensiling, ranged from 1.31 to 9.93%. The percent holocellulose loss in hays during ensiling was lower than in straws and stalks. Ensiling of barley, triticale, wheat straws, and cotton stalk significantly increased the conversion of holocellulose to sugars during subsequent hydrolysis with two enzyme combinations. Enzymatic hydrolysis of ensiled and untreated feedstocks by Celluclast 1.5 L-Novozyme 188 enzyme combination resulted in equal or higher saccharification than with Spezyme® CP–xylanase combination. Enzyme loadings of 40 and 60 FPU/g reducing sugars gave similar sugar yields. The percent saccharification with Celluclast 1.5 L-Novozyme 188 at 40 FPU/g reducing sugars was 17.1 to 43.6%, 22.4 to 46.9%, and 23.2 to 32.2% for untreated feedstocks, feedstocks ensiled with, and without enzymes, respectively. Fermentation of the hydrolysates from ensiled feedstocks resulted in ethanol yields ranging from 0.21 to 0.28 g/g reducing sugars.

Keywords Carbohydrate · Ensiling · Enzyme · Feedstocks · Fermentation · Hydrolysis · Lignin · Pretreatment

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Introduction

A growing economy, increasing population, and rising standard of living have put tremendous pressure on United States energy resources. It has been projected that the US energy consumption will increase by about 32% by 2020 [1]. To meet the growing demand for energy and replace diminishing fossil fuels, it is necessary to explore the millions of tons of crop residues such as straws, stalks, and cobs that are generated as agricultural byproducts each year [2]. Currently, a very limited amount of lignocellulosic feedstock is effectively utilized for ethanol production while most of it is disposed as waste, causing environmental problems such as air pollution and global warming. One promising technology to reduce agricultural wastes is to convert this abundant and renewable biomass to ethanol through an enzyme-based process [3]. However, effective conversion of lignocellulose into ethanol is challenging due to the complex structure of the plant cell wall. Thus, pretreatment is required to alter the structural and chemical composition of lignocellulosic biomass to facilitate rapid and efficient hydrolysis of carbohydrates to fermentable sugars [4].

Pretreatment of lignocellulosic feedstocks can be carried out physically (mechanical comminution, pyrolysis), chemically (dilute acid, alkaline pretreatment), physicochemically (steam explosion), and biologically (fungal delignification) [5]. These methods open up the lignocellulosic multicomponent matrix and render the carbohydrate components more accessible to hydrolytic enzymes [6]. Typically, hydrolysis yields in the absence of pretreatment are less than 20% of theoretical yields, whereas yields after pretreatment often exceed 90% of the theoretical yields [5]. However, because of the need for high energy, chemicals, and corrosion-resistant, high-pressure reactors, pretreatment is one of the most expensive steps in cellulosic ethanol production, accounting for 33% of total processing costs in the base-case National Renewable Energy Laboratory (NREL) design [7, 8]. Moreover, production of toxic substances (furfurals and various lignin-related phenolics) during conventional pretreatments has been reported to inhibit the fermentation step [9, 10]. Inclusion of detoxification steps to inactivate or remove toxic substances before fermentation significantly increases the overall capital cost of pretreatment. There is therefore a need to explore low-cost alternatives to traditional pretreatment techniques.

Ensiling is a forage crop preservation method which has been in use for centuries and is a common practice even in modern agriculture [11]. It is a natural choice for the stable storage of feedstocks. During ensiling, the low pH caused by fermentation of free sugars inhibits microbes that decompose polysaccharides, therefore effectively minimizing the degradation of carbohydrates in the feedstocks [12]. The acidic environment produced by ensiling also serves as a pretreatment that can result in enhanced yields of reducing sugars [13]. Linden et al. [14] reported that ensiling fresh sorghum resulted in hydrolysis of 70% cellulose to fermentable sugars. The enhancement in hydrolysis can be attributed to the solubilization of hemicellulose by acids produced during ensiling, which may change the composition of cell wall constituents in feedstocks [15]. Compared with more commonly used pretreatment methods, the ensiling process involves lower capital investment and energy input and produces fewer substances that may be inhibitory to the subsequent hydrolysis and fermentation processes.

