

Formation and Characterization of Pseudo-Lignin Microspheres during High-pressure Water Pretreatment

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Microspheres were generated after subjecting corn stem rind to high-pressure water pretreatment. These microspheres can be divided into two categories according to their location: free microspheres and adsorbed microspheres. The formation and characterization of both microsphere types were studied. For the free microspheres, the maximum formation was reached at 180 °C; when the temperature was too high (200 °C) or too low (120 °C), no free microspheres were observed. As pretreatment temperature was varied, the morphologies of the free microspheres were different. For the adsorbed microspheres, their formation occurred over a wider temperature range, and their density increased with rising pretreatment temperature. Using Fourier transform infrared spectroscopy, many C=C and C=O bonds were identified on the surface of the free microspheres, indicating the presence of functional groups similar to those of lignin. Compared with untreated corn stem rind, the lignin signal of the residue surface was enhanced, which may be attributable to the increase in adsorbed microsphere quantity.

Keywords: Pretreatment; Microsphere; Pseudo-lignin; Morphology

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INTRODUCTION

The biotransformation of lignocellulose, an abundant renewable resource, into chemicals and fuels, is an important step towards sustainable economic development (Kruse *et al.* 2013). Because of the compact structure of lignocellulose, pretreatment is a necessary step before enzymatic hydrolysis (Ding *et al.* 2012). A variety of pretreatment methods has been designed; however, each has disadvantages in some respect (Alvira *et al.* 2010; Wörmeyer *et al.* 2011). For example, alkaline-based pretreatment methods such as ammonia fiber explosion (AFEX) are less satisfactory for processing recalcitrant substrates such as softwoods (Chandra *et al.* 2007). Acid-based pretreatment processes cannot effectively reduce the lignin content of lignocellulose, and they are relatively expensive (Mosier *et al.* 2005). For effective improvement of existing pretreatment methods, an in-depth understanding of what occurs in the cell wall during pretreatment is required. Microspheres or droplets have been observed on the surface of the solid residue after thermochemical pretreatment of lignocellulose (Selig *et al.* 2007; Pu *et al.* 2013). This interesting phenomenon is worthy of attention, and is a promising lead in understanding the reactions inside the cell wall.

Donohoe and co-workers found that the droplets were spherical with a flattened surface contacting the cell wall, and they tended to accumulate in pits, corners, and delamination zones (Donohoe *et al.* 2008). These droplets have been shown to contain lignin and to form when thermochemical pretreatments reach temperatures above the range for lignin phase transition, at which point lignin begins to coalesce into larger molten bodies and migrate out of the cell walls. However, Sannigrahi and co-workers (2011) demonstrated that the microspheres can also be generated from carbohydrates without significant contribution from lignin during dilute acid pretreatment, especially under high severity pretreatment conditions. As the spheres could yield a positive Klason lignin value, they were termed pseudo-lignin. A formation mechanism for pseudo-lignin has been proposed: polysaccharides undergo acid-catalyzed dehydration, fragmentation, rearrangement, and polycondensation and/or polymerization reactions to produce pseudo-lignin (Hu *et al.* 2012). The impact of pseudo-lignin on subsequent cellulose hydrolysis is cause for attention. It has been demonstrated that pseudo-lignin can significantly inhibit the enzymatic hydrolysis of cellulose (Selig *et al.* 2007; Kumar *et al.* 2013; Li *et al.* 2014); thus, pretreatment should be performed to avoid its formation (Hu *et al.* 2013). However, there have been no detailed reports to date on the generation of pseudo-lignin spheres under different pretreatment conditions (Chundawat *et al.* 2011).

