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Predicting Digestibility of Ammonia Fiber Explosion (AFEX)-Treated Rice Straw

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Abstract

The enzymatic digestibility of ammonia fiber explosion (AFEX)-treated rice straw was modeled by statistically correlating the variability of samples to differences in treatment using several different analytical techniques. Lignin content and crystallinity index of cellulose affect enzymatic hydrolysis the most. X-ray diffraction was used to measure the crystallinity index (CrI), while fluorescence and diffuse reflectance infrared (DRIFT) spectroscopy measured the lignin content of the samples. Multivariate analysis was applied to correlate the enzymatic hydrolysis results of the various samples with X-ray diffraction and spectroscopic data. Principal component analysis (PCA) and multilinear regression (MLR) techniques did not accurately predict the digestibility of the rice straw samples. The best correlation (*R* value of 0.775) was found between the treatment conditions of the AFEX process and the concentration of xylose at 24 h after enzymatic hydrolysis.

Index Entries: Lignocellulose; enzymatic digestibility; lignin; crystallinity; AFEX; correlation; pretreatment; X-ray diffraction; fluorescence; DRIFT.

Introduction

Numerous methods have been tried to efficiently utilize the excess lignocellulosic residues generated each year. A recently developed technique, ammonia fiber explosion (AFEX), is a physicochemical pretreatment method that efficiently treats nonwoody lignocellulosic residues and residues such as rice straw *(1)*. Studies have shown that AFEX treatment increases the digestibility of the biomass, probably owing to depolymerization of lignin, disruption of plant biomass, decreased crystallinity of cellulose *(1)*, and increased wettability of the rice straw *(2)*. High lignin content

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and high crystallinity index (CrI) of cellulose have been shown to be important deterrents to the enzymatic hydrolysis of lignocellulosic residue *(3)*.

Because lignin content and percentage crystalline cellulose vary with different treatments and with different feedstocks, the digestibility of the biomass also varies with different treatments and with different feedstocks. Therefore, to predict the digestibility of the AFEX-treated biomass, several analytical techniques were tested to obtain quantitative information on the lignin content and CrI within these lignocellulosic residues. X-ray diffraction, fluorescence spectrometry and diffuse reflectance infrared fourier transform (DRIFT) spectrometry methods were chosen because these techniques were rapid, on-line, and inexpensive. Previous research has shown that the CrI of pretreated biomass samples could be measured by X-ray diffraction *(4)*. Research done by Billa et al. *(5)* gave a good correlation between the fluorescence data and the lignin content of wheat straw while Ferraz et al. *(6)* found a high correlation between lignin content and DRIFT data of wood. Multivariate analysis using principal component regression (PCR) analysis was applied to correlate the hydrolysis results of the AFEX-treated rice straw samples with the analytical data obtained by X-ray diffraction, DRIFT, and fluorescence spectrometry.

Methods

Raw Materials

Rice straw samples were treated at Texas A&M University using the ammonia fiber explosion process *(7)*. The rice straw samples were pretreated in a batch reactor with high-pressure liquid ammonia under varying conditions and treatment times. After treatment, the samples were released into a flash tank by opening a valve. The instantaneous drop of pressure in the flash tank caused the ammonia to flash to vapor, which in turn caused the explosive decompression of the rice straw and considerable fiber disruption. From the flash tank the treated samples were sent to a drier to remove residual ammonia.

A limited set of pretreatment conditions was considered. Temperature for the treatment was set at 80 or 90°C while moisture varied among 20, 40, and 60 percent (kg water/kg dry straw) at a reaction time of 5 or 10 min. The ammonia ratio was set at 0.1, 0.5, 0.75, 1.0, 1.5, or 2.0 (kg of ammonia/kg of dry straw). Combinations of the above mentioned conditions were used to treat the rice straw.

The AFEX-treated rice straw samples were powdered using a mortar and pestle. Untreated rice straw and delignified rice straw samples were used as controls. The rice straw was delignified using the Van Soest method *(8)*. All the samples were sieved through an opening of 106 μm (150 mesh) to obtain a homogeneous powder.

