# Green Chemistry

Accepted Manuscript






ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/greenchem

# Investigating the role of lignin on biphasic xylan hydrolysis during dilute acid and organosolv pretreatment of corn stover

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## Abstract

One of the key objectives of biomass pretreatment is to maximize xylose yields. However, the kinetics of xylan hydrolysis appear to be governed by two parallel first-order reactions with one reaction much faster than the other thereby limiting both the rate and extent of xylan hydrolysis. Here, we investigate the influence of lignin on xylan hydrolysis kinetics during dilute acid pretreatment of corn stover rind (CSR) by modifying either the substrate or the pretreatment conditions. Dilute acid pretreatment was conducted to test the hypothesis that association of a fraction of the xylan with lignin causes this fraction to hydrolyze at a slower rate resulting in biphasic kinetics. Additionally, CSR was pretreated under organosolv (OS) conditions, where xylan and lignin were solubilized simultaneously, to decouple the hydrolysis of xylan from the lignin redistribution process that occurs during dilute acid pretreatment. Dilute acid pretreatment of CSR delignified under mild conditions still exhibited biphasic kinetics, although the fraction of slow hydrolyzing xylan decreased and the rate of fast hydrolyzing xylan increased by 60% resulting in achieving more than 95% total xylose yield. Pretreatment of CSR under OS conditions also appeared to exhibit biphasic xylan hydrolysis kinetics. Unexpectedly, the solubilization of xylan and lignin appeared to occur at similar rates. The increases in rate and fraction of fast hydrolyzing xylan observed by removing the majority of the lignin supports the hypothesis that the slow hydrolyzing xylan is caused by its association with lignin.

To further investigate the role on lignin on xylan hydrolysis, the raw and pretreated CSR samples were labeled with a monoclonal antibody (containing a fluorescent dye) that binds xylan specifically so that the location of xylan in the cell wall could be imaged by confocal laser scanning microscopy (CLM). CLM of pretreated delignified samples showed a similar, intense signal pattern as exhibited by the raw and pretreated control, indicating that the majority of the remaining xylan was located at both cytosolic and middle lamellar cell wall edges. OS pretreated CSR did, however, show a diminution in signal intensity at cell wall edges compared to CSR pretreated under standard dilute acid conditions.

**Keywords**: Xylan, biphasic kinetics, delignification, dilute acid pretreatment, organosolv pretreatment, corn stover rind, cell wall, confocal laser scanning microscopy, scanning electron microscopy

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## Introduction

A key technical barrier to the commercialization of fuels and chemicals from biomass via a sugar platform route is the high cost and relative inefficiency of producing sugars from lignocellulosic biomass [1-4]. Pretreatment of biomass with dilute acid has long been recognized as a promising technology for rendering the cellulose in biomass more accessible to saccharifying enzymes [1, 4-6]. Dilute acid pretreatment of biomass is an effective method of hydrolyzing the hemicellulosic components of biomass and increasing the accessibility of the remaining cellulose to enzymatic hydrolysis, thus increasing enzymatic hydrolysis rates and extents of hydrolysis compared to unpretreated biomass substrates [7-9]. However, a deeper understanding of hemicellulose hydrolysis kinetics during biomass pretreatment is required to optimize the overall conversion process [10, 11].

One may ask: why is the understanding of hemicellulose hydrolysis kinetics important to the conversion of biomass into biofuels? The answer is that a better understanding of the factors that govern hemicellulose hydrolysis kinetics may allow us to design pretreatment conditions where more of the xylan, the principal hemicellulose present in hardwoods and herbaceous species, can be hydrolyzed at the higher rate [12]. Moreover, sugar degradation products, furfural and polymeric products, are formed when sugars, particularly xylose, are exposed to the harsh conditions necessary to achieve high levels (> 90%) of xylan hydrolysis, which requires operating pretreatment during the slow phase of xylan hydrolysis. If xylan could be hydrolyzed more quickly, less xylose would be lost and less degradation products would be formed. Smaller, less costly pretreatment reactors would also be a consequence of this achievement [13].

Various researchers [14-20] in their studies of xylan hydrolysis have observed that the solubilization rate decreases significantly after reaching about 70% conversion. To explain this initially fast and then slow solubilization of xylan, they postulated that xylan consists of two fractions; that is, one fraction is easy to hydrolyze (fast solubilizing) and the other fraction is difficult to hydrolyze (slow solubilizing). This biphasic kinetics of xylan hydrolysis has been observed for several types of biomass, including hardwoods, grasses and agricultural residues [14, 17, 20, 21]. There have been a number of hypotheses for the heterogeneity in xylan hydrolysis (fast and slow solubilizing) [22-24]. First, a fraction of the xylan is located within the cell wall in such a way that it is rendered easily accessible to the action of acid, whereas a second fraction is located deep inside the cell wall possibly in greater association with cellulose chains that hydrolyzes slowly. Second, the ratio of acetyl groups and uronic acid moieties to xylose influences the susceptibility of the xylan fraction towards solubilization [14, 25]. Third, due to the presence of lignin-carbohydrate bonds, a portion of xylan could be intimately associated with or embedded in the lignin matrix [14, 26]. The reported values for the fraction of fast-solubilizing xylan vary for different biomass species and range from 60 to 80% [14, 15, 22, 24, 27].

The overall purpose of this work was to determine the cause of the biphasic kinetic behavior that has been observed for xylan hydrolysis under dilute acid pretreatment conditions. Our objective in performing this research has been to gain a more detailed understanding of the mechanism of xylan hydrolysis within the plant cell wall during dilute acid pretreatment.

In this work, the influence of lignin on xylan hydrolysis kinetics during dilute acid pretreatment of corn stover rind (CSR) was assessed by modifying either the substrate or the pretreatment conditions. First, a series of dilute acid pretreatment experiments were performed on CSR samples that had been 70% delignified prior to pretreatment by mild treatment with acid chlorite, a bleaching agent. Second, the effect of lignin on xylan hydrolysis kinetics was also studied by conducting pretreatments under novel organosolv (OS) conditions[28], in which the lignin and xylan

were solubilized simultaneously [28-30]. The use of organosolv conditions was meant to decouple the hydrolysis of xylan from the lignin redistribution process that occurs in dilute acid pretreatment [31, 32]. It was expected that operation under conditions where lignin and xylan were simultaneously solubilized would decrease if not remove the biphasic phenomenon. Finally, another test of the biphasic kinetics behavior was made by treating isolated xylan (extracted from corn stover) and beechwood xylan under conditions similar to those used in dilute acid pretreatment.

## Materials and Methods

### Corn stover rind

CSR was used throughout this study as a model lignocellulosic substrate because of its relatively uniform composition and structure compared to corn stover or even isolated stems. Corn stems were hand-cut from a single field on the Gustafson Farm in Weld County, CO in mid-January 2005. The corn plants were a Round-Up Ready Pioneer hybrid, 36N18, and had completely field-senesced. Stalks were air dried to a moisture content of approximately 10% prior to use. CSR "matchsticks" (0.5 cm × 2 cm) were cut by hand after peeling rind from corn stems cut between nodes. Extractives-free CSR was prepared according to the NREL procedure [33] using the Dionex ASE system. Delignified CSR was prepared using a modification of the acid chlorite method described elsewhere [34]. Initially, delignified CSR was prepared under the standard conditions, acid chlorite bleaching at 70°C for 60 min. Compositional analysis showed that the CSR was completely delignified, but there was also a 30% loss of xylan. In addition, inspection by SEM revealed that the morphology of the cells was significantly altered, with the cells appearing to be swollen (data not shown). Therefore in this work, it was decided to use a less severe delignification treatment that gave < 10% xylan loss and resulted in no observable change in cell morphology. In the modified method, 2 g of CSR was added to 20 g of de-ionized (DI) water containing 0.23 g $NaClO_2$/g water. The chips were vacuum impregnated and 0.2 mL of concentrated HCl was added to initiate the formation of $ClO_2$. The mixture was treated at 25°C for 4 h. Following the treatment, the delignified CSR was filtered on a Buchner funnel, washed extensively to completely remove any $ClO_2$ that had penetrated into the rind, and then freeze dried. It was found that using the modified method, 70% lignin removal could be achieved with < 10% loss of xylan. Control CSR was prepared by soaking 2 g of CSR in 20 g of DI water containing 0.2 mL of concentrated HCl at 25°C for 4 h followed by washing and freeze drying. Compositional analysis of the extractives-free, delignified-control, and delignified CSR is given in Table 1.

### Isolated xylan from corn stover

The method of extracting the xylan was selected so that the xylan was isolated in near native state with the majority of its acetate and phenolate groups still attached. Briefly, whole corn stover was milled, extracted, bleached with acid chlorite, and freeze dried according to the method described elsewhere [34]. The dried material was extracted with dimethyl sulfoxide (18 mL/g biomass) at room temperature overnight with constant stirring. The DMSO extract was removed by filtration through polypropylene filter (70 μm) and diluted to 20% (v/v) with anhydrous ethanol acidified with concentrated HCl in a ratio of 0.5 mL/ L of final solution. After thorough mixing, the precipitated xylan was allowed to settle at 4°C overnight. The xylan, which was a gel like material, was filtered, washed and then dried sequentially with 100% ethanol then diethyl ether. The ether was allowed to evaporate and the filtered material was macerated in warm water and filtered through a polypropylene filter with small additions of warm water. The resulting

filtrate (isolated xylan) was lyophilized and stored at room temperature for future experiments. The beechwood xylan was purchased from Sigma-Aldrich.