In our experiments, dilute acid and high-pressure water pretreatments of corn stem rind were performed under the same conditions (180 °C for 20 min), except for the addition of 1.2 mL of H₂SO₄ in the dilute acid pretreatment system. It was found that the two pretreatment methods yielded significantly different results. The slurry from the dilute acid pretreatment resulted in a deeper color than that from the high-pressure water pretreatment, which is attributable to more fuscous substances being produced when H₂SO₄ was added. The liquid phase was collected after the slurry was filtered through a sand core funnel. The liquid sample from the dilute acid treatment was found to be clear, but that from the high-pressure water treatment was turbid. The turbid liquid was dried and observed by SEM. A surprising observation was made: a large number of microspheres existed in the liquid sample. It has been reported that a certain amount of particles were formed and adsorbed on the surface of solids after pretreatment with dilute acid or high-pressure water (Sannigrahi *et al.* 2011). However, there have been no reports on the formation of free microspheres during pretreatment. The conditions of the dilute acid pretreatment were changed in an attempt to obtain free microspheres. However, no microspheres were generated in the liquid phase. To study the formation of microspheres, the high-pressure water treatment was selected for the subsequent experiments. In this manuscript, the formation and characterization of microspheres in the solid-liquid phase and effects on enzymatic hydrolysis of biomass were studied.

EXPERIMENTAL

Materials

Corn stem rind was chosen in this study because of its high mass and representative plant cell wall structure. Corn stover was obtained *via* hand-cut corn in farmland located in Shijiazhuang, Hebei province, China, in October 2011. The stover was air dried for about two years. The rind portion was then peeled from the corn stover and milled to 20-mesh in a cutting mill.

All hydrolysis experiments in this study were performed using the *Trichoderma reesei* cellulase system Cellulast 1.5L (Sigma, America) that had a protein content of 65mg/ml and a titer of 210 FPU/mL. The commercial β -glucosidases (cellobiases) preparation Novozyme 188 (Sigma, America) with a protein content of 60 mg protein/ml and a titer of 250 CBU/mL was added to reduce end-product inhibition due to cellobiose accumulation.

Methods

High-pressure water pretreatment

High-pressure water pretreatment of the corn stem rind was performed in a 150-mL (working volume 120 mL) reactor (Weihai Automatic Control Reaction Kettle Co., Ltd., China) with an electric heater. The rind loading was 5 wt%. The heating rate ranged between 2 and 4 °C/min. Discharged the air in the reactor when the temperature reached to almost 100 °C. The time at which the temperature reached the desired value was recorded as the start of the reaction. The reaction lasted for 20 min. Following pretreatment, the reactor was cooled to below 50 °C in a room temperature water bath, and the slurry was then filtered through a sand core funnel (G-3 grade). A liquid sample was collected and centrifuged for 10 min at 4930×g to separate the microspheres from the liquid. The microspheres were washed twice with deionized water and stored in 1 mL of deionized water at 4 °C. The solid samples were washed with 100 mL of water, then separated by filtration and air dried for subsequent analysis. Composition analysis of the solid was performed according to NREL standard laboratory analytical procedures (Sluiter *et al.* 2007).

Scanning electron microscopy (SEM) and particle size analysis

The microspheres and solid samples obtained from pretreatment were characterized using SEM after mounting dry samples on aluminum specimen stubs and sputter coating with gold. Images were acquired with a JEOL-1530 Thermally Assisted Field Emission SEM (Japan) at a beam accelerating voltage of 5 kV and various resolving powers. The microspheres obtained from pretreatment could disperse well, such that their particle size could be readily analyzed by a particle size analyzer (Malvern Laser Sizer 2600, United Kingdom). According to instrument requirements, it was necessary to adjust the microsphere content of the analysis system by adding a certain amount of deionized water.

Fourier transform infrared (FT-IR) spectroscopy

The surface properties of the microspheres and solids were characterized by FT-IR (Perkin Elmer, Wellesley, MA). To improve the dispersion of the rind, the untreated rind and solid residue were ground with a mortar and pestle. The microspheres present in the water were dried at 60 °C and then ground. Samples were prepared by mixing 2 mg of Tamarix powder with 200 mg of KBr. The FT-IR spectra were obtained by averaging 32 scans from 4000 to 400 cm^{-1} at a resolution of 2 cm^{-1} .