Acid Hydrolysis

Theoretical sugar was determined using the acid hydrolysis method. Approximately 0.1 g of the dry rice straw samples dried overnight at 80°C

were hydrolyzed in 1 mL of a 72% sulfuric acid solution for 1 h in a 30°C water bath. The hydrolyzed mixture was quantitatively diluted by adding 28 mL of distilled water, autoclaved for 45 min, filtered through Whatman No. 1 filter papers using a celite funnel, and further diluted to a final volume of 100 mL.

Enzymatic Hydrolysis

Approximately 0.5 g of rice straw, according to dry weight, was added to a final volume of 10 mL 0.1 *M* citrate buffer (pH 4.8) containing 20 ppm of sodium azide. All solutions and the sieved rice straw samples were assumed to have a specific gravity of 1.0 g/L. The cellulase enzyme (Celluclast, Novo Nordisk) activity was 71 filter paper unit (FPU)/mL. Activity of the cellulase enzyme was determined by the method of Mandels *(9)* and Miller *(10)* using Whatman No. 1 filter paper as a substrate and was expressed as filter paper activity (FPA) in terms of FPU. Cellulase loading was 5 IU/g dry biomass while cellobiase (β-Glucosidase) loading was 30 IU/g dry biomass. The activity of the cellobiase was 150 IU/mL (β-Glucosidase, Novo Nordisk). Approximately 1 mL volume well mixed samples were taken at 3 h, 24 h and 48 h and diluted 100 fold prior to sugar analysis.

Sugar Measurements

All samples were filtered through 0.45 μm Gelman HPLC certified filters prior to sugar analysis. Dextrose and xylose were measured using the DIONEX system and high pressure liquid chromatography (HPLC). The HPLC was carried out with a Waters 600 HPLC solvent delivery system with a Waters 410 Differential Refractometer. A HP-X-87X Ion Exclusion column (300 × 7.8 mm) (Biorad Laboratories, Hercules, CA) was used to separate the sugars in the HPLC. The mobile phase consisted of 0.01 *N* H_2SO_4 set at a rate of 0.4 mL/min and a temperature of 60°C. The procedure for the DIONEX system was set to the specifications given in DIONEX Technical Note 20 *(11)*. For the DIONEX system, a CarboPac PA-10 (4 × 250 mm) (Dionex Corp. Sunnyvale, CA) analytical column was used to analyze the sugars. Percentage yield of sugar (dextrose or xylose) was determined by using Eq. 1. The percentage yield of sugar is based on the amount of theoretical sugar obtained from acid hydrolysis:

$$\text{Percentage Yield of Sugar (\%)} = \frac{\text{Amount of sugar from enzymatic digestion}}{\text{Amount of sugar from acid digestion}} \times 100 \quad (1)$$

X-Ray Diffraction Method

Crystallinity index (CrI) was measured using the Rigaku Rotaflex 200B X-ray diffractometer. The sample was placed vertically and analyzed using the horizontal goniometer. The sample was scanned at 1°/min from

$2\theta = 10°$ to $26°$ with a step size of 0.5°. The crystallinity index was determined as defined by Segal et al. *(12)*:

$$\text{Crystallinity Index (\%)} = \frac{(I_{002} - I_{am}) \times 100}{I_{am}} \quad (2)$$

where I_{002} is the intensity of the peak at 22.8° and I_{am} peak is the intensity of the peak at 18°.

Fluorescence Spectroscopy Method

Fluorescence spectra were recorded using a SPEX-3 Fluorolog. Autoemission spectra were obtained at an excitation wavelength of 350 nm with an interval of 0.5 nm. The excitation and emission slit widths were set at 3 and 5 nm, respectively. The solid sample holder was filled with the powdered sample and was held in place with a quartz cover slip. The mode of detection was set at front face. Each sample was measured three times. All the fluorescence data were normalized as shown in Eq. 3:

$$\text{Normalized data} = \frac{\text{Intensity} - \text{Intensity of baseline}}{\text{Maximum Intensity} - \text{Intensity of baseline}} \quad (3)$$

DRIFT Spectroscopy Method

A Perkin Elmer System 2000 FT-IR with the DRIFT accessory was used for DRIFT analysis of rice straw. The samples were analyzed using the method described previously by Ferraz et al. *(6)*. Reflectance spectra were transformed to Kubelka–Munk (KM) units to minimize scattering contributions to the absorption measured *(13)*.