While ensilage has considerable potential as a cost-effective pretreatment method, processing strategies have not been established for various lignocellulosic feedstocks such as crop residues and forage hays. Therefore, this study was undertaken to (1) determine the chemical compositions of ensiled cotton stalks, wheat, barley, and triticale straws (residue after grain harvesting), and triticale hay (harvested when crops are green); (2) investigate

suitable ensiling pretreatment conditions for different feedstocks; (3) compare hydrolysis efficiency of ensiled and untreated feedstocks; and (4) determine ethanol yield using selected yeast fermentation.

Materials and Methods

Feedstocks Triticale hay and straw samples used for this study were obtained from a cropping system study that compared biomass production potentials of an irrigated single cropping to a double cropping system on a producer's land in Central Montana. Wheat and barley straw samples were collected from the nearby fields at the Central Agricultural Research Center of Montana State University. The fields were under typical dryland single cropping production practices. Cotton stalks, harvested in early October 2003, were obtained from Cunningham Research Station in Kinston, NC, USA. The stalks were shredded and bailed in the field soon after the cotton was picked.

The feedstocks (cotton stalks, triticale hay, barley, triticale, and wheat straws) were chopped, oven dried, and ground to pass a 1-mm sieve in a Thomas Wiley Laboratory Mill (Model No. 4, Thomas Scientific, Philadelphia, PA, USA) for composition analysis. The bulk densities of barley straw, cotton stalk, triticale hay, triticale straw, and wheat straw were found to be 151.2, 188.8, 166.1, 143.2, and 119.6 kg/m^3, respectively [16]. Moisture content of the feedstocks ranged from 4.5 to 7.7%. Table 1 shows the proximate composition of the various feedstocks. Dried samples were stored in sealed plastic bags (approximately 500 g per bag without vacuum) at room temperature until use. The feedstock moisture contents were low enough to prevent preensiling of biomass.

Ensiling of Feedstocks To test the effect of enzymes on ensiling, each chopped feedstock was split into two subsamples and water was added to the dry feedstocks to bring the moisture content to 60%. An additive for corn silage called SI-LO-FAME 500 CS (BioTechnologies, Eagle Grove, IA, USA), which contains hemicellulase (851 U/g), fungal

Table 1 Summative percent composition of untreated and ensiled feedstocks.

Feedstocks	Condition	DNS sugars	ASL	AIL	Ash
Barley straw	Untreated	58.6 (2.10) A	2.18 (0.04) A	25.4 (1.35) A	5.26 (0.08) A
	With enzyme	50.9 (1.04) B	2.56 (0.25) A	24.9 (0.43) A	6.52 (1.13) A
	Without enzyme	52.4 (2.70) B	2.22 (0.06) A	23.7 (0.42) A	6.76 (0.86) A
Triticale straw	Untreated	59.8 (0.56) A	2.07 (0.13) B	23.0 (0.32) B	8.23 (0.08) A
	With enzyme	49.8 (0.85) B	2.71 (0.16) B	26.7 (0.15) A	8.16 (1.66) A
	Without enzyme	50.2 (2.91) B	3.53 (0.09) A	26.0 (0.70) A	7.88 (1.32) A
Wheat straw	Untreated	62.5 (1.26) A	2.22 (0.04) A	23.2 (0.24) B	3.64 (0.03) A
	With enzyme	53.8 (1.47) B	2.63 (0.13) A	26.5 (0.58) A	3.05 (0.18) B
	Without enzyme	54.6 (1.92) B	2.43 (0.11) A	26.7 (0.58) A	2.51 (0.39) B
cotton stalk	Untreated	51.2 (2.01) A	2.04 (0.31) A	33.3 (2.01) A	6.51 (0.05) A
	With enzyme	47.1 (1.28) B	1.69 (0.01) A	36.9 (1.97) A	6.84 (0.51) A
	Without enzyme	46.6 (1.04) B	2.01 (0.30) A	36.4 (0.51) A	6.79 (0.14) A
Triticale hay	Untreated	54.3 (1.90) A	3.96 (0.18) A	22.1 (0.43) B	9.62 (0.38) B
	With enzyme	50.5 (1.25) B	3.17 (0.71) A	26.4 (1.22) A	6.63 (0.41) A
	Without enzyme	53.0 (1.32) A	3.53 (0.15) A	24.93 (0.19) B	5.46 (0.47) A

Values in parentheses are standard deviations. For each feedstock, values in columns followed by the same capital letter are not significantly different ($p>0.05$).

alpha-amylase (7,023 U/g), bacterial alpha-amylase (8,087 U/g), and cellulase (1,596 U/g) was added at the suggested rate of 10 U/g dry cellulosic feedstock to one subsample from each of the five feedstocks [17]. The additive was sprinkled at a rate of 0.0038 g/g wet silage and mixed thoroughly in a bucket. The feedstocks were then packed into quart size canning jars. The lids of the jars were heated to aid in creating a seal and the jars were stored at room temperature for 96 days.