Effect of microspheres on enzymatic hydrolysis of biomass

The substrates of enzymatic hydrolysis were water-insoluble solids from pretreatment at 180 °C and 20 min. Batch hydrolysis was carried out in 50 mL Erlenmeyer flasks containing 2% substrate (w/v) in 15 mL of 50 mM citrate buffer (pH 4.8). These flasks were placed horizontally in a shaker at a rotation of 100 rpm and 50 °C. The typical enzyme loading was 20 FPU, 20 CBU per gram of substrate. Prior to the addition of

enzymes, a certain amount of free microspheres were added to hydrolysis system. A sample of hydrolysis liquid (1.0 mL) at time intervals of 6, 12, 24, 48 h was withdrawn and was immediately frozen at -20 °C until analysis.

Analysis methods

The concentration of glucose was determined using high performance liquid chromatography with an Aminex HPX-87P column (300 mm × 7.8 mm) (Bio-Rad Laboratories Inc., America). The mobile phase was 5 mmol/L H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C.

The cellulose digestibility was calculated as follows,

$$CD = (\text{Glu} \times 0.9 \times 100) / (\text{SDM} \times \text{CC}) \quad (1)$$

where CD is cellulose digestibility (%), Glu is glucose (g) in the hydrolysate, SDM is the weight of solid dry matter (g), CC is the cellulose content in the solid dry matter (%), and 0.9 is the factor considering the molecular mass ratio between anhydroglucose contained in cellulose and free glucose.

RESULTS AND DISCUSSION

Free Microspheres

An interesting phenomenon observed in our present research piqued our interest: many microspheres were observed in the liquid phase, which conflicts with prior reports stating that the spheres were adsorbed on the surface of solids. Firstly, the effect of the pretreatment temperature on the formation of free microspheres was studied. From the results, as shown in Fig. 1, it is clear that the pretreatment temperature had an obvious effect on the amount of microspheres formed.

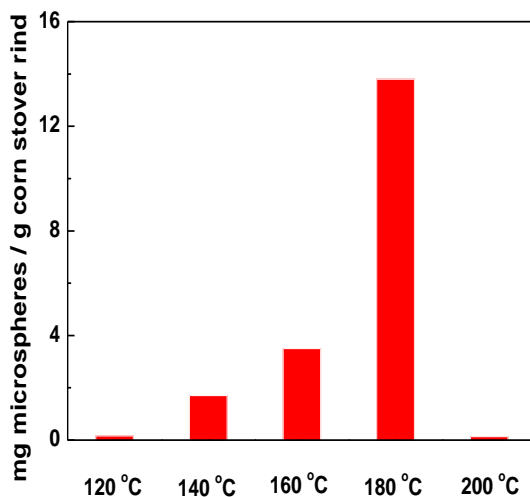


Fig. 1. The effect of pretreatment temperature on the amount of free microspheres formed

When the treatment temperature was 120 °C, barely any microspheres existed in the liquid phase. As the temperature was increased to 140 °C, free microspheres were observed in the liquid with a mass of 1.7 mg/g corn stem rind. The amount of free

microspheres increased with temperature: at 160 °C, it reached 3.5 mg/g corn stem rind, and at 180 °C it substantially increased to 13.8 mg/g corn stem rind. However, when the temperature was further raised to 200 °C, no microspheres were detected in the liquid. In earlier reports, in order to increase pretreatment effectiveness, temperatures above 200 °C were often selected for high-pressure water pretreatment (Lasera *et al.* 2002; Pérez *et al.* 2008). No free microspheres were observed at such high temperature, which may be the reason for the lack of previous reports on the formation of free microspheres in high-pressure water pretreatment.