Statistical Modeling

Multivariate linear regression (MLR) was done between each *X* variable and the concentration or percentage yield of sugar (dextrose, xylose, or total sugar at 3, 24, and 48 h). The *X* variables were AFEX treatment conditions, CrI, chosen fluorescence intensities (400, 483, 527 nm), or the area under the fluorescence peak (400–650 nm).

Principal component regression (PCR) was done by first performing principal component analysis (PCA) on the spectral data (fluorescence and DRIFT) followed by MLR between the concentration or percentage yield of sugar (dextrose, xylose, or total sugar at 3, 24, and 48 h) and the chosen principal components (PCs). The fluorescence and DRIFT spectral data were analyzed using PCA on MatLab. The spectra were normalized and then mean scaled (covariance about the mean) by subtracting each spectrum from the mean spectrum. Ten principal components were chosen for MLR analysis. Also, DRIFT PCs and Fluorescence PCs were each combined with CrI data and then MLR was performed on the combined data versus the hydrolysis results.

Forty-nine different treatment conditions were considered for both MLR and PCR analysis. The analysis of variance (ANOVA) for each model gave probability values that were used to estimate the quality and validity of the models.

Results and Discussion

Hydrolysis Results

Only dextrose and xylose sugars with negligible (<1%) amounts of other sugars were detected from acid hydrolyzed samples. Therefore, total theoretical sugar is composed of primarily xylose and dextrose sugars. Triplicate samples taken at 3, 24, and 48 h time points did not give highly reproducible results. The reproducibility of the concentration of dextrose from enzymatic digestion was ±30% while reproducibility at the concentration of xylose from enzymatic digestion was ±25%. Reproducibility of ±10% was seen in acid digestion analysis. Possible sources of error in reproducibility include variations in rice straw composition, non-uniform conditions in the AFEX reactor and variations in hydrolysis and sampling.

Trends in the percentage yield of dextrose and xylose for some of the treated samples are plotted in Figs. 1 and 2, respectively. Both figures show a similar trend in the rate of hydrolysis and the percentage yield of theoretical sugar except for delignified rice straw. Delignified rice straw had a 55% yield of dextrose, but only a 25% yield of xylose. The delignification process is moderately effective at improving the yield of dextrose but is not very effective in improving the yield of xylose. Although Celluclast is reported to have both xylanse and beta-xylosidase activities, we did not measure these activities. Removal of lignin can significantly increase the accessible surface area of cellulose for enzymatic hydrolysis. An important conclusion drawn from Figs. 1 and 2 is that AFEX treatment greatly increases the digestibility of the rice straw. Sample B150 had an 80% yield of both dextrose and xylose, while untreated rice straw had only an 18% dextrose yield and a 7% xylose yield. The rate of hydrolysis of the untreated rice straw is slow and maximum digestibility is nearly reached within 24 h while AFEX treated samples have greater rates of hydrolysis before and after 24 h compared to untreated samples. Some treatment conditions (sample B150) had greater yields of both dextrose and xylose than obtained from hydrolysis of delignified rice straw. This suggests that although lignin has an important effect on the hydrolysis of the rice straw, it does not fully control the extent of hydrolysis, at least for rice straw.