### Dilute acid pretreatment

CSR (approximately 0.5 g per experiment in the form of 0.5 cm × 2 cm ‘‘matchsticks’’) was pretreated in small glass reactors (10 mL) with 1.2 wt% dilute sulfuric acid (approximately 6.4 mL of 0.12 M). The glass reactors were sealed with rubber septa and then placed in small steel autoclaves along with sufficient water (10-15 mL) so as to balance against the pressure generated within the glass reactors during heating. The autoclaves were rapidly heated to the desired reaction temperature (i.e., 130, 140 or 160°C) in approximately 90 s by immersion in a sand bath at 40°C above the target temperature. The reactor was then rapidly transferred to a second sand bath maintained at the target temperature where it remained for the desired reaction period (2.0 min). The autoclave was then removed from the sand bath and rapidly cooled to room temperature by immersion in cold water. After the autoclave had cooled, the glass reactors were removed, opened, and the hydrolyzate was removed. Fresh acid was then added to wash the treated CSR. The original hydrolyzate and wash were combined and then brought to 25 mL in a volumetric flask. The combined hydrolyzate and wash were analyzed for the sugars solubilized by the acid treatment. For reactions that were continued past 2 min, fresh acid solution was added to the reactor and CSR and the autoclaves/reactor then went through another cycle of heating, cooling, emptying, washing, and refilling. These 2 min cycles were repeated as many times as were necessary to reach the desired reaction time; that is, for a 10 min reaction time, the 2 min cycle was repeated five times. Xylan isolated from corn stover was also treated with dilute sulfuric acid at 120, 140 and 160°C in 2 min reaction time increments using the same process as adopted for the treatment of CSR except the treatments were conducted in glass centrifuge vials (10 mL). Hydrolyzate obtained after treatment of the xylan was removed after centrifuging the vials at 1,500 rpm for 3 min at room temperature in a swing-bucket centrifuge.

Acid catalyzed pretreatment experiments on beechwood xylan were carried out at temperatures ranging from 70 to 100°C in batch mode using a Discover S-Class microwave reactor (CEM Explorer, Matthews, NC) outfitted with a 48-position auto-sampler. This reactor system is equipped with a computer-controlled temperature and pressure feedback system, which is used to regulate the microwave power for rapid heating and constant temperature. The temperature inside the reactor was monitored by measuring the infrared emission from the bottom of the reactor tube and pressure was measured with a transducer placed on the top of the reactor. Pressure and temperature values read into the software were then used in a feedback control algorithm to maintain a constant temperature. Microwave frequency radiation of 230 MHz and a maximum pulsed-power of 200 W were used to heat the samples. Temperature, pressure and microwave power profiles were acquired using CEM’s Synergy software version 1.27. Samples were pretreated at 1% solids in 1.2 wt% $H_2SO_4$ (0.122 M $H_2SO_4$, Ricca Chemical Company) in glass reactor tubes. After addition of acid, the reactor tube was capped, weighed and then placed in a vacuum oven (Fisher Scientific Model 280) with 19 inches Hg vacuum pressure (0.6 bar) at room temperature for 24 h to remove air pockets within the wood and enable adequate soaking of acid into the biomass particles. During microwave pretreatment, samples were pre-stirred at the low speed setting for 30 s and continuously stirred throughout the experiment. The temperature was maintained within ±2 °C of the target temperature. After completion of the reaction, samples were cooled to 60 °C via a stream of compressed air that was blown onto the reactor tubes at the end of each run. Samples were kept at room temperature until the sequence was complete after which the samples were stored at 4°C for further analysis. The work-up of the hydrolyzates obtained after treatment of the isolated and beechwood xylan was the same as for products from dilute acid pretreatment of CSR.

### Organosolv (OS) pretreatment

The OS pretreatment was performed following the same experimental procedure as described above for the dilute acid pretreatment. To perform the OS pretreatment experiments, a solvent was used which consisted of water and acetone in combination with methyl isobutyl ketone (MIBK) in the following ratio: MIBK/acetone/water (11/44/44, g/g/g, 100 mL). The sulfuric acid concentration remained at 1.2 wt%. The work-up of the hydrolyzates obtained after OS pretreatment experiments was different from the dilute acid pretreatments because of the lignin dissolved in the pretreatment hydrolyzate in addition to the hemicellulose sugars. After the pretreatment, the lignin in each sample of collected hydrolyzate was separated by forcing the MIBK to phase separate from the acetone and water. This was achieved by adding MIBK in a 1:1 ratio with the volume of the pretreatment liquor. A dark upper phase was formed containing lignin and MIBK which was removed. The bottom layer was further extracted with a second equivalent of MIBK and then the combined MIBK extracts were extracted three times with equivalent volumes of water to ensure that all sugars were removed from the MIBK. The water washes were combined with the aqueous acetone liquor and analyzed for sugars as previously. The MIBK extract was dried over anhydrous magnesium sulfate and then evaporated under a stream of nitrogen at 40°C followed by a final drying under vacuum. The dried extract was weighed and the weight was used to quantify the amount of lignin solubilized during the pretreatment.

### Hydrolyzate analysis

The concentrations of monosaccharides in the hydrolyzates were determined using a Dionex ICS-2500 Ion Chromatograph with a Carbopac PA-20 anion exchange column and a pulsed amperometric detector (ED-50). Separation of the monosaccharides was accomplished using a potassium hydroxide concentration of 2 mM provided by an EG-50 eluant generator. After elution of the monosaccharides (about 15 min), the column was regenerated with 100 mM potassium hydroxide for 5 min. The column was then allowed to re-equilibrate with 2 mM potassium hydroxide for 15 min before the next injection. Total sugar concentrations were determined by a modification of the standard analytical protocol [35]. Duplicate 2 mL samples of hydrolyzate were acidified to 4 wt% sulfuric acid by addition of 72 wt% sulfuric acid (68.6 μL). Sugar recovery standards were prepared on the same scale. The samples were placed in glass vials that were then sealed with crimp caps and hydrolyzed in an autoclave for 1 h at 121°C. The hydrolyzed samples were also analyzed on the Dionex ICS-2500 system. The pH of the hydrolyzate was measured after each reaction cycle and fresh acid was added to the reactor and CSR to ensure adequate availability of acid during each reaction cycle. The plot of pH vs. time for pretreatment hydrolyzates of corn stover pretreated under dilute acid and organosolv conditions is provided in Figure S1. This figure clearly shows that the pH was constant after each reaction period and acid available in adequate amount for the reaction.

### Embedding and sectioning

Several 3 mm wide sections of pretreated CSR were cut and dehydrated in a graded ethanol series moving in 10% increments from 50% to 100% ethanol. LR White resin was serially added, and the sections were left overnight in 100% LR White. For optimal infiltration, samples were placed in a vacuum microwave oven for 1 and 5 min during each ethanol and resin concentration rinse, respectively. After 2 days, the samples were transferred to flat-bottomed ‘‘BEEM type’’ capsules and filled with 100% LR White. Polymerization occurred overnight in a vacuum oven set to 60°C. Resin blocks were trimmed with a Leica EM Trim trimmer and 1.8 mm sections were cut using glass knives and a Leica ultracut UCT ultra microtome. Glass knives were made from a 6 mm glass bar and cut on a LKB Bromma 7800 KnifeMaker.

### Confocal Laser Scanning Microscopy (CLM)

CLM images of stained CSR samples magnified 600 x were obtained using a Nikon Eclipse E800 Laser Confocal Microscope.

### Scanning Electron Microscopy (SEM)

SEM images of CSR samples were obtained using a FEI Scanning Electron Microscope.

### Immunolocalization of Xylan

For confocal microscopy, 1.8 mm sections of CSR were first blocked with BSA to minimize non-specific binding and then incubated with the monoclonal antibody against xylan, LM11 [36] and an Alexa fluor 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA). The fluorescence-labeled xylan signals were observed using the Nikon confocal microscope. Autofluorescence and non-specific signal was minimized by utilizing samples washed with the Alexa fluor 488 secondary only and photo multiplier tube (PMT) gains were set so that autofluorescence from the plant tissue; as well as any non-specific antibody binding were invisible in the image.

The epitope for LM11 consists of four xylose residues and seems to be able to accommodate both unsubstituted xylan backbone; as well as substituted xylan backbones, such as arabinoxylan. The LM10 epitope was found to be just two xylose residues, which limits its targets to unsubstituted xylan backbones [36].

## Kinetic models for xylan solubilization

The hydrolysis of xylan is a hydronium-ion catalyzed reaction, which results in a random cleavage of polymer chains [37]. As shown in Figure 1a, the simplest model used to describe the kinetics of xylan hydrolysis is based on the first-order reaction model proposed by Saeman [38] for cellulose hydrolysis. According to this scheme, xylan is hydrolyzed to xylose, which reacts further to degradation products, i.e., furfural, by dehydration. Based on this schematic of xylan conversion (Figure 1a), a differential equation for the hydrolysis and solubilization of xylan can be written assuming a first-order dependency of the reaction rate on xylan concentration. First-order reaction kinetics for xylan solubilization is thus described by the following equation:

$$\frac{-d\left[Xylan\right]}{dt} = k_1\left[Xylan\right] \tag{1}$$

By integration of equation (1) we get,

$$\ln\left[\frac{\left[Xylan\right]_t}{\left[Xylan\right]_{t=0}}\right] = -k_1 t \tag{2}$$

or $$\left[-\ln\left(1 - Xylan\,Conversion\right)\right] = k_1 t \tag{3}$$

To determine whether xylan hydrolysis can be described by first-order reaction kinetics, xylan conversion data must be plotted as [– ln (1 – *Xylan Conversion*)] against pretreatment time (*t*). First-order reactions plotted in this way should give straight lines where the slope is the first-order rate constant ($k_1$).

The model used to describe the kinetics of xylan hydrolysis during dilute acid pretreatment in this and most others' work [14, 15, 17, 22] is based on the scheme shown in Figure 1b. In the development of the model, the following assumptions were made: (i) corn stover xylan is composed of two fractions, one being more easily hydrolyzed called "fast hydrolyzable xylan," and the other fraction being difficult to hydrolyze called "slow hydrolyzable xylan;" (ii) random cleavage of glycosidic bonds on the backbone of the xylan polymer results in the formation of only xylooligomers which, on further hydrolysis in the aqueous phase, give xylose monomer; (iii) xylose monomers undergo dehydration reactions to yield furfural; and (iv) all reactions are irreversible.