Carbohydrates in the liquid were determined with HPLC (Table 1). Xylose content increased obviously as the temperature rose, which indicated that hemicellulose was digested significantly.

Table 1. Chemical Composition of Corn Stem Rind after High-Pressure Water Pretreatments

Temperature (°C)	Carbohydrates in the liquid (g/L)			Compositions of water-insoluble solid (g/100g DM)			
	Glucose	Xylose	Arabinose	Glucan	Xylan	Arabinan	Klason lignin
120	0.5	2.3	0.3	41.3	23.1	3.1	22.7
140	0.8	4.6	0.3	40.9	19.3	2.0	23.4
160	1.0	6.3	0.5	40.5	10.5	0.9	25.0
180	1.5	10.2	1.3	38.2	6.1	0.3	26.3
200	1.8	12.1	1.5	36.4	5.8	0.2	29.6

The morphologies of the free microspheres were characterized by SEM. A majority of the free microspheres were observed to possess a regular spherical shape (Fig. 2). At 140 °C, the obtained free microspheres displayed large diameters ranges, with the largest microspheres being nearly 0.8 µm, while the smallest microspheres were less than 50 nm (Fig. 2A). Such features were also reflected by the wide-ranging particle size distribution (Fig. 2a). Furthermore, in addition to the regular spheres, gourd-shaped particles were observed, indicating that large spheres can be formed by the fusing of smaller spheres.

Because of the greater dilution of the liquid phase, there were fewer microspheres in the 160 °C samples than in the 140 °C samples. From the particle size distribution (Fig. 2b), it is apparent that the microspheres were uniformly sized, with diameters ranging from 100 to 500 nm. The majority of the microspheres had a smooth surface; however, a very small percentage of the spheres had a rough surface (Fig. 2B), which might be due to the combination of lignins and polysaccharides (Donohoe *et al.* 2008).

The size of the free microspheres obtained at 180 °C showed obvious variance, similar to that at 140 °C. The difference between the spheres obtained at 180 and 140 °C is that at 180 °C, the spheres had a larger diameter, with a range of 1 µm to 100 nm (Figs. 2C and 2c). As the microspheres were sprayed with a layer of gold nanoparticles during SEM sample preparation, the measured value of the microsphere diameter tended to be larger than the actual value. However, this creates no confounding effects when comparing differences in sphere size between treatment temperatures.

FT-IR was used to analyze the chemical structure of the microsphere surface. The FT-IR spectra of the three kinds of free microspheres showed similar profiles but different intensities of the adsorption bands (Fig. 3).