Enzymatic Hydrolysis Celluclast 1.5 L produced by *Trichoderma reesei* and Novozyme 188 produced by *Aspergillus niger* were purchased from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Spezyme® CP and Multifect® xylanase of *T. reesei* origin were obtained from Genencor International Inc. (Palo Alto, CA, USA). The enzymatic activities, optimum pH, and optimum temperature of the various enzymes are summarized in Chen et al. [18].

Enzymatic hydrolysis of 1 g (dry weight) ensiled and untreated samples was performed in 250 mL Erlenmeyer flasks. Wet, ensiled samples and untreated samples were mixed with 30 mL citrate buffer (pH 4.8, 50 mM) containing 40 μg/mL tetracycline hydrochloride (added to avoid microbial contamination). Sample flasks were preincubated in a shaking water bath at 55°C and 150 rpm for 10 min before addition of enzymes. Two sets of enzymes were used for hydrolysis experiments: Celluclast supplemented with Novozyme 188 at a ratio of 1:1.75 (*v*/*v*, C/C) and Spezyme® CP supplemented with Multifect® xylanase (S/X) at the same ratio. The enzymatic hydrolysis was performed at two enzyme loadings, 40 and 60 FPU/g reducing sugars for 72 h. Control samples which did not contain hydrolytic enzymes were incubated under similar conditions to determine the background sugar concentrations in untreated and ensiled feedstocks. Two-milliliter aliquots of hydrolysates were taken at the termination of enzymatic hydrolysis, immediately chilled on ice, and centrifuged at 5,000×*g* for 10 min. The supernatant was analyzed for sugar content. Percent saccharification was calculated as a ratio of reducing sugars in hydrolysate supernatant and holocellulose (cellulose plus hemicellulose derived sugars) in untreated or ensiled samples.

Fermentation Saccharomyces Cerevisiae (ATCC 24859) used for hydrolysate fermentation was obtained from the Microbiological Engineering Laboratory in the Department of Agricultural and Biological Engineering at Pennsylvania State University. The yeast strain was grown at 30±1°C in 100 mL medium containing 20 g glucose, 8.5 g yeast extract, 1.32 g NH_4Cl, 0.11 g $MgSO_4$, and 0.06 g $CaCl_2$, per liter of deionized water. The culture was allowed to grow under aerobic condition in a shaker incubator (150 rpm) for 24 h. Cells were harvested by centrifugation at 5,000×*g* at 4°C for 10 min and washed three times with 0.1% peptone water to remove excess media and resuspended in 30 mL peptone before use. One-milliliter liquid sample was taken to determine the dry matter (%) of the inoculum by weighing it before and after drying at 105°C [19]. Dry matter (%) content was then used to determine the volume of yeast used to inoculate the hydrolysate at a cell concentration of 10 g dry matter/liter [20].

Hydrolysates, from hydrolysis treatments identified as optimal on the basis of reducing sugar recovery, were centrifuged at 5,000×*g* for 10 min. Twenty milliliter of supernatant was transferred to 100 mL serum bottles and pH was adjusted to 7.0 by adding 2 N NaOH before inoculation with *S. cerevisiae*. All samples were then incubated in airtight serum bottles at 30±1°C for 72 h and analyzed for ethanol content at the end of fermentation.

Analysis Methods The total solids, acid soluble lignin (ASL), and acid insoluble lignin (AIL) content of the untreated and ensiled feedstocks were determined by Laboratory Analytical Procedures from the NREL [21–23]. Total solids were determined by weighing

the feedstocks before and after drying in a convection oven at 105°C. To measure lignin, extractive free feedstock samples were sequentially treated with sulfuric acid at 72 and 4% (*w*/*w*) acid followed by autoclaving for 60 min at 121°C. The recovered solids were ashed at 550°C for AIL determination and the liquid phase was used to determine ASL through absorbance measurements at 205 nm. Ash content for the feedstocks was determined gravimetrically by placing the feedstocks in a muffle furnace at 550°C overnight based on a method developed by Han and Rowell [24]. The holocellulose content of untreated and ensiled feedstocks was determined by measuring reducing sugar with the 3, 5-dinitrosalicylic acid (DNS) assay [25]. Three milliliters of the DNS reagent was mixed with 500-μL sample and boiled at 100°C for 5 min. The mixture was quickly cooled in an ice bath and the absorbance measured at 540 nm. A standard curve was prepared with α-D-glucose at various concentrations. Reducing sugars in the hydrolysates from enzymatic hydrolysis were also determined by DNS assay.