Correlation between AFEX-Treatment Conditions and Hydrolysis Results

The highest correlation (R = 0.775) was observed from regression analysis between AFEX-treatment conditions and concentration of xylose at 48 h after enzymatic hydrolysis. Figure 3 compares the actual measured xylose concentration vs the predicted xylose concentration for nine random AFEX-treated samples. The higher correlation for concentration of

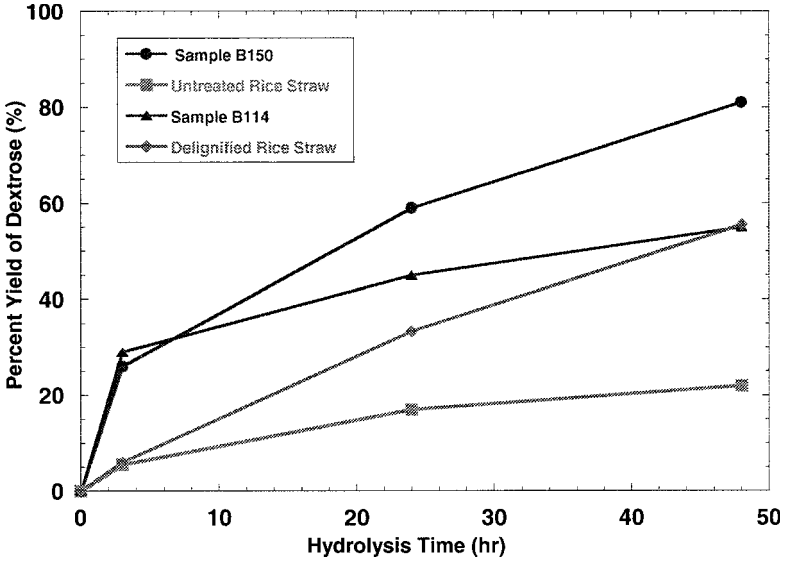

Fig. 1. Trends in percentage yield of dextrose at 3, 24, 48 h: Sample B150 [40 percent moisture (100×kg water/kg dry rice straw), 2 ammonia to straw ratio (kg ammonia/ 1 kg dry rice straw), 90°C, 5 min]; Sample B114 [20 percent moisture (100×kg water/ kg dry rice straw), 1.5 ammonia to straw ratio (kg ammonia/ 1 kg dry rice straw), 80°C, 5 min].

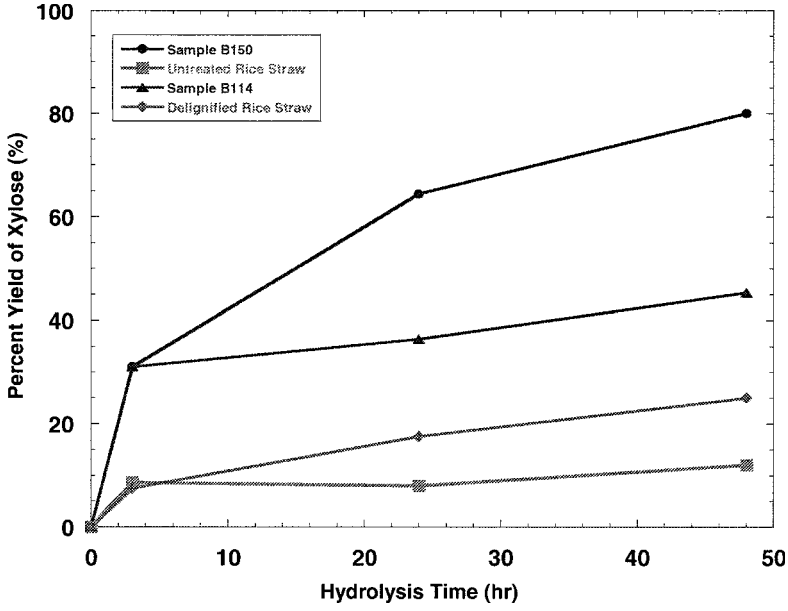

Fig. 2. Trends in percentage yield of xylose at 3, 24, 48 h: Sample B150 [40 percent moisture (100×kg water/kg dry rice straw), 2 ammonia to straw ratio (kg ammonia/ kg dry rice straw), 90°C, 5 min]; Sample B114 [20 percent moisture (100×kg water/ kg dry rice straw), 1.5 ammonia to straw ratio (kg ammonia/kg dry rice straw), 80°C, 5 min].

