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Characterization of Rice Straw Prehydrolyzates and Their Effect on the Hydrolysis of Model Substrates Using a Commercial *endo*-Cellulase, β-Glucosidase and Cellulase Cocktail

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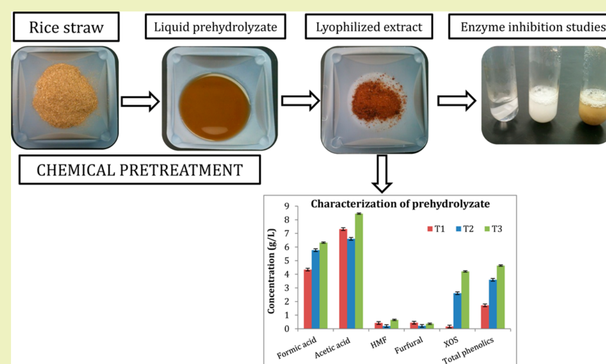
ABSTRACT: Pretreatment and enzymatic saccharification are two major upstream processes that affect the economic feasibility and sustainability of lignocellulosic biofuel production. Cellulase-inhibiting degradation products, generated during dilute acid pretreatment, increase enzyme usage, and therefore, it is essential to mitigate their production. In an attempt to elucidate the most deleterious degradation product to enzymatic hydrolysis, hydrolyzates were generated from rice straw, and their effect on enzyme activity was determined. Ground rice straw was subjected to the following pretreatments having a combined severity factor of 1.75: T1−160 °C, pH 1.7; T2−180 °C, pH 2.25; and T3−220 °C, pH 7.0. The liquid prehydrolyzates were freeze-dried, and their inhibitory effects on the activities of a commercial cellulase cocktail, *endo*-cellulase, and β-glucosidase were determined using filter paper, carboxymethyl cellulose, and cellobiose, respectively. Addition of 15 g L^{-1} of T1, T2, or T3 freeze-dried prehydrolyzates resulted in 67%, 57%, and 77% reduction in CMC-ase activity of *endo*-cellulase, respectively. In the presence of 35 g L^{-1} of T1, T2, or T3 prehydrolyzates, the filter paper activity of the cellulase cocktail was reduced by 64%, 68%, and 82%, respectively. Characterization of the freeze-dried prehydrolyzates showed that T3 had significantly higher xylo-oligosaccharides and total phenolic content than T2 and T1.



KEYWORDS: *Rice straw, Prehydrolyzate, endo-Cellulase, β-Glucosidase, Filter paper activity, Cellobiase activity, Xylo-oligosaccharides, Phenolics, Enzyme inhibition*

INTRODUCTION

The estimated production of lignocellulosic fuel in the United States in 2012 was less than 0.5 million gallons, a fraction of the revised projection of the Renewable Fuel Standards (RFS2) of the Energy Independence and Security Act, 2007.[1] Start-up cellulosic biofuel industries are afflicted with problems ranging from feedstock availability to high production costs to scalability.[2] Cellulolytic enzymes, used in saccharification, account for 14% of the total production cost and next to the biomass cost is the highest input cost.[3] Carbohydrate degradation products, such as acetic acid and formic acid, and furan derivatives, such as furfural and 5-hydroxymethyl furfural, are formed during pretreatment of the lignocellulosic biomass and have been reported to inhibit the cellulase cocktails.[4−6] In addition, xylo-oligosaccharides and lignin-derived compounds, released during biomass pretreatment also inhibit commercial cellulase cocktails.[7,8] Lignin model compounds, such as tannic acid, gallic acid, vanillin, cinnamic acid, ferulic acid, *p*-coumaric acid, sinapic acid, syringaldehyde, and *p*-hydroxybenzoic acid, were reported to inhibit and deactivate the cellobiose activity of β-glucosidases and filter paper activities of commercial cellulase cocktails through precipitation and competitive binding.[9,10] Similarly, the inhibitory effects of pure xylobiose and higher DP xylo-oligosaccharides have been tested against commercial cellulases on Avicel substrates. It was reported that initial glucose yields were reduced by 82% in the presence of 12.5 g L^{-1} of xylooligomers as a result of competitive inhibition of cellulases.[11] Thus, these degradation products play a significant role in reducing the efficiency of enzymatic hydrolysis and increasing the input cost.