Ethanol content in the fermentation broth was analyzed by an enzymatic assay [26]. All samples were centrifuged at 5,000×*g* for 10 min before analysis. The ethanol yield was determined both as gram of ethanol produced per gram of reducing sugars present in hydrolysates and gram of ethanol per gram of ensiled feedstock. Ethanol standards (0–5 mM) and fermented samples were mixed with 1-mL standard solution containing glycine, hydrazine sulfate, Na_2EDTA, NaOH, β-nicotinamide adenine dinucleotide, and alcohol dehydrogenase. The mixture was incubated at 37°C for 1 h and absorbance measured at 340 nm using a spectrophotometer (UV-1700, Shimadzu, Columbia, MD, USA).

Data Analysis All treatments were conducted in triplicate. Differences between treatments were evaluated by performing Tukey simultaneous tests under PROC MEANS in SAS (version 8.0, SAS Institute Inc., Cary, NC, USA) at a 95% confidence level.

Results and Discussions

Effect of Ensiling on Feedstock Characteristics

Untreated and ensiled (with and without enzymes) feedstocks were characterized for holocellulose, lignin, and ash content and the compositions are shown in Table 1. The holocellulose content of feedstocks indicated by the DNS reducing sugar contents was significantly higher in the untreated feedstocks than in ensiled feedstocks ($P<0.05$). Holocellulose losses ranging from 1.31 to 9.93% were observed as a result of the ensiling process. The loss of holocellulose can be attributed to the preferential degradation of cellulose and hemicellulose by the ensiling microflora [12]. Percent holocellulose loss in straws and stalks decreased with the increase in feedstock lignin content (Fig. 1), indicating that the presence of lignin protects some of the carbohydrate in plant residues from microbial attack and decay [27].

The percent holocellulose loss in triticale hay during ensiling was however significantly lower ($p<0.05$), possibly due to variation in feedstock composition. Hays are premature forage crops that typically contain significant amount of nonfiber carbohydrates such as starch, simple sugars like glucose or fructose held together by alpha chemical bonds, and pectin that are easily accessible to microbes and enzymes [28]. In the early stage of ensiling of green forage, lactic acid bacteria grow along with aerobic microbes including yeast and molds [29]. After the depletion of oxygen from interstitial voids, the available free sugars in green forages are fermented into lactic and acetic acids under anaerobic conditions, causing

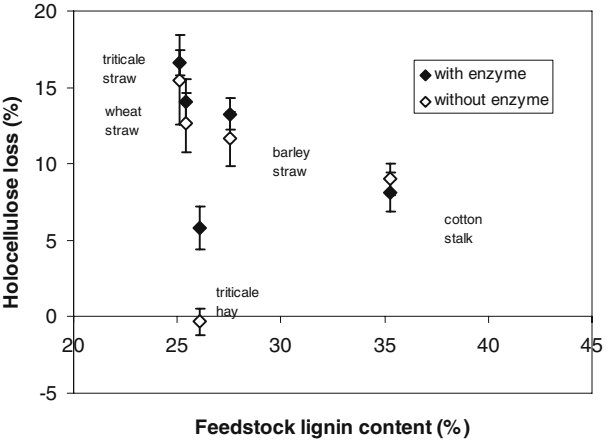

Fig. 1 Correlation of feedstock lignin content with loss of holocellulose during ensiling