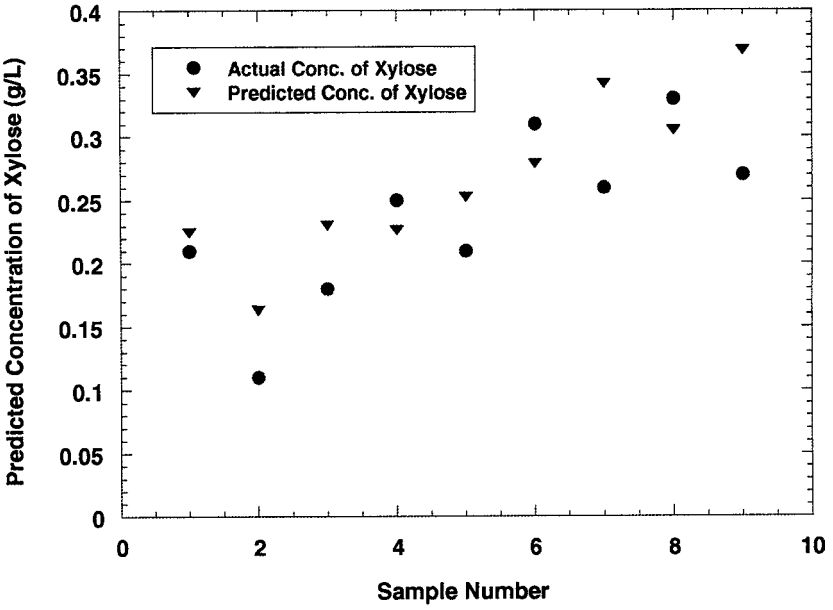

Fig. 3. Comparison between actual and predicted concentration of xylose at 48 h enzymatic hydrolysis. Equation obtained for predicted concentration of xylose at 48 h [(R =0.775): Y_X = -0.234 + 0.000379 X_1 + 0.0104 X_2 + 0.00263 X_3 + 0.127 X4 Y= concentration of xylose (g/L); X_1 = treatment moisture (kg water/kg dry straw) × 100; X_2 = reaction time (min); X_3 = reaction temperature (°C); X_4 = ammonia:straw ratio (kg ammonia/kg dry straw)].

xylose at 48 h could be due to solubilization of hemicellulose, deacetylation of hemicellulose *(3)*, and disruption of the lignin molecules.

Predicting Ultimate Yield of Sugar

The concentration of neither dextrose nor xylose at 48 h could be well predicted using the 3 h concentration of dextrose. We obtained an R value of 0.728 for predicting the concentration of xylose, while we only obtained an R value of 0.51 for predicting the concentration of dextrose. One of the reasons that there is poor correlation between ultimate (48 h) and initial (3 h) sugar yields may be because some of the samples may not have been fully hydrolyzed by 48 h. Also, factors such as crystallinity of cellulose and the content of lignin could affect the rate and extent of hydrolysis differently at different stages of hydrolysis.

X-ray Diffraction

The CrI of the AFEX treated rice straw samples was reproducible with an error of + 0.3% for four-sample replicates. AFEX treatments have a definite impact on CrI of the various rice straw samples, which varied from 18.75 to 42.9%. A few of the treatments apparently increased the CrI of the samples, but most of the AFEX-treatment conditions decreased the CrI of the rice straw. Contrary to what we might have assumed, the samples with

a lowest CrI (18.75%) had only a 40% yield of sugar as compared to the 80% yield of sugar for samples with a CrI of 28%. A few of the AFEX treatments caused a slight increase in CrI (41.7–42.9%) compared to the untreated rice straw (40.7%). The treatment conditions that caused the increased CrI were 60 percent moisture (100×kg water/kg dry rice straw) for 10 min at 80 or 90°C with an ammonia ratio of 1.5 or 1 (kg ammonia/kg dry rice straw). As expected, hydrolysis of these samples resulted in lower yields of dextrose (29%) and xylose (26%).