For the satisfactory performance of the saccharification cocktail, inhibitory compounds present in prehydrolyzates need to be mitigated prior to enzymatic hydrolysis. Rinsing the pretreated biomass with 10X or more volumes of water have been reported to remove the degradation products and improve the enzymatic saccharification on a laboratory scale.[8,12,13] At the demonstration scale, estimates of water consumption in the cellulosic ethanol industry are 23−38 L of water per liter of ethanol; detoxification with such water volumes would further decrease its sustainability.[14] Therefore, efforts were made in this

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study to characterize the prehydrolyzates and to determine which group of compounds affected saccharification. The long-term objective of this work is to identify which components in the crude prehydrolyzate are most inhibitory to the cellulase cocktail as a whole and to its individual enzymes, such as *endo*-cellulase and *β*-glucosidase.

Agricultural residues constitute the bulk of potential feedstock available in the United States for cellulosic fuel production.[15] Globally, rice straw alone represents 23% of all agricultural wastes and is a major feedstock source for cellulosic biofuel production in countries such as India where extensive land use changes would be unsustainable.[16] Because of its importance on the world scale, rice straw was used as the feedstock in this study.

■ MATERIALS AND METHODS

Chemicals. Commercial standards of glucose, arabinose (Alfa-Aesar, Ward Hill, MA), xylose, 5-hydroxymethyl furfural (HMF), furfural, *p*-coumaric acid, *trans* ferulic acid, protocatechuic acid, acetosyringone (Sigma-Aldrich, St. Louis, MO), syringaldehyde, 4-hydroxybenzoic acid, vanillin, salicylic acid, gallic acid (TCI chemicals, Montgomeryville, PA), and formic acid (Amresco, Solon, OH) were used in the characterization of the prehydrolyzates. Xylo-oligosaccharide standards (DP2 to DP6) were purchased from Megazyme International (Wicklow, Ireland). The enzymes used in this study were Cellic CTec2, a saccharification cocktail from *Trichoderma reesei*, NS 22118, a *β*-glucosidase (Novozymes North America Inc., Franklinton, NC), and *endo*-1,4-*β*-D-glucanase (*endo*-cellulase) from *Aspergillus niger* (Megazyme International). The saccharification cocktail had a filter paper activity of 62 U mL^{-1}. The *endo*-cellulase had a carboxymethyl cellulase (CMC-ase) activity of 2000 U mL^{-1}. NS 22118 had a cellobiase activity of 105 U mL^{-1}. Filter paper (Grade 1 Whatman), carboxymethyl cellulose (CMC), and cellobiose (≥98%) were purchased from VWR International (Houston, TX), EMD Millipore (Bedford, MA), and Alfa-Aesar (Ward Hill, MA), respectively.

Sulfuric acid (≥95.0%), hydrochloric acid (≥98.0%), and glacial acetic acid (HPLC) were purchased from EMD Chemicals (Gibbstown, NJ). Folin and Ciocalteau's (F−C) phenol reagent, 2-hydroxy-3,5-dinitrobenzoic acid, ammonium hydroxide, and potassium sodium tartrate tetrahydrate, analytical grade, were purchased from Sigma-Aldrich (Milwaukee, WI). Citric acid anhydrous, sodium citrate dihydrate, sodium acetate trihydrate, and calcium chloride dihydrate were procured from Alfa-Aesar (Ward Hill, MA). Water was prepared with a Direct-Q system (Millipore, Billerica, MA) that had 18.2 MΩ resistivity.

Raw Material and Composition. Rice straw was obtained from the University of Arkansas Cooperative Extension Service, Little Rock, AR. The straw was a Clearfield hybrid rice variety and was originally harvested from a location corresponding to 34°58′28.3152″ N longitude and 92°0′59.5224″ W latitude (Cabot, Lonoke County, Arkansas). The biomass was stored at 4 °C in a walk-in refrigerator until use. It was ground using a Thomas Willey Mini mill (Swedesboro, NJ) and sieved with a 20-mesh screen, such that particle size was uniformly reduced to 0.84 mm.[17] The total solids, structural carbohydrates, lignin, ash content, and total extractives in the rice straw were determined per the NREL (National Renewable Energy Laboratory, Golden, CO) protocols.[18−21]