rapid decrease in pH of the amendment solution [30]. While the lactic acid bacteria flourish and become dominant due to their better tolerance to low pH [31], other microbes that decompose plant polysaccharides are inhibited at this low pH and holocellulose degradation is greatly reduced [12]. Straws and stalks, on the other hand, are senesced; extremely fibrous residues left after crops have been harvested. During their maturation and field drying, the free sugars are largely consumed, and the in situ free sugar contained in the feedstock is usually too low to provide sufficient substrate for rapid organic acid production at the initiation of ensiling [32]. Hence, in the ensiling of straws and cotton stalks (with and without enzymes) which contain limited amount of free sugar, significant degradation of accessible structural carbohydrates could have been carried out by the active microorganisms that were not inhibited by the inadequate drop in pH. The lowest pH values of 3.7 and 4.0 were obtained during ensiling of triticale hay with and without enzymes, respectively, while the pH values during ensiling of senesced crop residues were significantly ($p<0.05$) higher (Table 2). The variation in pH supports the above hypothesis that during feedstock ensiling, availability of free sugars affects the level of acidification attained, which in turn correlates with the loss of holocellulose. Results of this study are consistent with those obtained by Thompson et al. [12], who observed that addition of molasses (a mixture of sucrose, fructose, glucose, and dextrose) decreased cellulose and hemicellulose loss by 34.1% during ensiling of wheat straw. Richard et al. [33] also observed that acid production was lower during ensiling of corn stover (<3%) than green plant materials (5–15%).

Table 2 pH values of amendment solution after ensiling of feedstocks.

Feedstocks	With enzyme	Without enzyme
Barley straw	4.2 (0.06)	4.3 (0.06)
Triticale straw	5.0 (0.02)	5.3 (0.04)
Wheat straw	5.0 (0.07)	5.0 (0.33)
Cotton stalk	5.3 (0.04)	5.3 (0.19)
Triticale hay	3.7 (0.02)	4.0 (0.06)

Values in parentheses are standard deviations.

To promote in situ hydrolysis of feedstocks, an additive for corn silage which contains hemicellulase, fungal and bacterial alpha-amylase, and cellulase was added to samples during ensiling. Henk and Linden [17] reported a maximum reducing sugar yield at 10 U/g dry matter and obtained 250 g reducing sugar per kilogram of dry matter in 18 days compared to 50 g reducing sugar per kilogram of dry matter in the control with 0 U/g. In this study, however, except for triticale hay, no statistically significant difference ($p<0.05$) was observed between the holocellulose content in feedstocks ensiled with enzyme and those without enzyme (Table 1). It is speculated that supplemental enzymes could not access the hard-to-digest portion of straws and cotton stalks and were unable to further increase the release of sugars [17]. For triticale hay, addition of enzymes increased holocellulose loss by 2.52% ($p<0.05$), a result that could be attributed to the ability of enzymes to access the easily digestible holocellulose in immature feedstocks and release more sugars.

The ensiling process did not significantly affect ($p>0.05$) the lignin content of barley straw, cotton stalk, and triticale hay ensiled without enzyme, but slightly increased ($p<0.05$) the lignin content in triticale straw, wheat straw, and triticale hay ensiled with enzyme. The apparent increase in lignin content could be a result of repolymerization of the lignin–protein complex. When treated with concentrated sulfuric acid, proteins in ensiled feedstocks originating from ensiling microflora and supplemental enzymes can condense and become insoluble [34]. It is speculated that combination of lignin and condensed proteins increased acid-insoluble lignin content of the ensiled feedstocks [35]. Although lignin biodegradation is extremely slow under strictly anaerobic conditions [36], presence of oxygen during the initial stage of ensiling may result in lignin degradation [12]. Due to the lack of practical and cost-effective field-scale ensiling methods [12], no particular efforts were made in this study to completely exclude oxygen except for filling up the silos with ensiled feedstocks. Hence, the impact of presence of oxygen on delignification could not be quantified.

Enzyme kinetics during ensiling were not examined in this study. However, Linden et al. [37] found that enzyme activity lasted about 32 days after which the reducing sugar yield reduced. The reason for reduction in enzymatic conversion is not clear but it was observed that the sugar content remained at a constant level up to 155 days. Therefore, enzyme-assisted-ensiling not only serves as a method for in situ pretreatment but it also serves as an ideal storage method for feedstock.