Correlation between CrI and Sugar Yields

Although the AFEX treatment conditions affect the CrI, the association between treatment conditions and CrI is not very significant ($R < 0.55$). No correlation ($R < 0.32$) was found between CrI of the samples and the concentration or percentage yield of sugar at 3, 24, 48 h. Therefore, we were not able to predict the relationship between CrI and hydrolysis results. CrI alone is not a good predictor of digestibility perhaps because of the lignin barrier, which also affects hydrolysis. A major obstacle to the regression studies was the lack of reproducibility in the hydrolysis results, which further reduces our ability to find a correlation.

Fluorescence Analysis

The fluorescence profile of each AFEX treated sample was very reproducible. The variability of the fluorescence profiles among samples with different treatment conditions is shown in Figure 4. The dissimilarity among the samples is seen at 400nm, 483nm and at 527nm. No correlation was found between these intensities and hydrolysis results.

PCR Analysis between Fluorescence Data and Sugar Yields

The coefficient of multiple correlation for the PCR analysis between the fluorescence data and hydrolysis results for 99% confidence level is presented in Table 1. PCR analysis is done in a two-step procedure. The first step involves a PCA on the fluorescence data matrix. In the second step, MLR is done between chosen fluorescence PCs vs the hydrolysis results. Dextrose and xylose yield are defined in Equation 1. Total sugar is the sum of xylose and dextrose.

From Table 1 we conclude that there is not much correlation between the chosen principal components (PCs) of the fluorescence data matrix and the concentration or percentage yield of sugar (dextrose, xylose, or total sugar at 3, 24 and 48 h).

Since both CrI and lignin content affect digestibility of biomass, we combined the CrI and the fluorescence PCs. The combined data were then regressed against the concentration and percentage yield of sugar (dextrose, xylose, or total sugar at 3, 24, and 48 h). Table 2 provides the *R* values from the correlation studies. Regression of the combined data against the hydrolysis results gave higher correlations (*R* values). The highest *R* value (0.73) for xylose was obtained for correlation between the combined data and xylose yield at 48 h.

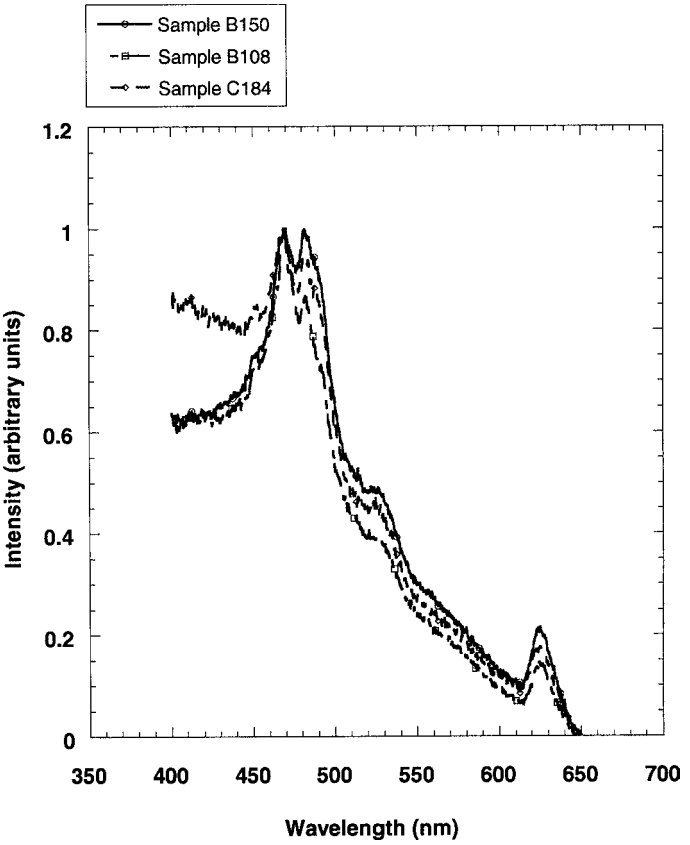

Fig. 4. Effect of AFEX-Treatment on the fluorescence profile of rice straw: Sample B150 [40 percent moisture (100×kg water/kg dry rice straw), 5min, 90°C, 2 ammonia to straw ratio(kg ammonia/kg dry rice straw)]; Sample B108 [60 percent moisture (100×kg water/kg dry rice straw), 10 min, 90°C, 1 ammonia to straw ratio (kg ammonia/kg dry rice straw)], Sample C184 (60 percent moisture (100×kg water/kg dry rice straw), 10 min, 90°C, 1.5 ammonia to straw ratio (kg ammonia/kg dry rice straw)].