Pretreatment. Rice straw, 25 g, and water, 225 mL, were loaded in a 1 L Parr 4525 reactor (Moline, IL) and agitated at 144 rpm until the end of the reaction. The three treatments were T1 (160 °C, 48 min, and pH 1.7), T2 (180 °C, 44 min, pH 2.25), and T3 (220 °C, 52 min, and pH 7.0). The conditions for pretreatment were calculated based on the combined severity factor (CSF) given by

$$\mathrm{CSF} = \log\left\{t \times \exp\left[\frac{T_H - T_R}{14.75}\right]\right\} - \mathrm{pH}$$

where t is the duration of pretreatment, T_H is the pretreatment temperature, and T_R is the reference temperature (100 °C).[22] Concentrated sulfuric acid was used at 1.0% (w/v) and 0.1% (w/v) to adjust the pH of T1 and T2, respectively, prior to the Parr reaction. The pretreatment duration was calculated only at the desired temperature. Once the required duration had elapsed, the heating jacket was removed, and the reactor was cooled with cooling coils. The liquid prehydrolyzates were separated from the pretreated biomass using a Buchner filtration apparatus fitted with Whatman #1 filter paper and stored frozen at −20 °C in 100 mL polypropylene bottles.

Lyophilization. The frozen prehydrolyzates were quick frozen with liquid nitrogen at −196 °C to ensure thorough freezing and then lyophilized in a FreeZone 18 L console freeze-dry system (Labconco, Kansas City, MO). The lyophilization conditions were −44 °C, 7.7 Pa, and 72 h. Inhibitor stock was prepared by mixing the lyophilized T1, T2, and T3 prehydrolyzates with buffer at a concentration of 100 g L^{-1}. This stock was used for further enzyme assays and for characterization studies using liquid chromatography techniques.

Characterization of Prehydrolyzate. *High Performance Liquid Chromatography (HPLC).* The monosaccharide composition of the lyophilized rice straw prehydrolyzates was determined using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) equipped with a SP-G precolumn, SP0810 analytical column (Shodex, Kawasaki, Japan), and refractive index detector (Model 2414, Waters Corporation, Milford, MA). The xylo-oligosaccharide concentration was determined by equipping the HPLC described above with a Bio-Rad Aminex-HPX 42A analytical column (Bio-Rad, Hercules, CA) and a Micro-Guard deashing precolumn. Calibration curves for the xylo-oligosaccharides (DP 2 to DP 6) were determined using pure (>95.0%) reference compounds. The analytical columns and the detector were maintained at 85 and 50 °C, respectively. Millipore water was used as eluent at a flow rate of 0.2 mL min^{-1}, and the sugars were quantified using in-house calibration curves.

Analyses of formic acid, acetic acid, HMF, and furfural were performed using the Waters Alliance HPLC system fitted with a Bio-Rad Aminex HPX-87H ion exclusion analytical column (Life Sciences Research, Hercules, CA) and photodiode array detector (Model 2996, Waters Corporation, Milford, MA). The samples were eluted with a 5 mM sulfuric acid at a flow rate of 0.6 mL min^{-1} and were detected at 280 nm.[23]

Total Phenolics Assay. The total phenolic content of the rice straw prehydrolyzates was determined using the Folin and Ciocalteau's (F−C) method modified from a previously published protocol.[24] The F−C assay relies on the transfer of electrons from the phenolic compounds in an alkaline medium to phosphomolybdic/phosphotungstic acid complexes to form a blue color, whose absorbance is determined at 765 nm.[25] The 100 g L^{-1} of prehydrolyzate solutions were diluted to 1.25 g L^{-1}, and a 100 μL aliquot was mixed with 200 μL of 0.2 N F−C reagent and incubated in the dark for 5 min. Then 700 μL of 7.5% sodium carbonate solution was added to the mixture and incubated in the dark at room temperature for 2 h. After the incubation period, the samples were diluted 4X with water, and their absorbance at 765 nm was determined using a spectrophotometer (Model 517601, Beckman Coulter Inc., Indianapolis, IN). Gallic acid standards (0.5 to 2.5 g L^{-1}) were used to build a standard curve; the results were expressed in gallic acid equivalent.

LC/ESI−MS Analysis. The lyophilized rice straw prehydrolyzates were dissolved in methanol to a concentration of 100 g L^{-1} and analyzed in a Hewlett 1100 HPLC system (Hewlett-Packard, Palo Alto, CA) using a Supelco C18 column (15 cm × 4.6 mm, 5 μm particle size, 300 Å pore size) coupled online to a quadrapole ion trap electron spray ionization-mass spectrometer (ESI−MS) (Bruker Esquire 2000, Bruker, Billerica, MA). The samples were eluted in a gradient system using 0.1% formic acid in water and methanol from 85:11 to 50:50 v/v in 35 min at a flow rate of 0.4 mL min^{-1}. The solvent system was selected based on a previously published method.[26] Mass spectrometry parameters were adjusted to optimize the m/z signal in the range from 50 to 800. The mass spectrum was obtained in a positive ion mode. The chromatogram peaks were identified based

on the retention times of the reference compounds and their corresponding m/z values.