The xylan hydrolysis reaction is modeled as two parallel pseudo-first order reactions [14]. Equations (4) and (5) are individual mass balance equations for the concentrations of fast and slow hydrolyzing xylan, respectively, that are assumed to have a first-order dependence of the reaction rate on reactant concentration, where $[X_f]$ and $[X_s]$ are the concentrations of fast and slow hydrolyzing xylan, respectively, in the solid phase after any reaction time ($t$), and $[k_f]$ and $[k_s]$ are the first-order rate constants for the fast and slow reactions, respectively.

$$\frac{dX_f}{dt} = -k_f X_f \tag{4}$$

$$\frac{dX_s}{dt} = -k_s X_s \tag{5}$$

Equations (4) and (5), upon integration and rearranging, yield

$$\left(1\text{-Conversion}\right) = z \cdot \exp\left(-k_f t\right) + \left(1 - z\right) \cdot \exp\left(-k_s t\right) \tag{6}$$

where $z$ is the fraction of original xylan identified as the fast-hydrolyzing xylan.

## Results and discussion

### *Dilute acid pretreatment of extractives-free CSR*

The dilute acid pretreatment experiments with extractives-free CSR were conducted at temperatures of 130, 140 and 160°C with 1.2 wt% sulfuric acid. The experimental data for xylan solubilization obtained during pretreatment of extractives-free CSR is presented in Figure 2a. From Figure 2a it can be seen that for all three temperatures, xylan solubilization was initially rapid and then slowed down after achieving 60 to 80% xylan removal. These results of initially fast- followed by slow-hydrolyzing xylan corroborate earlier findings[14, 15, 17].

The kinetics of xylan hydrolysis for all the pretreatment experiments are shown in Figure 3. The points show the experimental xylan conversion data plotted as [– ln (1 – *Xylan Conversion*)] against pretreatment time ($t$) (using equation 3). The lines show predictions for xylan solubilization obtained using the biphasic rate equation (6). From Figure 3a (dilute acid pretreatment only) it can be seen that for all three pretreatment temperatures, xylan solubilzation exhibited biphasic behavior, i.e., xylan solubilization could be described by two simultaneous first-order reactions, an initial fast reaction followed by a slower reaction. The kinetic parameters, i.e., the rate constants and the fractions of fast hydrolyzing xylan are reported in Table 2. The kinetic parameters are calculated by fitting equation (6) to the xylan conversion data by

minimization of the sum of the squares of the deviation between model prediction and experimental data. For the optimization, the SOLVER function of MS-EXCEL was used. The data in Table 2 show that as expected $k_f$ (rate constant for the hydrolysis of the fast-fraction) increased as the pretreatment temperature was increased; however, it was also observed that the fraction of fast hydrolyzing xylan ($z$) also increased. Because the fraction of rapidly hydrolyzing xylan increased with pretreatment temperature, it is unlikely that the cause of biphasic kinetics is due to the presence of two chemically distinct xylans that have different hydrolysis rates. It seems more likely that the biphasic phenomenon might be due to mass transport limitations [8, 39, 40], that retard xylan hydrolysis. Based on this evidence another plausible reason for slow xylan hydrolysis may be its accessibility [8], i.e., a portion of xylan may be intimately associated with other cell wall materials [41].

### *Dilute acid pretreatment of delignified CSR*

To test the hypothesis that association between lignin and xylan, due to the presence of lignin-carbohydrate bonds, has a role in the biphasic behavior of xylan hydrolysis kinetics, dilute acid pretreatment experiments were performed on delignified CSR. The experimental data and the kinetics of xylan hydrolysis obtained during pretreatment of control and delignified CSR are presented in Figures 2b and 3b, respectively. By comparing Figures 2a and 2b it can be seen that pretreatment of extractives-free CSR at 160°C for 2 min resulted in the solubilization of only 60% of the initial xylan; whereas the similar pretreatment of delignified CSR resulted in solubilization of 80% of the initial xylan. It is evident from Figures 2a and 2b that during the pretreatment of delignified corn stover rind, xylan is solubilized at a much faster rate after removing the lignin by acid chlorite bleaching.

The kinetic parameters obtained for dilute acid pretreatment of control and delignified CSR are given in Table 2 and the kinetics plots are shown in Figure 3b. From Table 2 and Figure 3b, it can be seen that delignification increased the fraction of fast hydrolyzing xylan ($z$). Moreover, a three to five fold increase in the rate constant for the hydrolysis of the slow xylan ($k'_s$) fraction was observed and up to 60% increase in the rate constant for the hydrolysis of the fast-fraction ($k'_f$). Although the fraction of fast hydrolyzing xylan increased and the hydrolysis rate constants also increased resulting in achieving more that 95% total xylose yield, the presence of biphasic kinetics was still observed. This result may be explained by the presence of residual lignin (about 30% of the initial insoluble lignin) and its association with the xylan. Additional pretreatment experiments with more extensively delignified CSR were not attempted because more severe delignification also resulted in hydrolysis and loss of a significant amount of the xylan.

### *Organosolv (OS) pretreatment of CSR*

#### Xylan hydrolysis during OS pretreatment of CSR

To further explore the hypothesis that lignin has a role in the biphasic behavior of xylan hydrolysis kinetics, pretreatment experiments were performed on CSR under OS conditions. The experimental data for xylan solubilization obtained during OS pretreatment are presented in Figures 2c and 3c. From Figure 2c, it can be seen that at 160°C xylan solubilization levels off at about 71%; whereas at 140°C, a maximum xylan solubilization of 77% was achieved. It can be observed from Figures 3a, 3b and 3c that for dilute acid pretreatment of extractives-free CSR, delignified CSR, and OS pretreated CSR, respectively, xylan hydrolysis shows biphasic behavior even when a majority of the lignin has been removed or is being solubilized at the same time as the xylan. The estimated kinetic parameters for xylan hydrolysis during OS pretreatment of CSR

are given in Table 2. It can be seen that OS pretreatment causes a slight increase in the rate constant for the fast-hydrolyzing fraction ($k_f$).

### Lignin solubilization during OS pretreatment of CSR

One of the primary advantages of conducting an OS pretreatment is that it results in the simultaneous solubilization of xylan and lignin. The solubilization of lignin was also examined to see if it followed first-order kinetics. As can be seen in Figure 4, it is apparent that the solubilization of lignin does not follow first-order kinetics, but shows similar kinetics to xylan hydrolysis. Lignin solubilization was treated as if it followed biphasic kinetics so that the kinetic parameters for lignin solubilization could be compared to those for xylan hydrolysis during organosolv pretreatment. These values are given in Table 3. From this analysis, the following two important observations can be made:

- The $z$ values for the fast hydrolyzable fraction for both xylan hydrolysis and lignin solubilization are comparable.
- The rate constant, $k_f$, for the fast hydrolyzable fractions of both xylan and lignin at 140°C are similar, indicating that at 140°C, xylan and lignin are initially solubilized at the same rate.

The similarities in the rates of xylan and lignin solubilization suggest that the processes involved in separating these biopolymers from the cell wall are closely linked. In native corn stover, as in other plant materials, it is believed that parts of the xylan and lignin are covalently linked through ether and ester bonds involving phenolic acids that are generally considered part of the xylan [42, 43]. During hemicellulose hydrolysis, it is believed that these linkages are mostly severed and that glycosidic linkages in xylan are hydrolyzed to the point where soluble xylooligomers are formed resulting in xylan solubilization [43]. The results of our research suggest that the rapidly hydrolyzing xylan fraction is not associated with other cell wall components, whereas the slowly hydrolyzing fraction is associated with lignin and possibly other cell wall components. The purpose of the OS pretreatment experiments and the pretreatments of delignified CSR were to test this hypothesis. It was expected that if the hypothesis was correct, then removal of the lignin prior to pretreatment or the simultaneous removal of lignin by OS pretreatment would result in removal of the biphasic phenomenon and that normal first-order kinetics would be observed. This was not observed, in both cases biphasic kinetics remained, although the kinetic rates were changed. In the OS pretreatments, the similarity of the rates of lignin and xylan solubilization suggest that under OS conditions, lignin and xylan remain linked during their separation from the cell wall [43]. In the pretreatment of delignified rind, we may have been limited by incomplete delignification. It is possible that the lignin that was removed came from the middle lamella (probably the most easily removed lignin) where there is little xylan. The lignin remaining behind may have been the lignin in the secondary cell wall that is most closely associated with xylan.

## *Dilute acid treatment of isolated and beechwood xylan*

To further examine our hypothesis that lignin has a role in determining the kinetics of xylan hydrolysis, experiments were performed on xylan isolated from corn stover and beechwood xylan. The method of extracting the xylan was selected so that the xylan was isolated in near native state with the majority of its acetate and phenolate groups still attached. The experiments on isolated xylan were performed under conditions similar to those used for pretreatment of CSR, i.e., temperatures of 120, 140 and 160°C with 1.2 wt% sulfuric acid. The xylan solubilization data obtained from these experiments are shown in Figure S2a. It was observed that the maximum xylan solubilization at all temperatures was about 100%. At all temperatures hydrolysis of the

isolated xylan was very rapid with greater than 90% conversion achieved in less than 4 min even at the low temperature of 120°C.

Analysis of the kinetics of the hydrolysis of isolated xylan (Figure S2b) showed that it appeared to follow first-order kinetics at least up to 90% xylan conversion. As can be seen in Figure S1b, treatment of isolated xylan with dilute acid does not show the biphasic behavior of xylan hydrolysis (up to 90% conversion). The rate constants for the hydrolysis of isolated xylan are obtained by using equation (3) and are given in Table 4. The rate constants for xylan hydrolysis in Tables 2 and 4 show that the rate of hydrolysis of isolated xylan is much faster (two to three-fold) than obtained for the xylan still present in the cell wall.