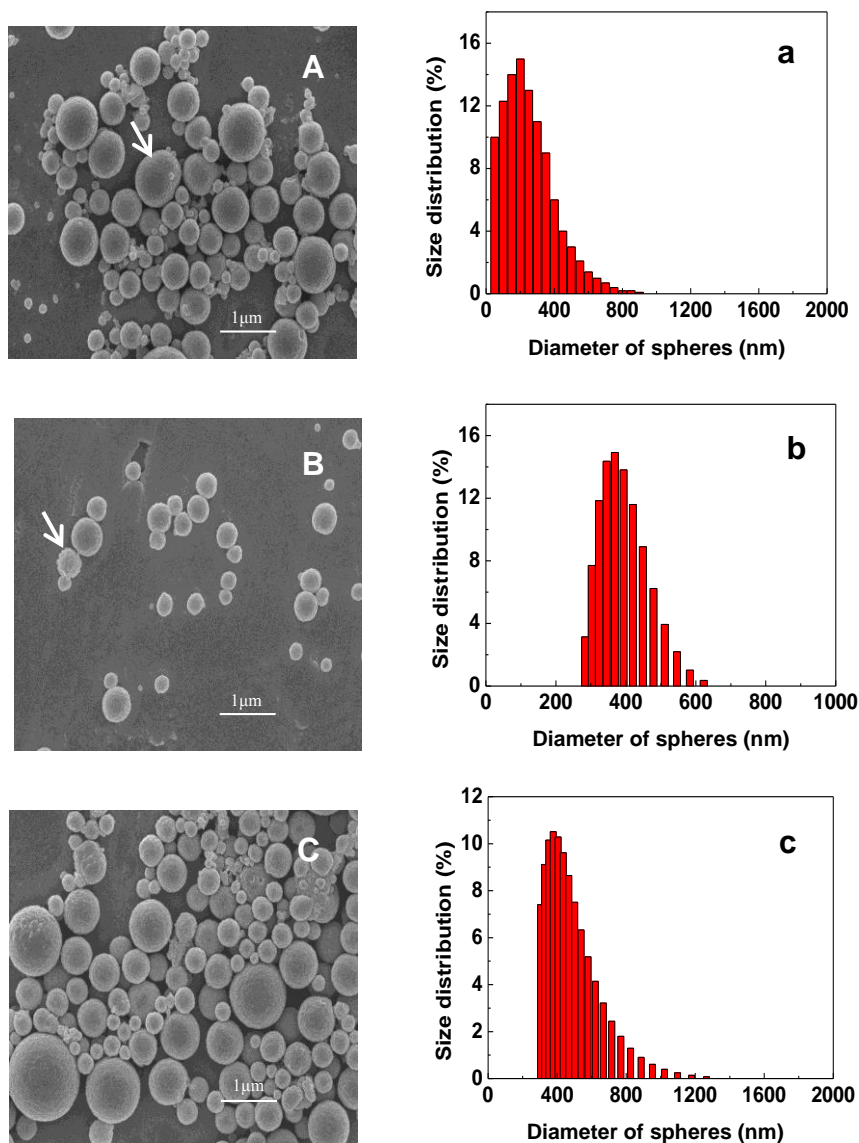


Fig. 2. SEM micrographs and size distribution of the free microspheres from different pretreatment processes. (A, a) at 140 °C; (B, b) at 160 °C; and (C, c) at 180 °C.

The bands at ~ 1645 , ~ 1600 , and ~ 1500 cm^{-1} can be attributed to C=O (*i.e.*, in carbonyl and/or carboxylic acid moieties) conjugated with an aromatic ring, whereas the bands in the 1000 to 1240 cm^{-1} region correspond to C-O stretching (*i.e.*, in hydroxyl, ether, or carboxylic acid moieties) (Kristensen *et al.* 2008; Kaparaju and Felby 2010; Xiao *et al.* 2011). These observations indicate that the free microspheres had a structure similar to that of lignin. This is believed to be due to the dehydration and aromatization reactions of carbohydrates that take place during the formation of free microspheres (Hu *et al.* 2012). In addition, the intensity of the carbonyl band at 1600 cm^{-1} became stronger with increasing reaction temperature. These results thus suggest that an increase in temperature could improve the formation of carbonyl groups.

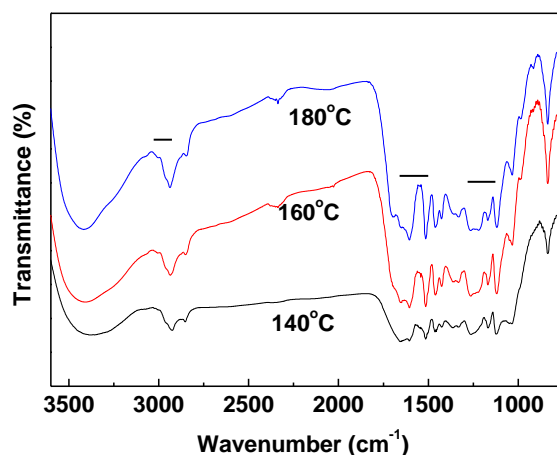


Fig. 3. The FT-IR spectra of free microspheres from high-pressure water pretreatment