Enzyme Assays. *Saccharification Cocktail Assay.* The filter paper activity of the cellulase cocktail was determined by mixing filter paper with 100 mM citrate buffer (pH 5.0), at 5% w/v loading in 16 mm × 100 mm glass test tubes.[27] For the inhibition studies, the inhibitor stock of T1, T2, and T3 prehydrolyzates were mixed with the buffer such that their concentrations were 15, 20, 25, 30, and 35 g L^{-1}. The filter paper, buffer, and inhibitor mixture were equilibrated at 50 °C for 5 min in a reciprocating water bath agitated at 100 rpm. Then the saccharification cocktail was added at 0.67 mg of enzyme per gram of filter paper and incubated at 50 °C for 30 min. Upon completion of the reaction, the enzyme was deactivated by boiling the mixture at 100 °C for 5 min. Then the reaction mixture was cooled in an ice bath and was centrifuged at 1286 g for 10 min (IEC Spinette centrifuge, Needham, MA) to separate residual filter paper from the supernatant. The supernatant was analyzed for glucose concentration using HPLC, and the filter paper units were determined.[27]

Endocellulase Assay. A 4% w/v carboxymethyl cellulose (CMC) solution was prepared with 50 mM acetate buffer (pH 4.5) and used as the substrate.[28] For the control, CMC was mixed with the 50 mM acetate buffer (pH 4.5) at 1.2% w/v loading and incubated with 0.04 mg of enzyme per gram of CMC. For the inhibition assays, 50, 100, 150, and 200 μL of the inhibitor stock, corresponding to prehydrolyzate concentrations of 5, 10, 15, and 20 g L^{-1}, respectively, were mixed with the buffer. All assay samples were incubated at 40 °C for 20 min in a reciprocating water bath agitated at 100 rpm. At the end of the reaction, 400 μL of dinitrosalicylic acid (DNS) reagent was added, and the color was developed by boiling the mixture at 100 °C for 10 min. The DNS reagent was prepared as previously reported.[28] After terminating the reaction by cooling the samples in an ice bath, their absorbances were determined at 530 nm using a spectrophotometer (Model 517601, Beckman Coulter, Inc., Indianapolis, IN), and the specific activity of the enzyme was determined.[29]

β-Glucosidase Assay. The cellobiase activity of NS 22118 was determined by mixing cellobiose with 100 mM citrate buffer (pH 5.0) at 1.0% w/v loading in 16 mm × 100 mm glass test tubes. For the inhibition studies, the inhibitor stock was mixed with the buffer such that their concentrations were 15, 20, 25, 30, and 35 g L^{-1}. The mixture was equilibrated at 50 °C for 5 min. Then β-glucosidase was loaded at 3.49 mg of enzyme per gram of cellobiose, and the mixture was incubated at 50 °C for 30 min in a reciprocating water bath agitated at 100 rpm. To terminate the reaction, the mixture was boiled at 100 °C for 5 min. Then the mixture was cooled in an ice bath, and the glucose concentration was determined using HPLC. The cellobiose units were determined as μmol cellobiose converted per minute per gram of the enzyme.

All samples were assayed in triplicate, and two blanks were prepared per assay for the sample and reagents. The control was prepared with only enzyme and substrate mixed with the buffers. The enzyme activities were determined as previously reported.[29] Analysis of variance and test of significance (Student's t-test) were performed using JMP Pro 9.0 (SAS, Cary, NC).

RESULTS AND DISCUSSION

Effect of Combined Severity on Prehydrolyzate Composition. The average composition of rice straw adjusted for its moisture content is presented in Table 1. The percent glucan (35%), xylan (17%), Klason lignin (11%), acid soluble lignin (0.4%), and total ash (15%), determined as per NREL protocols, aligned with previously reported compositions.[30,31] Ground rice straw was subjected to three pretreatment conditions (T1, T2, and T3) that displayed a combined severity factor (CSF) of 1.75. It has been previously reported that a CSF of 1.75 yielded the maximum quantities of xylose and glucose upon pretreatment and enzymatic saccharification, respectively.[30] The prehydrolyzates were lyophilized, and their compositions were determined using HPLC and colorometric analyses (Table 2). No significant differences were observed in composition of pre- and post-freeze-dried hydrolyzates, except for that of acetic acid, which showed 1.8 and 1.6 fold increases in T1 and T2, respectively.