Since the hydrolysis of the isolated xylan was very rapid with greater than 90% conversion achieved in less than 4 min even at the low temperature of 120°C, to investigate xylan hydrolysis kinetics at lower conversion yields additional experiments were performed at much lower temperature, i.e., 70 to 100°C. Also, since the presence of acetyl groups on isolated xylan from corn stover renders it water soluble, which may further influence its hydrolysis rates, low temperature hydrolysis were performed on beechwood xylan which is insoluble in water and behaves similar to biomass particles. The xylan solubilization data obtained from these experiments are shown in Figure 2d. It was observed that at 70 and 80°C, the xylose yields increase with the reaction time linearly; however, at 90 and 100°C, xylan undergoes rapid hydrolysis with more than 90% conversion achieved in the first 10 min of the reaction period. Analysis of the kinetics of the hydrolysis of beechwood xylan (Figure 3d) showed that at all four temperatures studied, first-order kinetics appeared to be followed thus corroborating the hydrolysis results obtained with corn stover isolated xylan. The rate constants for the hydrolysis of beechwood xylan are obtained by using equation (3) and are given in Table 5. A comparison of the rate constants for xylan hydrolysis in Tables 2 and 5 shows that the rate of hydrolysis of beechwood xylan at 100°C is in the same order of magnitude as the rate obtained for the xylan still present in the cell wall at 140°C, suggesting that plant cell wall components play a role in retarding the xylan hydrolysis rates.

The purpose of these experiments was to determine if xylan isolated from the cell wall, away from the influence of the other cell wall biopolymers and the possible effects of cell architecture, such as compartmentalization, would still exhibit biphasic behavior. In these experiments xylan hydrolysis did not exhibit biphasic kinetics which supports the hypothesis that lignin or other chemical or structural cell wall components play a role in causing the biphasic effect. In addition, in the corn stover isolated xylan as the xylan retained the majority of the acetate and phenolate groups it may be inferred that these groups are not the cause of biphasic kinetics obtained for the xylan still present in the cell wall.

### *Imaging of delignified and organosolv pretreated CSR*

Both CLM and SEM were used to study the effects of pretreatment on CSR. The presence of xylan was visualized in CLM utilizing the fluorescently labeled monoclonal antibody (LM11) which binds to surface exposed xylan present in sectioned pieces of rind.

#### Dilute acid pretreatment of delignified CSR

A feature of the delignified samples was an apparent increase in the brightness of the CLM images (Figure 5a-c), which was attributed to an increase in accessibility of the antibody to xylan due to removal of the lignin. Compared to control samples, the delignified samples had regions

of slightly higher signal intensity at the same gain levels indicating a higher level of bound antibody.

It was anticipated that the higher level of antibody signal around the edges of cell walls in CLM images of pretreated control samples might be diminished or even disappear in the pretreated CSR that had been delignified. Our hypothesis was that this phenomenon (bright cell wall edges) was caused by association of xylan with the lignin droplets that have been observed to adhere to the outer edges of cell walls in dilute acid pretreated CSR. CLM of the control samples that were just treated with HCl at room temperature for 4 h, and the acid chlorite delignified CSR that was pretreated with dilute sulfuric acid, are shown in Figure 6. In panels (a) and (c), the center of the cells of pretreated control samples show decreased signal intensity and the outer walls of the cells show higher intensity, which is a phenomenon that has previously been observed in CLM images of dilute acid pretreated rind samples [44]. In the pretreated control CSR shown in panel (a) (140°C for 2 min) the images have a much higher overall intensity. However, in the pretreated control CSR shown in panel (c) (160°C for 2 min) the higher level of xylan removal make the images quite dark. In the delignified CSR pretreated at 140°C for 2 min, shown in panel (b), signal intensity is generally higher and is more uniformly spread throughout the cell walls. This result has been interpreted as both an effect of mobilizing the xylan fraction out of the cell wall due to the pretreatment; as well as an increase in accessibility of the antibody to the xylan because of lignin removal. In the delignified CSR pretreated at 160°C for 2 min, shown in panel (d), signal intensity is decreased compared to both the control and the delignified CSR pretreated at 140°C. This is believed to be due to the much higher level of xylan removal at 160°C (77% xylan removal) compared to 140°C (56% xylan removal) resulting in a lower amount of xylan available for binding with LM11.

Previous work conducted at NREL has shown that the spherical droplets present on residual solids of dilute acid pretreated corn stover are, at least in part, lignin derived [32, 44]. In Figure 7, SEM images (500X) show the gross morphological changes due to dilute acid pretreatment of extractives-free, control and delignified CSR and OS pretreated CSR at 160°C for 2 min. Figure 7 clearly shows an increase in the gross accessibility of delignified and OS pretreated CSR shown in panels (c) and (d), respectively. The pretreated extractives-free and control samples (panels (a) and (b)) show vessel cells to which residues of parenchyma cells are still attached, whereas panels (c) and (d) show clean well-separated vessel cells due to more selective removal of the cell wall components (xylan and lignin) from the composite middle lamella that occurred during the dilute acid pretreatment of delignified CSR and OS pretreatment. In Figure 8, SEM images (5000X) show changes in the dilute acid pretreated control and delignified CSR pretreated for 2 min at 140 and 160°C, respectively. In the control CSR pretreated at 140°C for 2 min, a slightly delaminated cell wall structure is observed that is covered with a fine layer of uniformly distributed lignin droplets [panel (a)]. The increased delamination and presence of broken parenchyma cells and larger lignin droplets in the control CSR pretreated at higher severity (160°C for 2 min) is clearly observed in panel (c). However, as shown in panels (b) and (d) increased delamination and defibrillation [zoomed section in panels (b) and (d)] was observed in the delignified CSR due to more cell wall component removal. In the delignified CSR pretreated at higher severity [160°C for 2 min– panel (d)], lignin droplets can be seen despite the lower amount of lignin (30% of the original lignin content) available for coalescence when compared to control CSR.

It is well known that the majority of the lignin is present in the middle lamella where it acts as a binder that holds the fibers together. There is; however, little xylan in the middle lamella and interactions between and xylan and lignin most likely occur in the secondary cell wall where they are closely bound together. It appears likely that the acid chlorite delignification used in this work resulted in removal of the majority of the lignin present in the middle lamella and little or

none of the lignin present in the secondary walls. Thus, there was still sufficient lignin remaining for droplet formation; however, the removal of the lignin present in the middle lamella resulted in less recalcitrant plant cell walls with enhanced susceptibility for xylan hydrolysis during pretreatment. Consequently, pretreatment of delignified CSR resulted in more xylan removal and increased xylan hydrolysis rates. Unfortunately, the presence of lignin in delignified CSR (~33%) and its association with xylan, probably in the secondary cell wall, prevented us from using this approach as a definitive test of the role of lignin in causing biphasic xylan hydrolysis kinetics.

### Organosolv pretreatment of CSR

In Figure 9, comparing panels (b) and (d) with panels (a) and (c), respectively, there appears to be a change in the distribution of signal intensity in the OS pretreated CSR. Moreover, there appears to be more signal throughout the cell wall with less intensification of the signal at the cell wall edges than is observed in CSR pretreated without the organic solvent.

In contrast to the dilute acid pretreatment of delignified CSR, OS pretreatment produced the anticipated decrease in signal intensification around the edges of cell walls. It appears that by solubilizing lignin and xylan simultaneously, there has been at least a decrease in association between xylan and lignin. However, the kinetics of xylan hydrolysis still showed evidence of biphasic behavior, although there was a slight increase in the rate of xylan hydrolysis in the OS pretreatments.

There is a distinct difference in the appearance of cells in SEM images that have undergone delignification either prior to or during pretreatment. In Figures 7 and 10 panels (a) and (b) the associated cells appear contiguous and although there is some slight cell wall delamination, they are not greatly changed. In images of the pretreated delignified CSR and OS pretreated CSR samples (panels (c) and (d), respectively) the cells have largely separated into individual vessel cells indicating that the lignin and associated structures holding them together have been extracted. The residual cell structure is somewhat degraded but is mostly still intact, as mentioned earlier the majority of the lignin that is in the middle lamella which holds the cells together has been extracted producing the separated structure observed. The difference between images (c) and (d) is that in the pretreated delignified sample it is still possible to see droplets formed on the surface of the cells, whereas in the organosolv pretreated sample, the cells are clear of droplets. As was expected during organosolv pretreatment, lignin separates from the cell matrix and then dissolves in the aqueous organic solvent so that no droplets form. However, this approach did not prove to be sufficient to remove the biphasic effect from xylan hydrolysis kinetics, instead the rates of xylan hydrolysis and lignin dissolution appeared to be closely linked.

## Conclusions

The objective of performing this research was to test the hypothesis that association of a fraction of the xylan with lignin caused this fraction to hydrolyze at a slower rate, resulting in biphasic kinetics. Experiments performed where lignin was removed either before or during pretreatment were expected to remove or at least significantly diminish the kinetic biphasic effect. In addition, if the hypothesis were correct, the relatively intense signal observed at the cell wall edges, in CLM images of pretreated CSR labeled with a fluorescently tagged xylan antibody, should have disappeared or at least significantly diminished. The following important conclusions can be drawn from this work. Dilute acid pretreatment of CSR delignified (by 70%) under mild conditions with acid chlorite still exhibited biphasic kinetics, although the fraction of slow hydrolyzing xylan decreased and a three- to five-fold increase in the rate constant for the

hydrolysis of the slow fraction of xylan ($k_s$) was observed resulting in achieving more than 95% total xylose yield. CLM of the pretreated delignified CSR still showed brighter signals at the edges of the cell walls, as did the CLM of untreated and control pretreated CSR, although this was not conclusive because of the general brightening of images that had been delignified. Higher levels of delignification could not be obtained without significantly affecting the xylan content of the delignified rind and the morphology of the cell walls. Pretreatment of CSR under OS conditions, where xylan and lignin were solubilized at the same time, also appeared to exhibit biphasic xylan hydrolysis kinetics with solubilization of xylan and lignin appearing to occur at similar rates. The use of OS pretreatment was meant to decouple the hydrolysis of xylan from the lignin redistribution process that occurs in dilute acid pretreatment; however, it appears that this approach was not conclusive. CLM of the OS pretreated rind did; however, show a diminution in signal intensity at the cell wall edges compared to CSR pretreated under standard dilute acid pretreatment conditions. Another approach to testing the biphasic kinetics phenomenon was made by reacting isolated xylan (extracted from corn stover) under pretreatment conditions and beechwood xylan at lower temperatures to yield low xylan conversions. In these experiments, xylan hydrolysis did not exhibit biphasic kinetics and it is likely that this is due to the absence of lignin or other chemical or structural cell wall components that might play a role in causing the biphasic effect.