Enzymatic Hydrolysis of Ensiled and Untreated Feedstocks

Effect of Ensiling and Enzymes Addition Ensiling of barley, triticale and wheat straws, and cotton stalk had a significant ($p<0.05$) impact on the conversion of holocellulose to sugars. Ensiled straws and cotton stalks (hydrolyzed with C/C) released more sugars ($p<0.05$) than untreated feedstocks. Depending on feedstock, ensilage improved saccharification by approximately 5.2–9.3%. Addition of enzymes in the silage did not have a significant ($p<0.05$) impact on percent saccharification during subsequent hydrolysis of straws and stalks (Table 3), possibly due to the removal of easily accessible polysaccharides by undesirable fermentative microorganisms during ensiling. It is widely accepted that straws are rarely used directly for ensiling due to the low content of soluble carbohydrates [15, 33, 38]. However, degradation of lignocellulose in the straws could produce a slightly acidic environment (Table 2). Although the pH drop may not be significant to inhibit most deleterious bacteria and fungi and preserve potential fermentable carbohydrates [31], it could serve as a pretreatment, resulting in enhanced saccharification when the feedstocks are enzymatically hydrolyzed [13]. Linden et al. [14] studied the hydrolysis of forage

Table 3 Enzymatic hydrolysis of untreated and ensiled biomass.

Feedstock	Enzymes	Enzyme load (FPU/g reducing sugar)	Saccharification (%)[a]		
			Untreated	Ensiled with enzyme	Ensiled without enzyme
Barley straw	Control	0	0.0 (0.00) B, b	9.9 (0.37) B, a	5.2 (0.30) B, a
	C/C	40	23.5 (0.80) A, b	31.5 (2.01) A, a	32.2 (2.52) A, a
	C/C	60	23.2 (1.50) A, b	33.6 (0.93) A, a	31.1 (1.91) A, a
	S/X	40	23.4 (1.85) A, b	33.0 (0.56) A, a	30. 9 (1.38) A, a
	S/X	60	20.6 (1.51) A, b	33.0 (1.33) A, a	33.7 (1.45) A, a
Triticale straw	Control	0	0.0 (0.00) C, b	6.3 (0.39) C, a	7.2 (0.16) B, a
	C/C	40	23.8 (0.89) A, b	33.1 (0.46) A, a	30.8 (2.53) A, a
	C/C	60	24.2 (0.47) A, b	32.9 (0.54) A, a	33.1 (1.07) A, a
	S/X	40	19.4 (0.31) B, b	28.6 (1.19) B, a	29.2 (1.18) A, a
	S/X	60	20.6 (0.57) B, b	30.7 (2.43) B, a	29.3 (2.84) A, a
Wheat straw	Control	0	0.0 (0.00) C, b	1.4 (0.19) C, a	1.3 (0.05) C, a
	C/C	40	19.6 (1.22) A, b	26.7 (0.80) A, a	27.7 (1.54) AB, a
	C/C	60	19.2 (0.55) A, b	27.9 (1.32) A, a	29.3 (1.98) A, a
	S/X	40	18.2 (0.57) A, b	22.4 (0.97) B, a	23.5 (0.28) B, a
	S/X	60	17.9 (1.10) A, b	23.4 (0.75) B, a	25.4 (1.42) B, a
Cotton stalk	Control	0	0.0 (0.00) C, b	1.9 (0.07) C, a	2.3 (0.28) C, a
	C/C	40	17.1 (0.74) A, b	22.4 (2.27) A, a	23.2 (3.54) A, a
	C/C	60	19.1 (1.35) A, b	24.4 (0.83) A, a	24.2 (2.05) A, a
	S/X	40	11.8 (1.25) B, a	14.1 (0.90) B, a	13.6 (0.99) B, a
	S/X	60	13.2 (1.20) B, a	14.5 (1.10) B, a	14.4 (0.24) B, a
Triticale hay	Control	0	4.1 (0.16) C, b	11.2 (0.73) C, a	4.2 (0.56) C, b
	C/C	40	43.6 (0.72) A, a	46.9 (3.06) A, a	27.5 (2.45) A, b
	C/C	60	42.4 (1.95) A, a	49.9 (3.38) A, a	26.3 (2.34) A, b
	S/X	40	36.3 (0.65) B, a	32.1 (2.42) B, a	19.9 (1.49) B, b
	S/X	60	36.6 (2.47) B, a	34.1 (3.75) B, a	22.6 (3.29) B, b

Values in parentheses are standard deviations.

[a] For each feedstock, values in columns followed by the same capital letter are not statistically different ($p>0.05$). Values in rows followed by the same lowercase letter are not statistically different ($p>0.05$).

sorghums and indicated that ensiled sorghum resulted in 70% conversion of cellulose to fermentable sugars during subsequent enzymatic hydrolysis, in contrast with low conversion from unensiled fresh forage crops.