Table 1. Average Composition (± standard deviation) of Rice Straw

component	(%) dry weight
glucan	35.48 ± 0.99
xylan	17.14 ± 1.81
total ash	15.03 ± 1.32
total lignin[a]	11.53 ± 0.88
total extractives[b]	11.48 ± 1.33
total	90.66 ± 1.31

[a]Sum of Klason lignin and acid soluble lignin. [b]Sum of water and ethanol extractives.

Table 2. Average Composition (± standard deviation) of Freeze-Dried Rice Straw Prehydrolyzates Expressed as Percent Dry Weight of Biomass

compounds	T1	T2	T3
glucose	5.95 ± 0.21[d]	0.00 ± 0.00[d]	1.74 ± 0.65[d]
xylose	16.46 ± 0.75[d]	7.07 ± 0.54[d]	0.68 ± 0.02[d]
formic acid	4.34 ± 1.08	5.77 ± 1.16	6.32 ± 1.46
acetic acid	7.32 ± 1.98	6.60 ± 2.01	8.45 ± 0.59
HMF	0.42 ± 0.04	0.20 ± 0.03	0.65 ± 0.41
furfural	0.45 ± 0.05	0.21 ± 0.14	0.37 ± 0.29
xylo-oligosaccharides[a]	0.16 ± 0.00	3.08 ± 0.34	4.44 ± 0.11[c]
total phenolics[b]	1.34 ± 0.35[d]	2.98 ± 0.61[d]	4.64 ± 0.35[d]

[a]Sum of xylobiose (DP2) to xylohexaose (DP6). [b]Expressed as % gallic acid equivalent. [c]Significant difference between treatment groups (n = 3) at $P < 0.05$, $\alpha_{0.05}$. [d]Significant difference between treatment groups (n = 3) at $P < 0.01$, $\alpha_{0.05}$.

In T1 prehydrolyzates, theoretical maximum recoveries of glucose and xylose were 5.95% and 16.46%, respectively, which were similar to previously reported values.[30] The concentrations of degradation products in T1, such as acetic acid, furfural, and HMF, were 1.75 ± 0.31, 1.00 ± 0.09, and 0.16 ± 0.01 g L^{-1}, respectively, which were also similar to the previously reported values.[30] As shown in Table 2, despite having constant severities, T1, T2, and T3 yielded prehydrolyzates of varied compositions. Specifically, the proportion of monosaccharides and other degradation products were significantly different in T1, T2, and T3. The mass fractions of weak acids and furan derivatives were similar for T1, T2, and T3. However, the prehydrolyzate composition of total phenolics and xylo-oligosaccharides (DP2 to DP6) were significantly higher in T3 compared to T1 and T2.

It has been reported that pretreatment severity affected the kinetics of formation of degradation products.[32−34] The kinetics of xylo-oligosaccharides degradation have been previously reported to depend on the pH of the pretreatment; for example, the overall degradation rate constant of pure xylotetraose to xylose was 625.5 at pH 1.45 as opposed to 3.1 at pH 7.0, thus corroborating the presented results.[35] Lignin depolymerization, on the other hand, has been reported to increase as a function of temperature. During the dilute acid pretreatment of corn stover, increases in temperature at constant CSF and pH affected the resultant concentration of phenolics in the prehydrolyzates.[34] Moreover, raising the pretreatment temperature from 140 to 190 °C resulted in a

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67% increase in condensed phenolics in corn stover hydrolyzates.[36] Therefore, pretreatment conditions can affect the composition of resulting hydrolyzates, even at constant severities. In this work, the occurrence of significantly higher concentrations of xylo-oligosaccharides and total phenolics in T3 prehydrolyzates can thus be attributed to the neutral pH and higher pretreatment temperatures, respectively.