In conclusion, the increases in rate and weight fraction of fast hydrolyzing xylan observed by removing the majority of the lignin supports the hypothesis that the slow hydrolyzing xylan is caused by its association with lignin. However, the evidence is not conclusive, as we were unable to find conditions that completely removed the biphasic kinetic behavior, except when xylan was isolated from the cell wall. A clearer understanding of the interaction between the two cell wall components and its effect on xylan hydrolysis kinetics will; therefore, require continued investigation.

## Competing interests

The authors declare that they have no competing interests.

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## Acknowledgements

This work was supported by the U.S. Department of Energy (DOE) under Contract No. DE-AC36-08GO28308. Funding for the work was provided by the DOE Office of Energy Efficiency and Renewable Energy, Bioenergy Technologies Office (BETO).

## Electronic supplementary information (ESI) available

Figures S1 and S2 for pH vs. time for pretreatment hydrolyzates and xylose yields and 1st order kinetics for xylan hydrolysis during dilute acid pretreatment of xylan isolated from CSR, respectively. See http://www.rsc.org/suppdata/gc...

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Figure 10. SEM (5000X) comparing samples pretreated at 160°C. (a) Extractives-free CSR; (b) Control CSR; (c) Delignified CSR (d) OS pretreated CSR. (Scale bar 200 μm).

Figure S1. pH vs. time for pretreatment hydrolyzates of corn stover (♦), isolated xylan (▲) pretreated under dilute acid and corn stover pretreated under organosolv conditions (■)

Figure S2. Xylose yields (a) and 1$^{st}$ order kinetics for xylan hydrolysis during dilute acid pretreatment of xylan isolated from CSR (b)

## Tables

Table 1. Compositions of extractive-free, delignified-control, and delignified CSR. The errors reported are the standard deviation for the analyses conducted in triplicates.

| | Glucan, % | Xylan% | Klason lignin, % |
|---|---|---|---|
| Extractives-free | 42.4 ± 1.9 | 24.1 ± 1.0 | 23.3 ± 0.6 |
| Control | 43.0 | 23.3 | 22.1 |
| Delignified | 49.4 | 22.5 | 7.2 |
| Beechwood xylan | 0.7 | 64.4 | 0.3 |

Table 2. Kinetic parameters for xylan hydrolysis from CSR under dilute acid conditions (1.2% sulfuric acid) for extractives-free CSR, delignified and control CSR, and CSR pretreated under organosolv conditions.

| CSR Samples | Extractives-free | | | Control | | Delignified | | Organosolv | |
|---|---|---|---|---|---|---|---|---|---|
| Temperature, (°C) | 130 | 140 | 160 | 140 | 160 | 140 | 160 | 140 | 160 |
| $z$ (%) | 38 | 64 | 83 | 28 | 75 | 50 | 87 | 68 | 68 |
| $k_s$ ($min^{-1}$) | 0.033 | 0.024 | 0.022 | 0.081 | 0.080 | 0.075 | 0.107 | 0.021 | 0.12 |
| $k_f$ ($min^{-1}$) | 0.27 | 0.38 | 0.75 | 0.23 | 0.59 | 0.59 | 0.97 | 0.44 | 0.79 |
| $k'_s$ ($min^{-1}$) | - | 1 | 1 | 3.4 | 3.6 | 3.1 | 4.9 | 0.9 | 5.5 |
| $k'_f$ ($min^{-1}$) | - | 1 | 1 | 0.6 | 0.8 | 1.6 | 1.3 | 1.2 | 1.1 |

$k'_s$ ($min^{-1}$): hydrolysis rate for slow-reaction relative to $k_s$ for dilute acid pretreatment of extractives-free CSR
$k'_f$ ($min^{-1}$): hydrolysis rate for fast-reaction relative to $k_f$ for dilute acid pretreatment of extractives-free CSR

Table 3. Kinetic parameters for xylan hydrolysis and lignin solubilization from organosolv pretreatment of extractives-free CSR at 140 and 160°C

| | Xylan hydrolysis | | Lignin solubilization | |
|---|---|---|---|---|
| Temperature, (°C) | 140 | 160 | 140 | 160 |
| $z$ (%) | 68 | 68 | 62 | 65 |
| $k_s$ ($min^{-1}$) | 0.021 | 0.120 | 0.067 | 0.052 |
| $k_f$ ($min^{-1}$) | 0.44 | 0.79 | 0.47 | 0.54 |

Table 4. First-order kinetic rate constants for xylan hydrolysis from treatment of xylan, isolated from CSR, with 1.2% sulfuric acid at 120, 140 and 160°C

| Temperature, (°C) | $k$ ($min^{-1}$) |
|---|---|
| 120 | 0.79 |
| 140 | 1.10 |
| 160 | 1.38 |

Table 5. First-order kinetic rate constants for xylan hydrolysis from treatment of beechwood xylan, with 1.2% sulfuric acid at 70, 80, 90 and 100°C

| Temperature, (°C) | $k$ ($min^{-1}$) |
|---|---|
| 70 | $4.3\times10^{-3}$ |
| 80 | $8.4\times10^{-3}$ |
| 90 | $6.6\times10^{-2}$ |
| 100 | $1.8\times10^{-1}$ |

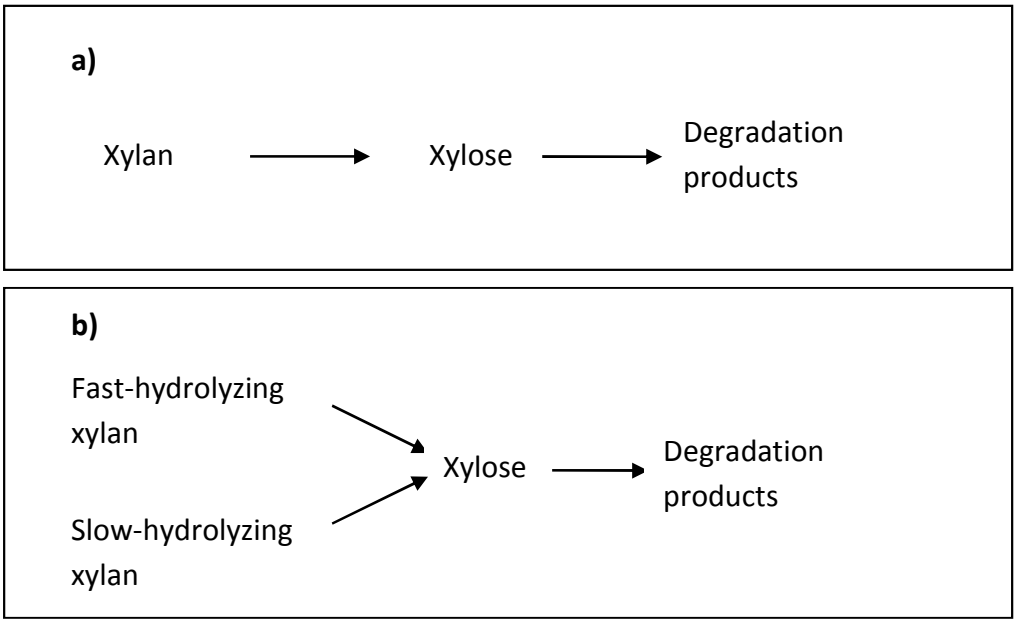


Figure 2. Xylose yields from xylan hydrolysis during dilute acid pretreatment of (a) Extractives-free CSR, (b) Delignified and control CSR, (c) Organosolv pretreatment of CSR, (d) Beechwood xylan