Adsorbed Microspheres

The solid residues were separated from the pretreatment slurry and then washed with water to eliminate any unadsorbed particles. As control, SEM micrographs of untreated corn stem rind are shown in Fig. 4. There was no obvious particle formed on the surface. Particles were detected on the surface of the residue after pretreatment at 120 °C for 20 min (Figs. 4A and 4a). Some parts of the surface exhibited aggregation of particles, while others were completely bare of particles. However, the adsorbed particles displayed good dispersion. The particles were mainly spherical and had a relatively uniform size of about 100 nm. The formation of the particles indicates that the cell wall structure was already destroyed at 120 °C, despite the lack of obvious changes in the composition of the corn stem rind.

After pretreatment at 140 °C, the stem rind was covered by a dense layer of particles with varied morphology. A small number of particles were of an irregular shape, but most were spherical and approximately 200 nm in diameter. Compared with the free microspheres, the adsorbed microspheres had a more uniform particle size. Meanwhile, adhesion between some of the spheres and the surface of the stem rind was found (Figs. 4B and 4b). It has been presumed that some substrates might migrate from the interior of the cell wall to the surface during pretreatment, and subsequently form microspheres when in contact with water due to their hydrophobicity (Trajano *et al.* 2013; Yu *et al.* 2014).

When the temperature was raised to 160 °C, the adsorbed spheres had a standard spherical shape and a 200 to 300 nm diameter, similar morphology to the spheres suspended in the liquid (Figs. 4C and 4c). However, when the temperature was raised to 180 °C, the particle size of the adsorbed spheres exhibited obvious differences. The smallest particles were only a few nanometers in diameter, whereas the largest particles reached up to 1 μm . It was observed that some spheres had an indentation on the surface (Figs. 4D and 4d), perhaps due to the rapid and sudden pressure drop upon cooling after completion of the reaction, which leads to air in the microspheres being extruded to form this feature. When the temperature was further raised to 200 °C, a large number of microspheres were still present on the stem rind surface (Figs. 4E and 4e), while barely any microspheres in the liquid.