Experimental Design and Enzyme Inhibition. During enzyme assays, the T1, T2, and T3 prehydrolyzates were lyophilized and mixed with 100 mM buffer solutions to raise the pH to 4.8. For T1, additional ammonium hydroxide was added in order to increase the pH to 4.8. This procedure effectively eliminated the inhibition effect due to pH differences. In previous studies, pure reference compounds reported to inhibit the saccharification cocktail were individually tested to determine their inhibitory effects.[9,10] Tannic acid, for example, at 0.005 mg per FPU, was reported to inhibit the filter paper activity of saccharification cocktail by 60%.[10] Instead of reference compounds, crude prehydrolyzates were tested in this study for their inhibition effect on commercially available cellulases, *endo*-cellulase, and β-glucosidase enzyme preparations. By using authentic hydrolyzates, a true to nature evaluation of their inhibitory effect could be determined. It has been previously reported that besides competitive inhibition the activity of cellulases was also reduced by low accessibility of recalcitrant substrates.[37] In order to eliminate the chances of reduction in enzyme digestibility related to biomass recalcitrance, filter paper, CMC, and cellobiose were used as substrates for the inhibition studies of cellulase cocktail, *endo*-cellulase, and β-glucosidase, respectively. The specific activity of the enzymes (U mg^{-1} enzyme) as a function of increasing T1, T2, and T3 prehydrolyzate concentrations are presented in Figures 1A, B, and C, respectively. The control consisted of 100% buffer solution and as expected exhibited the highest enzyme activity. For all three enzyme−substrate systems, decreases in enzymatic activity mirrored increases in hydrolyzate additions.

Effect of Inhibitors on β-Glucosidase, *endo*-Cellulase, and Saccharification Cocktail. β-Glucosidase was the least inhibited by the presence of prehydrolyzates. At an inhibitor concentration of 35 g L^{-1}, the cellobiase activity of NS 22118 was reduced by 53%, 62%, and 49% for T1, T2, and T3, respectively. Reductions in cellobiase activities were not significantly different between the treatment groups at any given inhibitor concentrations. It has been previously reported that β-glucosidase activity of Novozyme 188 was relatively unaffected by the derivatives of birchwood xylan.[11]

The CMC-ase activity of *endo*-cellulase decreased significantly as a function of increases in prehydrolyzate concentration. Addition of 15 g L^{-1} of T1, T2, or T3 prehydrolyzates resulted in 67%, 57%, and 77% reduction in CMC-ase activity, respectively. Addition of 20 g L^{-1} of T1, T2, or T3 prehydrolyzates to the enzyme system resulted in total loss of CMC-ase activity. The saccharification cocktail was equally susceptible to the addition of prehydrolyzates. Reduction in the filter paper activities of CTec2 was in the order of 64%, 68%, and 82% in the presence of 35 mg mL^{-1} of T1, T2, or T3 prehydrolyzates, respectively. Notably, the specific activity of both enzymes was significantly lower for prehydrolyzates stemming from T3 pretreatment compared to T1 and T2 ($P < 0.05$, $\alpha_{0.05}$), indicating that some phenolic and xylo-oligosaccharide compounds are more inhibitory to the cellulases.

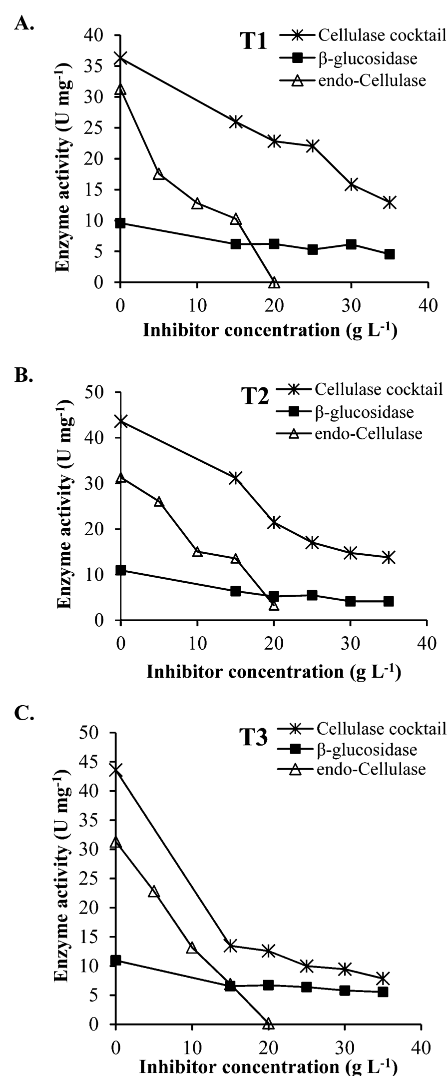

Figure 1. Specific activities of *endo*-cellulase (*A. niger*) at 0.04 mg enzyme g^{-1} of CMC, cellulase cocktail (Cellic CTec2) at 0.67 mg enzyme g^{-1} of filter paper, and β-glucosidase (NS 22118) at 3.49 mg enzyme g^{-1} of cellobiose in the presence of rice straw prehydrolyzates: (A) T1 (160 °C, pH 1.7), (B) T2 (180 °C, pH 2.25), and (C) T3 (220 °C, pH 7.0).