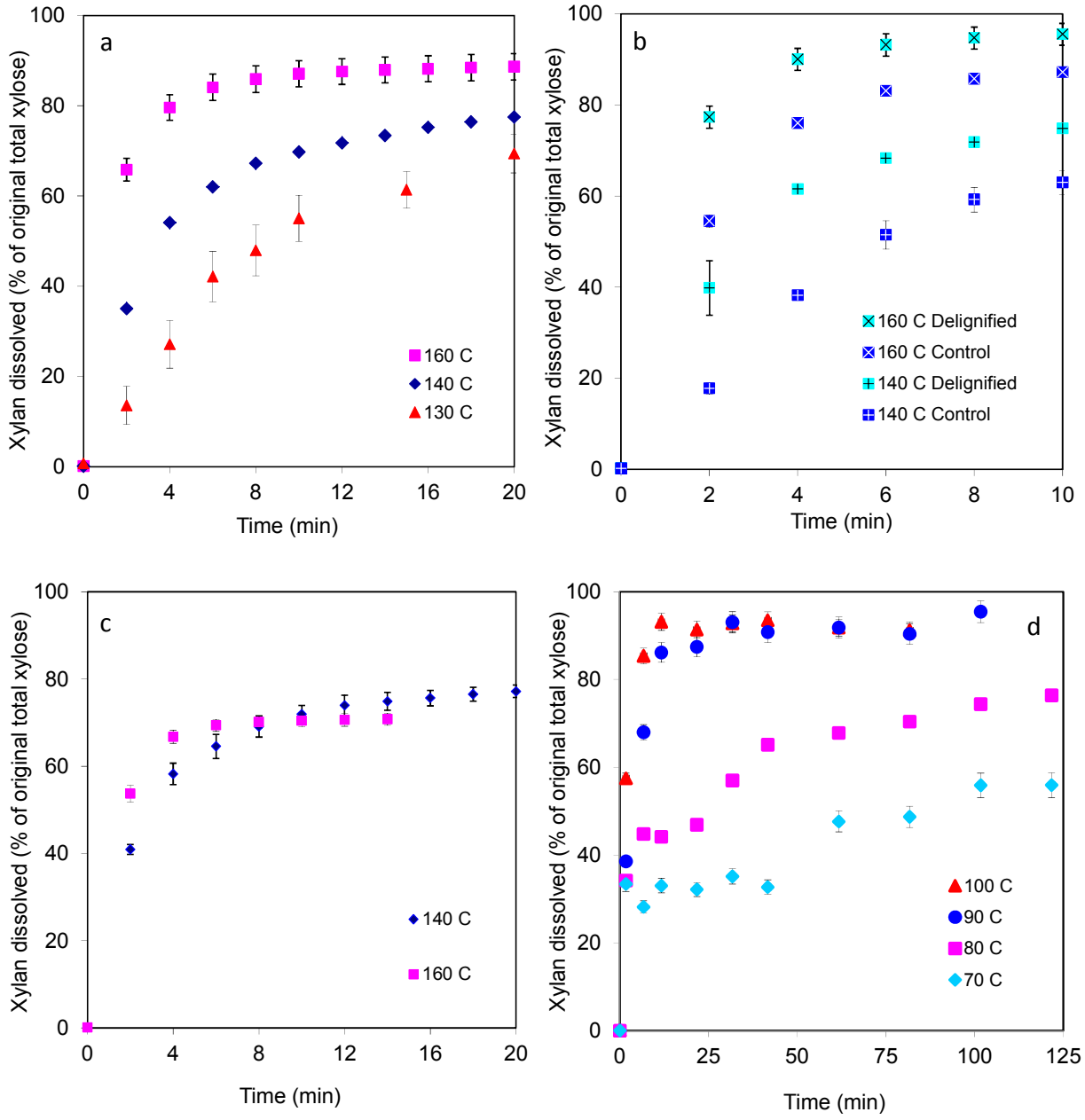
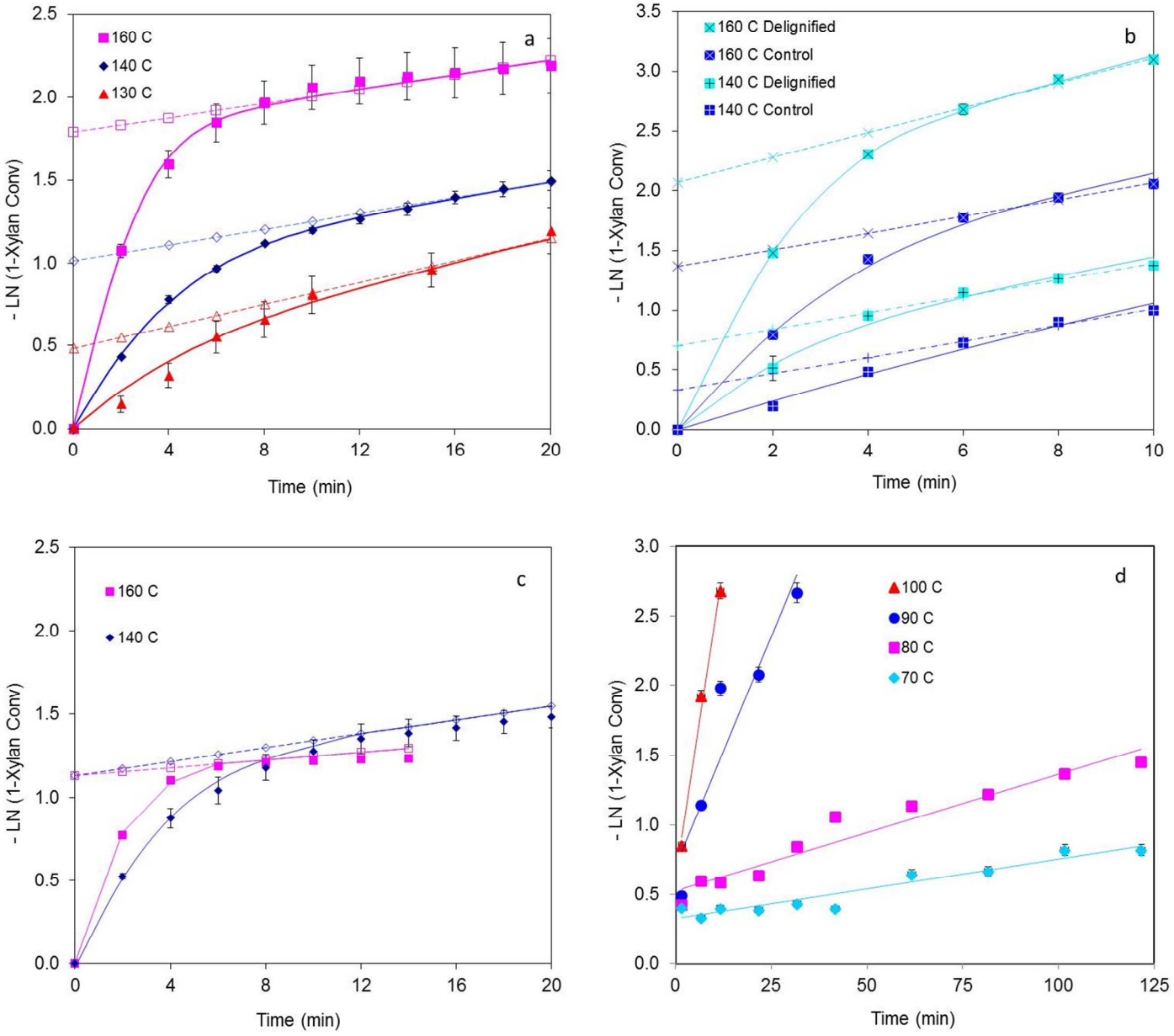
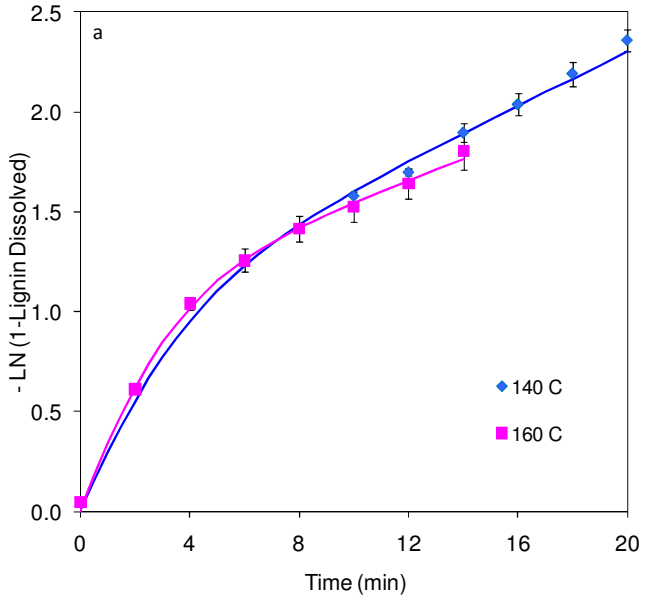
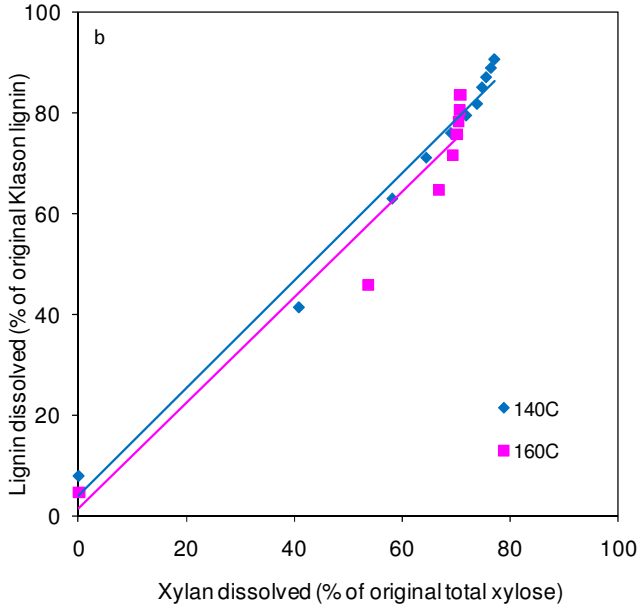
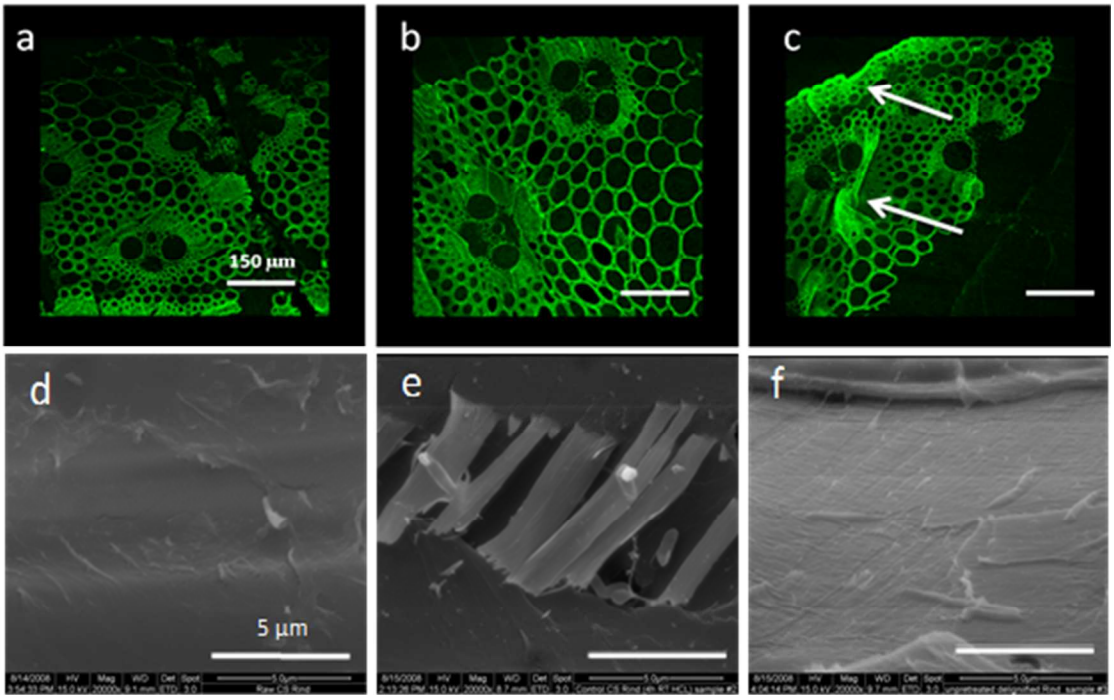