The treatment conditions of the AFEX process were also regressed against the fluorescence intensities at 400, 483, and 527nm. The highest *R* value was 0.72 for the correlation between treatment conditions vs the peak at 527 nm, but an *R* of 0.72 is not significant owing to high scatter.

The fluorescence technique did not give us very good correlations, probably because lignin fragments also fluoresce. Therefore, we do not know if the AFEX treatments are affecting the lignin within the rice straw. A major obstacle to measuring lignin, using fluorescence, is that plant cell walls contain other products such as cutin, suberin, sporopollenin, and cellulose, which fluoresce when excited with light *(14)*. Also, molecular structures that are not generally known to fluoresce may do so in the lignin structure or in the solid pulp matrix *(15)*. This was proved when delignified rice straw samples also fluoresced with the same intensity as AFEX treated samples.

Table 1
PCR Analysis Between Fluorescence Data and Hydrolysis Results

	R values					
	Dextrose conc.	Xylose conc.	Total conc.	Dextrose yield	Xylose yield	Total yield
3 h	0.48	0.46	0.48	0.41	0.39	0.38
24 h	0.6	0.28	0.51	0.6	0.41	0.58
48 h	0.48	0.24	0.36	0.58	0.43	0.55

Table 2
Regression Analysis: Fluorescence PCs and CrI vs Hydrolysis Results

	R values					
	Dextrose conc.	Xylose conc.	Total conc.	Dextrose yield	Xylose yield	Total yield
3 h	0.67	0.62	0.67	0.62	0.58	0.6
24 h	0.72	0.62	0.68	0.69	0.66	0.68
48 h	0.69	0.67	0.69	0.66	0.73	0.68

DRIFT Analysis

FTIR spectra of rice straw samples include several overlapped bands *(16–18)*. The only pure band is related to the aromatic components present in lignin, which gives a characteristic absorption near 1510 cm^{-1} and is usually used as the reference band *(17)*. This band is solely due to the aromatic skeletal vibration of the benzene ring in lignin. In our spectra, the internal reference band shifted a few degrees from 1510 cm^{-1} to 1507 cm^{-1}. Therefore, the spectra of the samples were normalized to the absorption intensity at 1507 cm^{-1}. Only the bands in the region 2987–3321 cm^{-1} and 1038–1209 cm^{-1} lacked reproducibility for replicate measurements of the same sample. Distortions in the region from 1038 to 1209 cm^{-1} are attributed to the specular radiation while the distortions in the region from 2987 to 3483 cm^{-1} are contributed by the O–H stretching *(19)*.

The effect of the AFEX treatment on the DRIFT spectra is shown in Fig. 5. In Fig. 5 the spectra of samples B111 and B126 mostly coincide, while the spectra of the untreated rice straw vary somewhat from the treated samples. Untreated rice straw varies most strongly in the region from 2300 to 3200 cm^{-1}.

Correlation between DRIFT Data and Sugar Values

The results of the PCR analysis between the entire DRIFT spectral data matrix (800–4000 cm^{-1}) and the concentration or percentage yield of sugar (dextrose, xylose, or total sugar at 3, 24 and 48 h) are given in Table 3. The