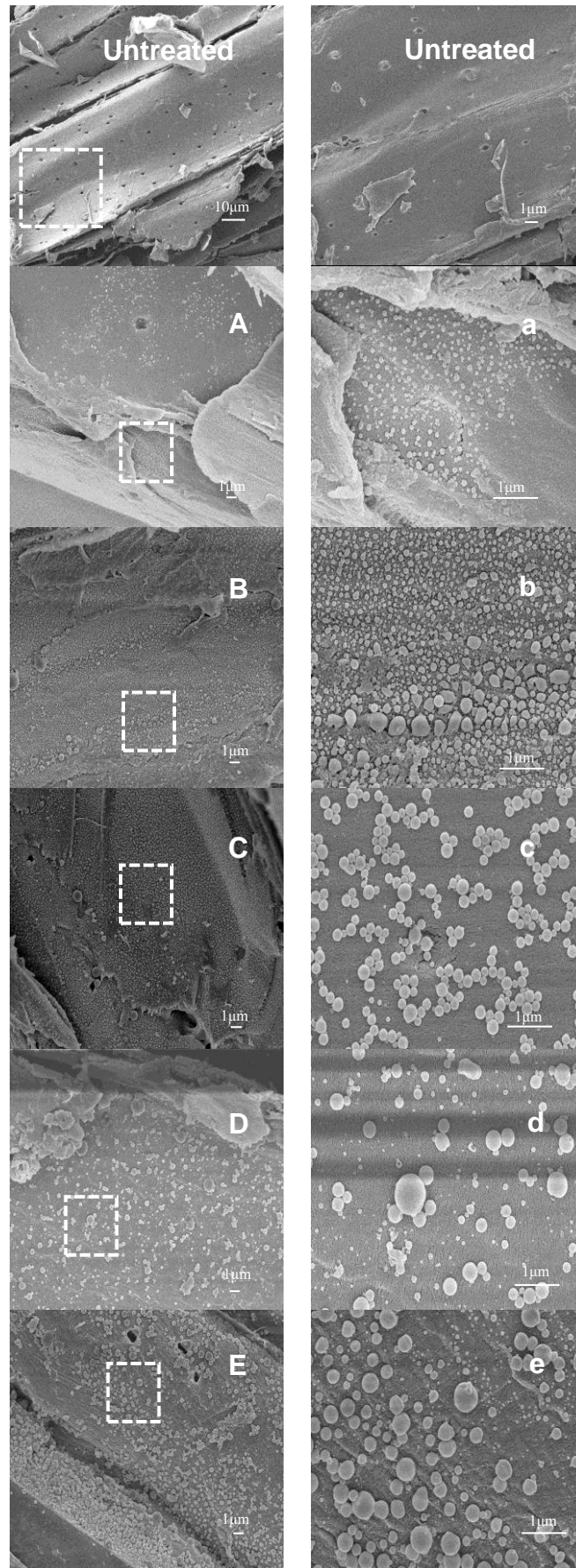


Fig. 4. SEM micrographs of corn stem rind surfaces, untreated and after high-pressure water pretreatment at (A, a) 120 °C, (B, b) 140 °C, (C, c) 160 °C, (D, d) 180 °C, and (E, e) 200 °C. Images a, b, c, d, and e are a higher magnification of the area boxed in A, B, C, D, and E, respectively.

According to the literature (Lasera *et al.* 2002; Pérez *et al.* 2008), the optimal temperature of high-pressure water pretreatment was usually about 220 °C. So a more severe pretreatment was implemented at 220 °C. There was also no free microsphere in the liquid, and a large number of microspheres were present on the solid surface, which was similar to the situation at 200 °C. The disappearance of free microspheres at more than 200 °C may be related to the surface properties of the microspheres and the rind, as well as, the microenvironment of the solution. The diameter of the adsorbed microspheres increased regularly with rising temperature; however, the deposition site of the microspheres did not display any obvious regularity.

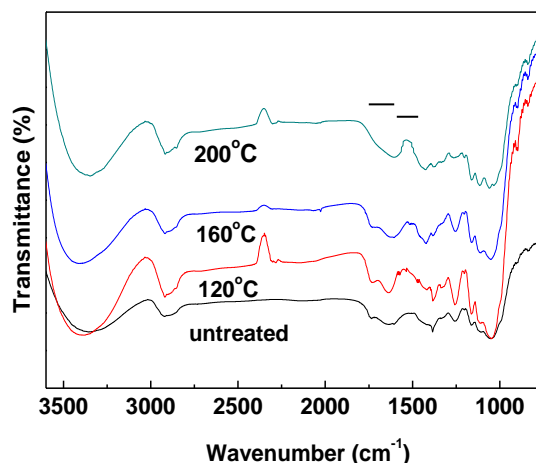


Fig. 5. The FT-IR spectra of corn stem rind residues after high-pressure water pretreatment

The analysis of chemical composition of water-insoluble solid showed that, as the temperature was raised, glucan and xylan were decreased, and klason lignin was, which was in agreement with previous published results. The current results were difficult to describe the characteristics of microspheres. So the changes in the prevalence of the main functional groups of the solid surface through FTIR were analyzed. The peaks at ~1740 and ~1501 cm⁻¹ underwent changes due to pretreatment (Fig. 5). The 1740 cm⁻¹ peak represents the acetyl group in hemicelluloses or the carbonyl on the carboxyl group (Kristensen *et al.* 2008; Kaparaju *et al.* 2010; Xiao *et al.* 2011). With an increase in pretreatment temperature, this peak decreased gradually, suggesting a reduction in the content of hemicellulose. Exhibiting a contrary trend, the peak at 1501 cm⁻¹, which was one of the typical peaks in the FT-IR spectra of free microspheres, became more prominent with an increase in reaction temperature, suggesting an increase in lignin content. The increase of lignin relative content (Table 1) and existence of the adsorbed microspheres could both be the causes of this phenomenon (Li *et al.* 2007; Sannigrahi *et al.* 2011).