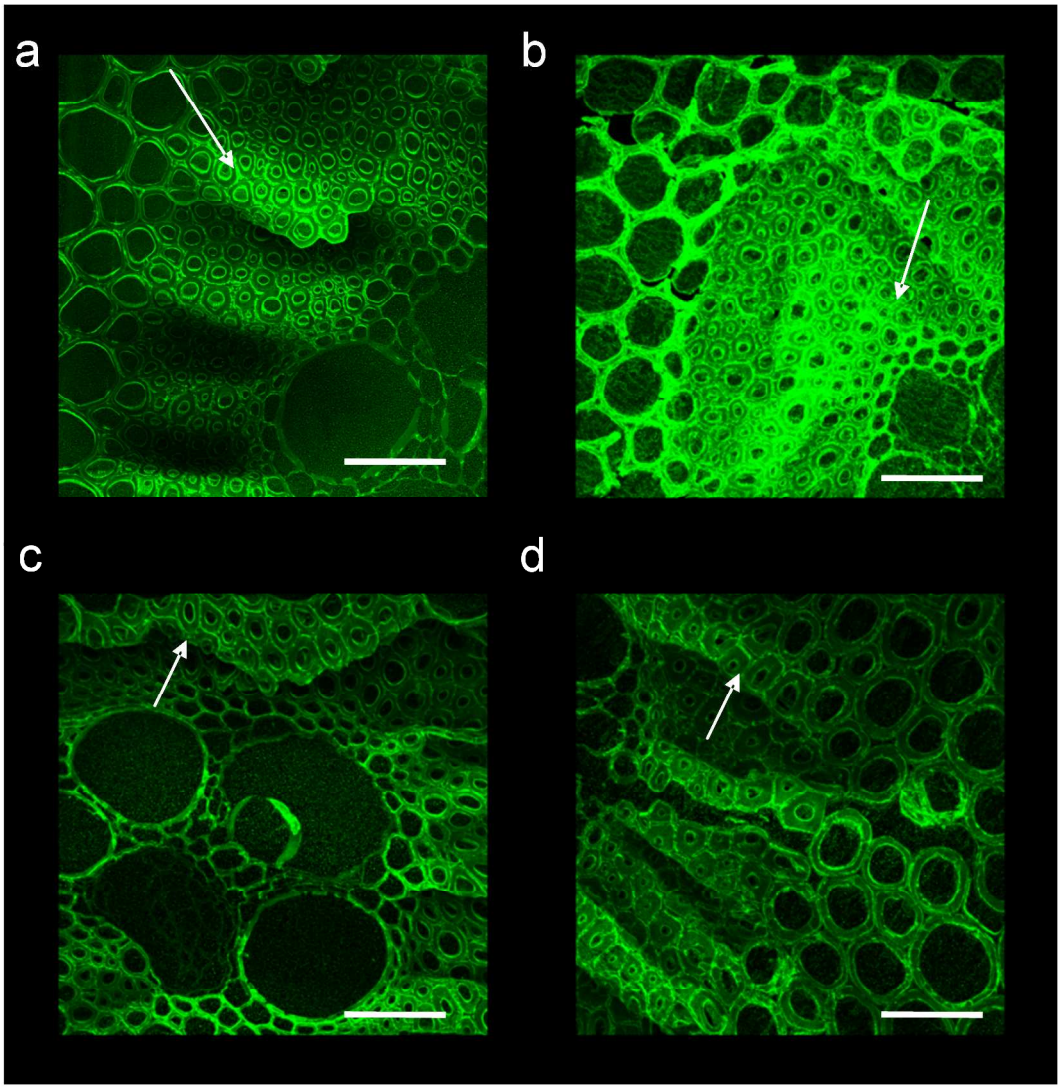
Figure 3. 1st order kinetics for xylan hydrolysis during dilute acid pretreatment of (a) Extractives-free CSR, (b) Delignified and control CSR, (c) Organosolv pretreatment of CSR, (d) Beechwood xylan