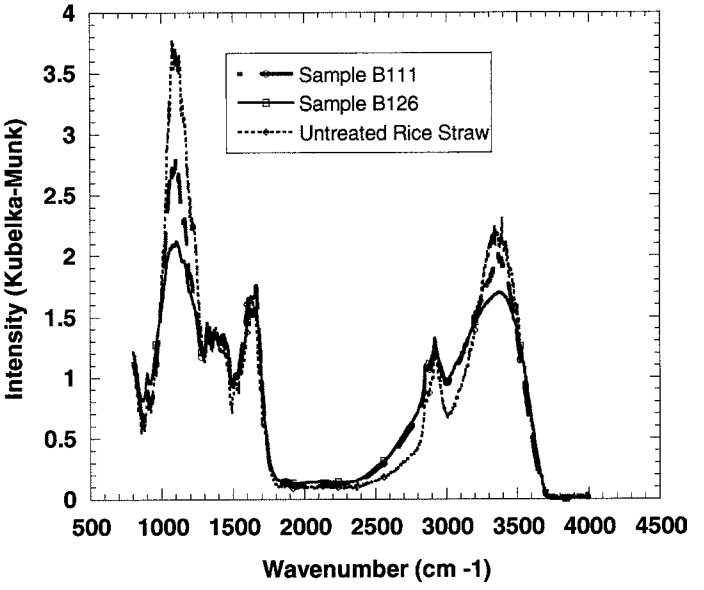

Fig. 5. Effect of various AFEX treatments on DRIFT spectra: Sample B111 [60 percent moisture (100×kg water/kg dry rice straw), 80°C, 10 min, 1 ammonia:straw ratio (kg ammonia/kg dry rice straw)]; Sample B126 [20 percent moisture (100×kg water/kg dry rice straw), 80°C, 10 min, 2 ammonia to straw ratio (kg ammonia/kg dry rice straw)].

Table 3
Regression Analysis: DRIFT PCs vs Hydrolysis results

	R values					
	Dextrose conc.	Xylose conc.	Total conc.	Dextrose yield	Xylose yield	Total yield
3 h	0.62	0.55	0.58	0.71	0.5	0.64
24 h	0.54	0.54	0.54	0.59	0.53	0.58
48 h	0.48	0.45	0.45	0.65	0.47	0.59

R values did not vary when PCR analysis was done using only the region from 1210 to 2986 cm^{-1} of the DRIFT spectral matrix. This was done to check if the inconsistency of the specular radiation region (1038–1209 cm^{-1}) and the O–H stretch region (2987–3483 cm^{-1}) for replicate runs of the same sample affected the regression analysis.

The highest *R* value of 0.71 was obtained from MLR between percentage yield of dextrose at 3 h and the DRIFT PCs. An *R* value of 0.71 reflects considerable scatter in the data set and, therefore, the DRIFT technique is not a good predictor of digestibility.

Table 4
Regression Analysis: DRIFT PCs and CrI Data vs Hydrolysis Results

	R values					
	Dextrose conc.	Xylose conc.	Total conc.	Dextrose yield	Xylose yield	Total yield
3 h	0.65	0.58	0.62	0.73	0.54	0.68
24 h	0.57	0.61	0.55	0.57	0.63	0.58
48 h	0.56	0.59	0.58	0.66	0.65	0.66

The PCR analysis between the combined data (CrI and DRIFT PCs) vs hydrolysis results slightly increased the *R* values as seen in Table 4. Again the highest *R* value (0.73) was seen for the correlation between the combined data and 3 h yield of dextrose.

Some reasons why high correlations were not observed may be (1) lignin content by itself is not a good predictor of digestibility; (2) lignin content may not vary much for these samples; (3) there may not be a difference between lignin monomers and the polymers as seen in the DRIFT spectra. The lack in reproducibility of the hydrolysis results themselves is also a major deterrent for predicting digestibility.

Conclusion

Our research goal was to predict the digestibility of the AFEX-treated rice straw using rapid, potentially on-line analytical methods. We have not yet been completely successful. The best correlation ($R = 0.775$) was found between the treatment conditions vs the concentration of xylose at 48 h enzymatic hydrolysis. Combining X-ray diffraction data (CrI) and DRIFT PCs did give us higher correlations, but these correlations were still not able to accurately predict the digestibility of the AFEX-treated rice straw samples. It may be that one or more of these analytical techniques could actually provide a useful prediction with additional development but due to sample variability we were not able to identify a suitable approach.

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