Characterization of Prehydrolyzates. The base peak chromatograms (BPC), which are obtained by plotting the signal of the most abundant ions, detected in each of a series of mass spectra as a function of retention time for T1, T2, and T3 are presented in Figures 2 and 3A. T1 and T2 prehydrolyzates had phenolic compounds like salicylic acid, syringic acid, vanillin, acetosyringone, *p*-coumaric, and *trans*-ferulic acid. Vanillin at 4 mg per g of protein reportedly caused 50% reduction in the cellulolytic activity of Spezyme CP (*T. reesei*).[9] Similarly, *p*-coumaric acid, vanillin, and ferulic acid at 1.5 mg per mg of protein were reported to cause 10%, 20%, and 30% reductions in cellobiase activity of Novozyme 188 (*A. niger*), respectively.[10] LC/ESI−MS analysis of T3 prehydrolyzate showed additional phenolic compounds compared to T1 and T2 as shown in Figure 3A. The compound 4-hydroxybenzoic acid that is also present in T3 was reported to be highly inhibitory to the cellobiase activity of Novozyme 188 at 1.5 mg per mg of protein.[9] Occurrence of 3,4-dihydroxybenzoic acid, 3-hydroxy-4-methoxycinnamic acid, and 2-furoic acid have been

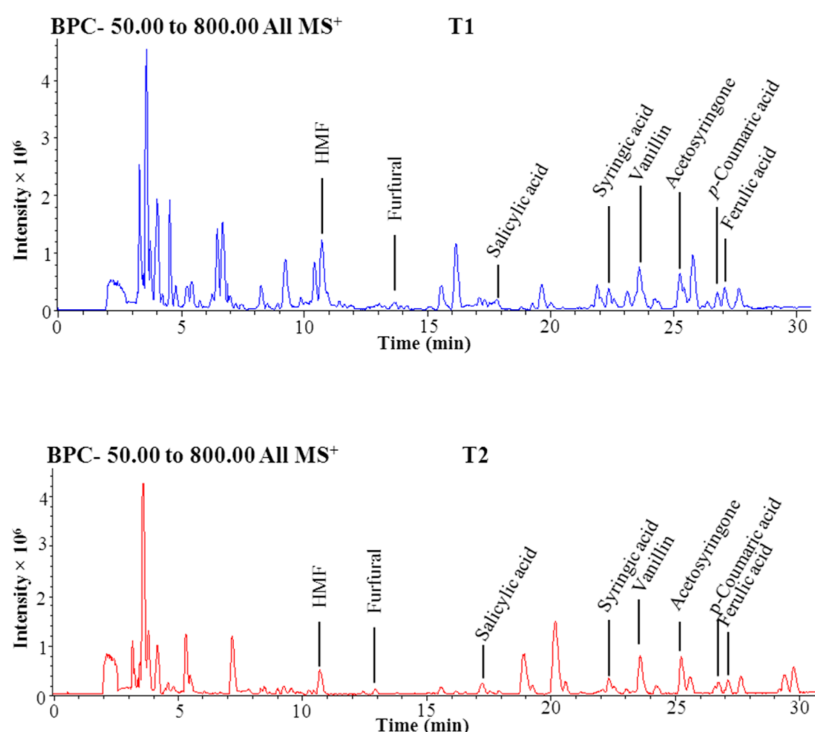

Figure 2. Base peak chromatograms (BPC) of freeze-dried rice straw prehydrolyzates, T1 (160 °C, pH 1.7) and T2 (180 °C, pH 2.25), dissolved in methanol and analyzed using LC/ESI−MS in a positive ion mode and m/z range from 50.00 to 800.00.