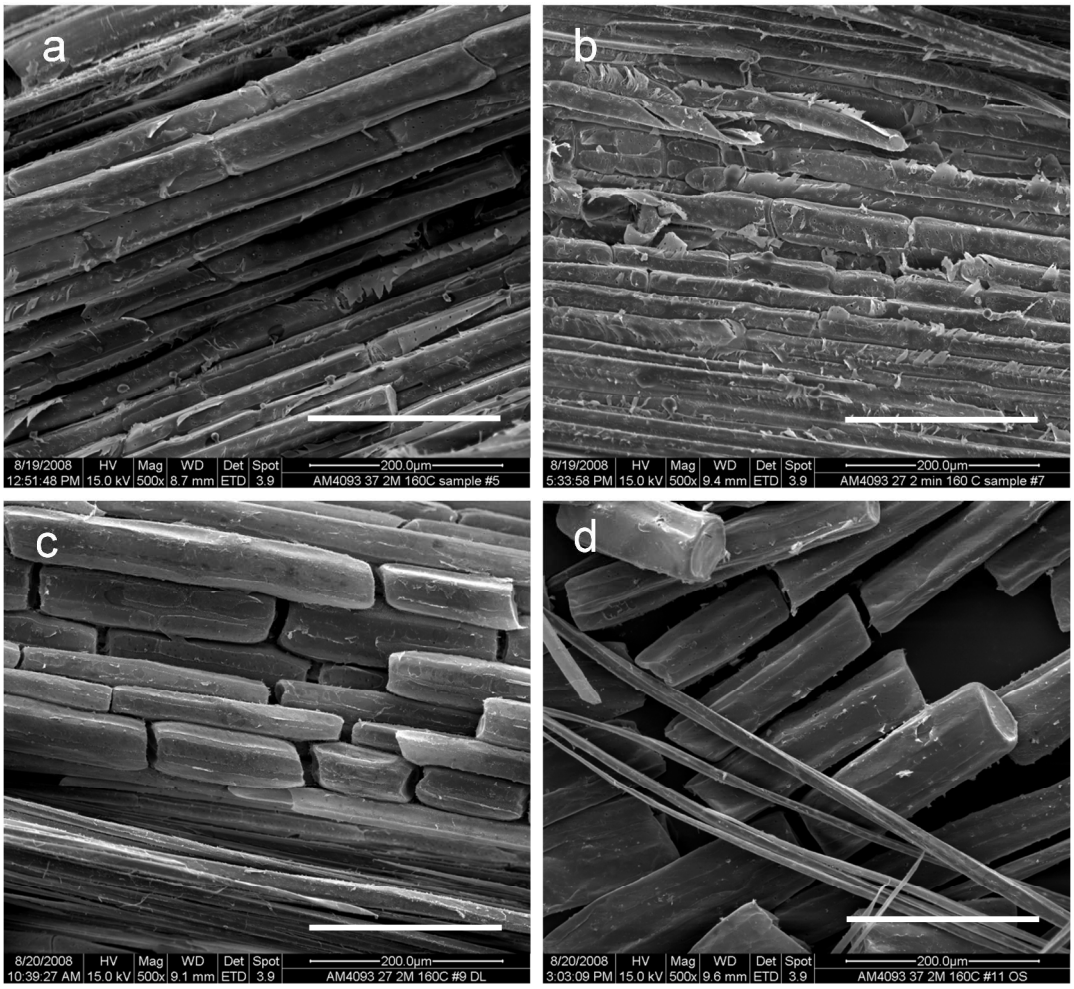


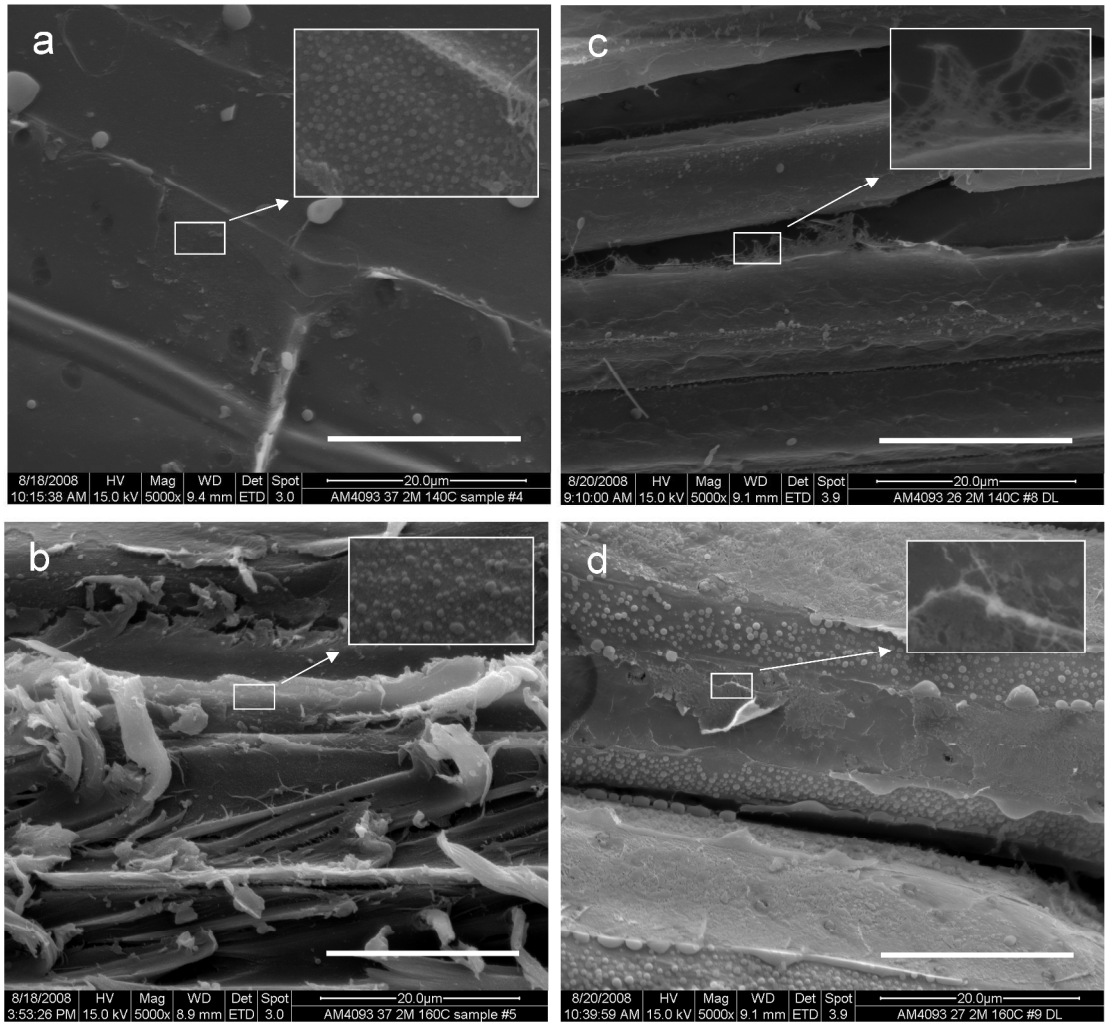


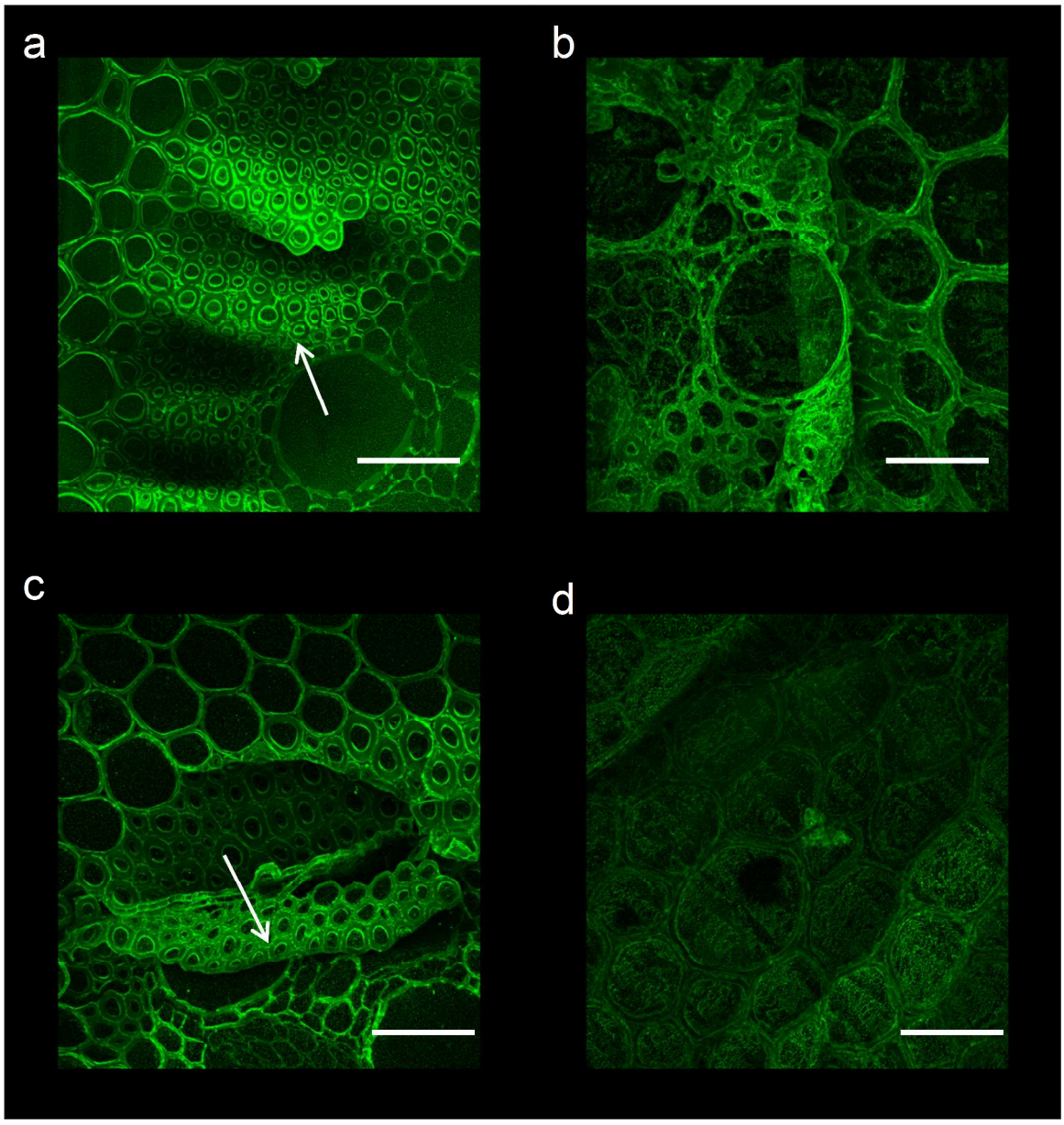



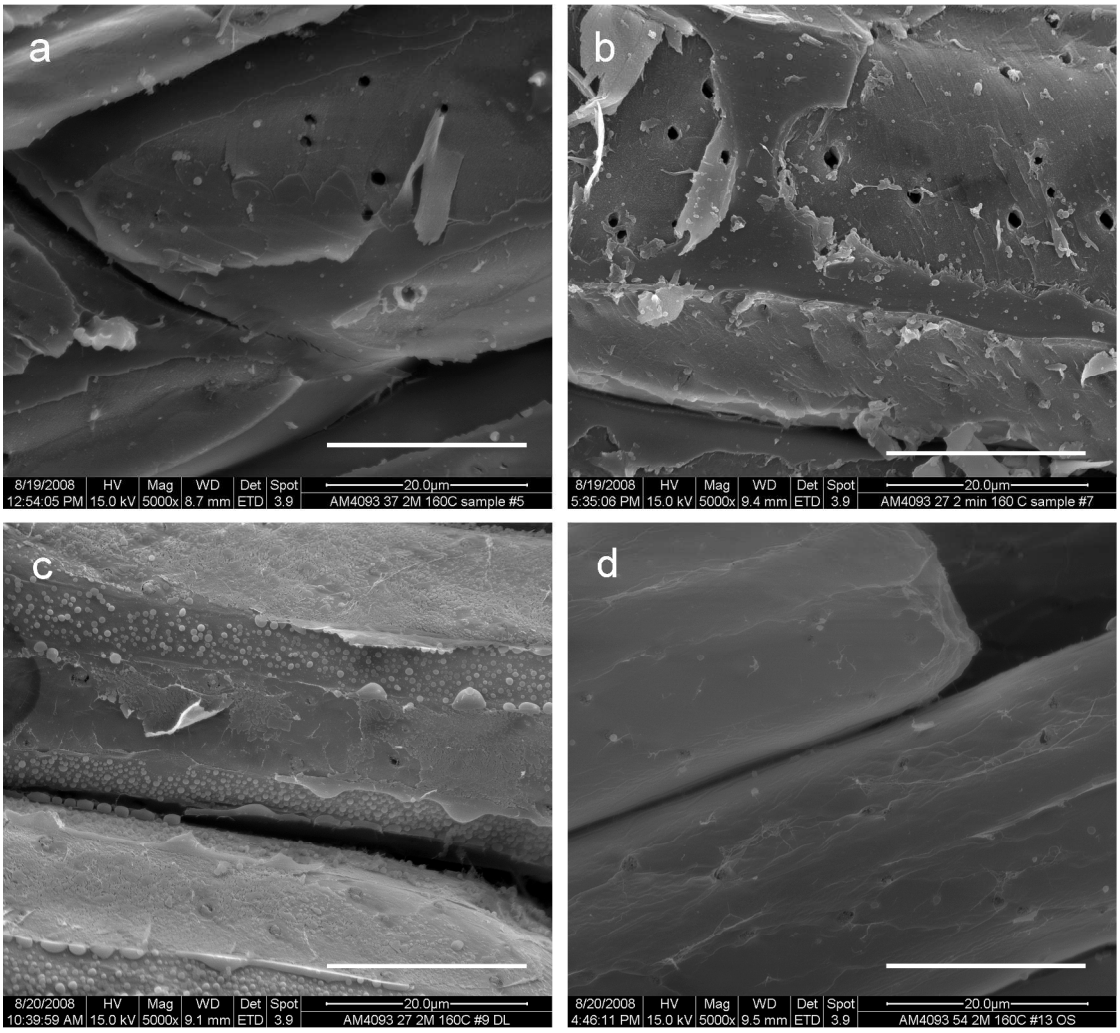












Untreated corn stover rind

Pretreated corn stover rind