There was no significant difference ($p>0.05$) between percent saccharification of untreated and ensiled (with enzyme) triticale hay. It is expected that untreated hay contained more carbohydrates than ensiled hay due to the absence of enzymes and carbohydrate-consuming microorganisms. Hence, in spite of the difficulty in accessing the holocellulose, relatively more sugar was available for saccharification. The presence of significantly high amounts of free sugars in untreated hay compared to other feedstocks was apparent from the high level of reducing sugars in the hydrolysate from triticale hay control (approximately 0.02 g/g dry biomass), while sugars in controls from other untreated feedstocks were undetectable.

Enzymatic hydrolysis of triticale hay ensiled with enzymes released approximately 20% more sugars than samples ensiled without enzyme. This may be attributed to the action of supplemental enzymes such as cellulase, hemicellulase, and amylases during ensiling which

may loosen the polysaccharides matrix surrounding the cellulose microfibril by weakening the hydrogen bonds between matrix components [39]. The cellulose therefore became more accessible to hydrolytic enzyme activity [17]. Improved accessibility may have compensated for the lower amount of available carbohydrates in triticale hay ensiled with enzymes, resulting in higher holocellulose conversion. In addition, lower pH (3.7) of the amendment solution during ensiling with enzyme addition served as a more effective acid pretreatment than that without enzyme (pH 4) and further enhanced the subsequent enzymatic hydrolysis. The low level of percent saccharification in triticale hay ensiled without enzymes was likely due to the absence of supplemental enzymes during ensiling and poor accessibility to holocellulose during hydrolysis.

Effect of Enzyme Combination The percentages of saccharification during hydrolysis of untreated and ensiled feedstocks are reported in Table 3. For all feedstocks, samples containing hydrolytic enzymes (C/C or S/X) released significantly more sugars than the controls ($p<0.05$), indicating low levels of freely available sugars in the untreated or ensiled controls. Statistical analysis showed that enzyme combination C/C resulted in equal or higher saccharification than the combination of S/X. The overall percent saccharifications for untreated and ensiled feedstocks hydrolyzed with C/C were 17.1–43.6% and 22.4–49.9%, respectively. Hydrolysis of untreated and ensiled feedstocks with S/X resulted in conversion of 11.8–36.6% and 13.6–34.1% holocellulose, respectively. The significantly ($p<0.05$) better (or similar) performance of C/C combination compared with S/X may be attributed to the provision of β-glucosidase activity by Novozyme 188, which can effectively reduce the inhibition of cellulase due to excess accumulation of cellobiose and result in high sugar yield [40]. In their study of enzymatic hydrolysis of steam-pretreated softwood, Tengborg et al. [41] observed an increase in cellulose conversion with increasing β-glucosidase addition at a given cellulase activity. However, beyond 50 IU cellulase/g cellulose, addition of β-glucosidase did not further improve cellulose conversion. Enzyme combination C/C also had considerable xylanolytic activity although no xylanase was supplemented. Duarte et al. [42] reported that Celluclast 1.5 L had β-xylanase and β-xylosidase activities of 100 and 0.53 U/g xylose, respectively. Saddler et al. [43] tested commercial cellobiase (Novozyme 188) and found that it had a xylanase specific activity even higher than its β-glucosidase activity. The endoglucanase, β-glucosidase, cellulase, and xylanase activities reported for Novozyme 188 are 2.1, 10, 0.2, 11.1 U/mg, respectively. Hence, the significant xylanolytic activity present in C/C is also speculated to enhance hydrolytic performance by promoting xylan to xylose conversion.

Effect of Enzyme Loading Increasing enzyme loading from 40 to 60 FPU/g reducing sugars did not significantly enhance saccharification ($p>0.05$) in any of the samples (Table 3). This indicates that cellulase loading might have reached saturation at 40 FPU/g reducing sugars or the remaining cellulose in ensiled feedstocks had low substrate reactivity. Substrate reactivity has been reported to be dependent on cellulose structure as well as its crosslinking with other components such as lignin and hemicellulose. Low substrate reactivity may significantly decrease the degradability of cellulose [44]. This result agrees with that obtained by Lloyd and Wyman [45], who reported that when the enzyme loading was reduced from 60 to 15 FPU/g glucan, glucose yield reduced slightly from 50.7 to 50.6%. Spindler et al. [46, 47] also investigated the correlation between cellulase activity (7–26 FPU/g cellulose) and ethanol yield from wheat straw and other herbaceous crops. Their studies showed an increase in ethanol yield when cellulase loading was increased, but reported that saturation was reached at 20 FPU/g cellulose.