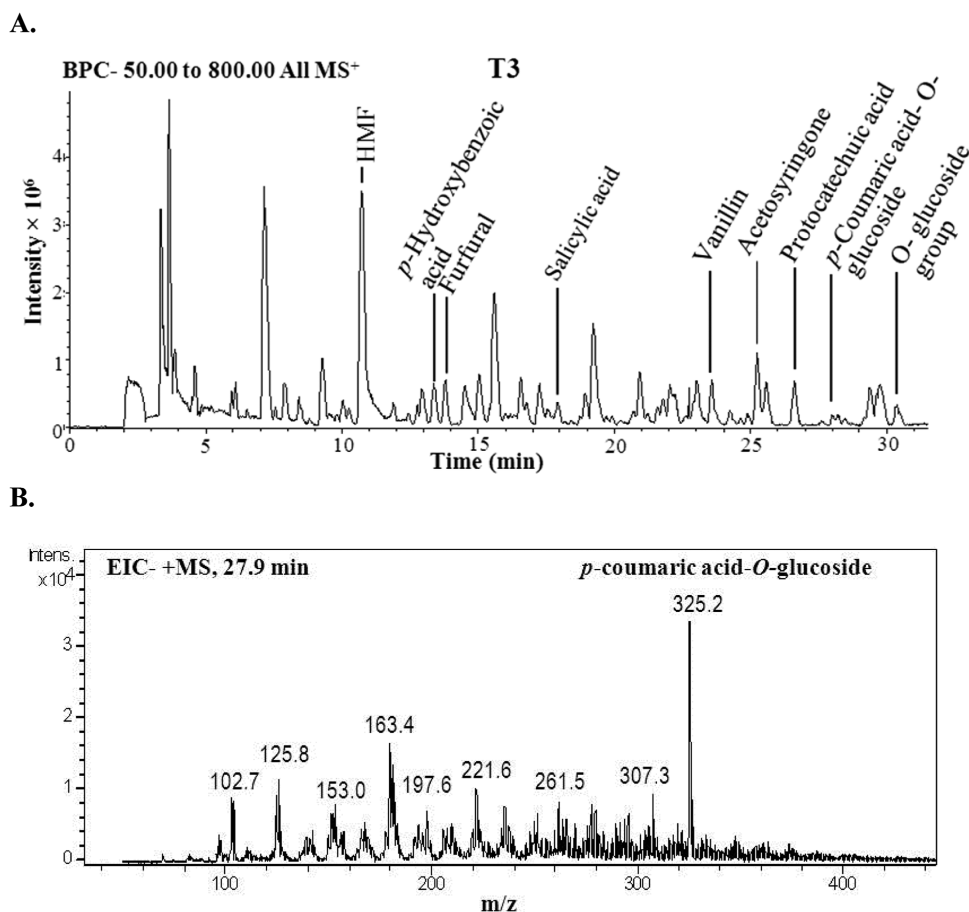

Figure 3. (A) Base peak chromatogram (BPC) for T3 rice straw prehydrolyzate (220 °C, pH 7.0). (B) Mass spectrum of p-coumaric acid-O-glucoside in sample T3 corresponding to BPC peak at 27.9 min.

reported in dilute sulfuric acid (0.7%) pretreated corn stover.[38] Compounds having identical $[M + H]^+$ ions were also detected in T3 prehydrolyzate at retention times of 11.85, 12.95, and 15.6 min, respectively. T3 also showed mass fragments corresponding to m/z 325 and m/z 163 as reported in Figure 3B. These masses can possibly be attributed to lignin−saccharide complexes, but further characterization will be required. Notably, compounds such as ferulic-*O*-glucoside and *p*-coumaric acid-*O*-glucoside detected in rice straw prehydrolyzates were reported to be possibly inhibitory to the cellulase system.[39] Moreover, lignin−carbohydrate complexes were reported in softwood prehydrolyzates, indicating that these complexes may be more prevalent than initially anticipated.[40] Therefore, the occurrence of lignin−carbohydrate complexes in rice straw (T3) prehydrolyzates and their effect on the activity of cellulases warrants further investigation.

■ CONCLUSION

The inhibitory effects of crude rice straw prehydrolyzates were determined using the filter paper activity of a saccharification cocktail, CMC-ase activity of *endo*-cellulase, and cellobiase activity of β-glucosidase. The β-glucosidase system was the least inhibited, and its cellobiase activity was not significantly different for all three pretreatments. On the other hand, both the cellulase cocktail and *endo*-cellulase showed significant reduction in their specific activities in the presence of only 15 g L^{-1} of either freeze-dried prehydrolyzates. It was determined that despite constant severities, the T1, T2 and T3 prehydrolyzates were heterogeneous in nature. T3, in particular, had significantly higher xylo-oligosaccharides and total phenolic content, which was attributed to the high temperature and neutral pH conditions of the pretreatment. On the basis of the prehydrolyzate composition of T3, xylo-oligosaccharides, phenolics, and their polymerization compounds could play important roles in inhibiting the cellulase cocktail.

Thus, characterization of crude prehydrolyzates and elucidation of their effects on individual cellulases will prove useful in identifying the key inhibitory compounds. Knowing which compounds inhibit enzymatic hydrolysis will enable the implementation of mitigation strategies that will result in an increase in process efficiency and sustainability.

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Notes

The authors declare no competing financial interest.

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