Microspheres Can Reduce the Cellulose Digestibility

Free microspheres were used to study its effect on enzymatic hydrolysis. In this experiment, water-insoluble solid and free microspheres were both from pretreatment system at 180 °C and 20 min. The water-insoluble solid was set as a control sample;

meanwhile, 0.5%, 1%, 2% (g/g water-insoluble solid) free microspheres were added to the hydrolysis system. From the results (Fig. 6), these free microspheres were detrimental to enzymatic hydrolysis. The cellulose digestibility declined as the free microspheres content increased. The control sample without free microsphere had the highest cellulose digestibility (67% after 48 h) among all samples, whereas sample at 2% addition level had the lowest (50% after 48 h). Donohoe *et al.* (2008) thought that lignin droplets act as a physical barrier to prevent enzyme access to the biomass during enzymatic hydrolysis. However, in the present experiments, the free microspheres had no contact with the surface of the biomass. So, a physical barrier could not have been the main reason for retarding hydrolysis. Non-producing binding of enzymes to lignin had been suggested to be responsible for reducing the cellulose digestibility (Yang and Wyman, 2006). Adsorption of BSA and purified CBHI on pseudo-lignin revealed that pseudo-lignin binds protein unproductively (Kumar *et al.* 2013). Thus, it might be the reason that microspheres bind protein unproductively and make less cellulase available for action on cellulose.

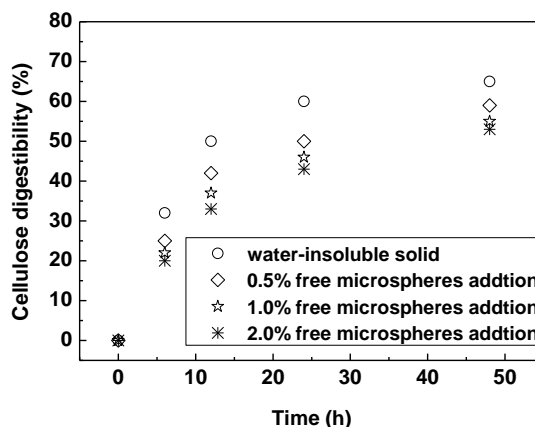


Fig. 6. Effect of added free microspheres on cellulose digestibility. Water-insoluble solid and free microspheres were both from pretreatment at 180 °C and 20min.

CONCLUSIONS

1. Microspheres were generated after subjecting corn stem rind to high-pressure water pretreatment. These microspheres can be divided into two categories according to their location: free microspheres and adsorbed microspheres.
2. The maximum formation of free microspheres was reached at 180 °C. The morphologies of the free microspheres differed according to pretreatment temperature. Relatively uniform free microspheres were formed at 160 °C. The effect of microspheres on the enzymatic hydrolysis was studied. The cellulose digestibility declined as the free microspheres content increased.
3. For the adsorbed microspheres, their formation occurred over a wide temperature range, and their density increased with rising pretreatment temperature.
4. Compared with untreated corn stem, the lignin signal of the residue surface was enhanced, which may be attributable to the increase in adsorbed microspheres. It is hypothesized that the formation of the microspheres may be a result of the degradation and polymerization of hemicellulose or/and lignin.

ACKNOWLEDGMENTS

The authors are grateful for the support of the National High Technology Research and Development Program of China, No. 2012AA022301 and No. 2012AA101800.

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Article submitted: November 26, 2014; Peer review completed: March 21, 2015; Revised version received and accepted: October 14, 2015; Published: November 2, 2015.
DOI: 10.15376/biores.10.4.8474-8486