Table 4 Fermentation of hydrolysate from enzymatic hydrolysis of ensiled feedstocks.

Feedstock	DNS sugars in the hydrolysate (g)	Ethanol yield (g/g reducing sugars)	Ethanol yield (g/g ensiled feedstocks)
Barley straw	0.47 (0.02)	0.24 (0.03)	0.17 (0.01)
Triticale straw	0.47 (0.02)	0.21 (0.02)	0.15 (0.02)
Wheat straw	0.41 (0.03)	0.26 (0.01)	0.15 (0.02)
Cotton stalk	0.29 (0.01)	0.22 (0.02)	0.10 (0.01)
Triticale hay	0.79 (0.01)	0.28 (0.01)	0.33 (0.01)

Values in parentheses are the standard deviations.

Fermentation of Hydrolysates Due to the high cost of enzymes, hydrolysates involving the least amount of enzyme at significantly higher percent holocellulose saccharification were selected for fermentation with *S. cerevisiae*. Hydrolysates from samples hydrolyzed with Celluclast 1.5 L and Novozyme 188 at an enzyme loading of 40 FPU/g reducing sugars were identified as optimal and were fermented, resulting in ethanol yields ranging from 0.21 to 0.28 g/g reducing sugars (Table 4).

The ethanol yields are lower compared to reported literature values [48, 49]. The variation in yield may be attributed to (1) differences in biomass characteristics due to factors such as location, cultivar, and harvest time; (2) difference in pretreatment methods utilized to render the feedstocks more accessible to cellulolytic enzymes [50, 51]; and (3) the limited substrate utilization spectrum of the yeast strain *S. cerevisiae*, making it incapable of fermenting C5-sugars released from hemicellulose [52].

Conclusions

This study investigated the potential of using ensiling as a cost-effective pretreatment method for bioethanol production from agricultural residues (barley, triticale, wheat straws, cotton stalks, and triticale hay). The following conclusions can be drawn:

- Ensiling caused holocellulose losses ranging from 1.31 to 9.93%, which could be attributed to the preferential degradation of cellulose and hemicellulose by the ensiling microflora. Percent holocellulose loss was observed to be correlated with lignin content of straws and stalks. The rapid pH drop during fermentation of free sugars in hay helped minimize the metabolic consumption of available carbohydrates, thereby reducing holocellulose loss compared to other feedstocks.
- The ensiling process did not significantly affect ($p>0.05$) the lignin content of barley straw, cotton stalk, and triticale hay ensiled without enzyme, but slightly increased ($p<0.05$) the lignin content in triticale straw, wheat straw, and triticale hay ensiled with enzyme. Change in lignin content is expected to have been impacted by the combined effect of lignin–protein repolymerization and delignification under aerobic conditions.
- Enzymatic hydrolysis of ensiled and untreated feedstocks by cellulase–cellobiase combination resulted in equal or higher saccharification than with cellulase–xylanase combination. Increasing enzyme loading from 40 to 60 FPU/g reducing sugars did not significantly increase sugar yield. The percent saccharification

during hydrolysis with cellulase–cellobiase at 40 FPU/g reducing sugars were 17.1 to 43.6%, 22.4 to 46.9%, and 23.2 to 32.2% for untreated feedstocks, feedstocks ensiled with, and without enzymes, respectively.

- Ensiling of barley, triticale, wheat straw, and cotton stalk had a significant ($p<0.05$) impact on the conversion of cellulose and hemicellulose to sugars. However, addition of enzymes during ensiling did not significantly ($p<0.05$) impact percent saccharification during subsequent hydrolysis.
- Ethanol yields from ensiled feedstocks range from 0.21 to 0.28 g/g reducing sugars.

Although not as effective as widely accepted chemical pretreatments, ensiling shows promise in enhancing hydrolysis of mature agricultural residues as a low-cost and energy conserving technique. SI-LO-FAME 500CS costs $210 per 500 g and for commercial silage operations, the manufacturer suggests using 50 g per 50 tons of wet silage which is equivalent to 0.004 U cellulase/g dry matter or $0.42 per ton silage. This study is a step towards the possible optimization of the ensiling process, but further research to develop relatively inexpensive silage additives, improve pretreatment efficiency of ensiling through the addition of readily available sugars, use of microorganisms capable of fermenting 5 C-sugars to ethanol, and optimization of fermentation procedures to eventually increase ethanol production, is needed.

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