A Sequential Combination of Laccase Pretreatment and Enzymatic Hydrolysis for Glucose Production from Furfural Residues

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Furfural residues (FRs) were pretreated with laccase or a laccase-mediator (1-hydroxybenzotriazole, HBT) system to produce fermentable sugar for bioethanol production. Compared to laccase-only pretreatment, laccase-mediator pretreatment dissolved more lignin. Approximately 10.5% of the initially present lignin was removed when FRs were treated with a laccase loading of 100 U/g of dry substrate in 1% (w/w) HBT at 48 °C for 24 h in an acetate buffer (pH 4.8). The enzymatic saccharification process was carried out by a combined laccase or laccase-mediator pretreatment without washing of the treated solids. The results showed that active laccase had a negative effect on the rate and yield of enzymatic hydrolysis. Laccase-oxidized HBT seriously reduced glucose yield. However, non-oxidized HBT increased glucose yield when laccase was deactivated at 121 °C for 20 min prior to enzymatic hydrolysis. The highest glucose yield, 80.9%, was obtained from the substrate pretreated with 100 U/g of dry substrate laccase and 1% (w/w) HBT at 48 °C for 24 h in an acetate buffer (pH 4.8). Furthermore, the structures of FRs before and after laccase-mediator pretreatment were characterized by scanning electron microscopy (SEM) and Fourier Transform Infrared spectroscopy (FT-IR).

Keywords: Laccase-mediator pretreatment; Furfural residues; Enzymatic hydrolysis; 1-hydroxybenzotriazole

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INTRODUCTION

Renewable lignocellulosic biomass sources such as agroindustrial residue, waste paper, and forestry wastes are promising resources for bioethanol production, as they can reduce greenhouse gas emissions and encroach only very little on food production (Hahn-Hägerdal *et al*. 2006; Ragauskas *et al.* 2006). In China, due to the lack of forest resources, significant research has been undertaken regarding agroindustrial residues (Chen and Qiu 2007). Furfural residues (FRs) are industrial wastes produced during the production of furfural from corncobs. They are composed mainly of cellulose and lignin, with about 45% of their weight being cellulose (Sun *et al.* 2010). It is worthwhile to retain such cellulose-rich wastes for the production of bioethanol.

The most crucial step of bioethanol production is the conversion of cellulose into fermentable sugar using cellulases. In plants, lignin forms a strong hydrophobic network that protects their cells from microbial and enzymatic attack (Li *et al.* 2010). The FRs have higher lignin content (around 45%) than native wood (20 to 35%) (Xing *et al.*

2012). In addition, the lignin structure in FRs becomes condensed under acidic conditions (5 to 8% dilute sulfuric acid) at high temperatures (170 to 185 °C) during the furfural production process (Bu *et al.* 2012). Research has shown that condensed lignin inhibits cellulases more strongly than native lignin (Berlin *et al.* 2006). Therefore, a pretreatment to separate or modify the lignin is a promising approach to improving the enzymatic digestibility of cellulose. Many pretreatment methods such as physical (chipping, milling, and grinding), chemical (alkaline pretreatment, acid hydrolysis, and oxidative delignification), and physicochemical methods (steam explosion and ammonia fiber explosion) have been developed to modify lignocellulosic biomass (Litzen *et al.* 2006; Wang *et al.* 2009). Unfortunately, these methods have disadvantages.

Physical pretreatment is based on increasing the surface area of cellulose by chipping, milling, or grinding the biomass into a fine powder in order to improve the efficiency of enzymatic hydrolysis. Physical pretreatment processes are expensive, and high glucose yield cannot be achieved (Galbe and Zacchi 2007). Chemical methods require high temperatures and pressures and highly concentrated chemicals to obtain a high delignification rate. Alkaline, sulfate, and sulfite cooking processes in particular pose serious environmental concerns (Taherzadeh and Karimi 2008). Steam explosion pretreatment is one of the most widely-used physicochemical methods because it partially degrades and dissolves lignin and hemicelluloses when the pressure rapidly decreases after exiting the pretreatment gun. In contrast, some soluble inhibitors (furfural, 5-hydroxymethylfurfural, formic acid, and others) of both the enzymes and the fermenting microorganisms are generated during such severe treatment. A high severity factor is required to obtain an enzymatic hydrolysis yield adequate for commercial bioethanol production (Litzen *et al.* 2006). A common problem with these methods is that the slurry obtained after the pretreatment process could be filtered and washed with water to obtain high glucose yield during enzymatic hydrolysis. From an economical and environmental standpoint, the filtration and washing steps should be avoided since they increase operational costs and generate wastewater. For these reasons, enzymatic pretreatment using laccase has been explored (Moreno *et al.* 2012; Rico *et al.* 2014).

Laccase (EC 1.103.2) is a copper-containing polyphenol oxidase that oxidizes a variety of phenolic units in lignin and a number of other phenolic compounds (Kudanga *et al.* 2011). Laccases can be generally divided into two major groups: those from higher plants and those from fungi (Mayer and Staples 2002). Laccases from higher plants act as a catalyst for the delignification process, while fungal laccases degrade lignin. The largest amounts of fungal laccases are produced by white-rot fungi (wood-decaying basidiomycetes) (Leonowicz *et al.* 2001). Fungal laccases are of particular commercial interest because they are secreted extracellularly in response to simple inducers, making their production and purification relatively simple (Giardina *et al.* 2010). Laccases are also able to catalyze the oxidation of non-phenolic lignin units (C4-esterified) into radicals, usually acting *via* a mediator such as 1-hydroxybenzotriazole (HBT), 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and others (Jeon and Chang 2013). Currently, laccases are mainly used for bleaching in the pulp and paper industry, as a stabilizer during must and wine processing, or as a dechlorinating agent (Rodríguez *et al.* 2006).

In the pulp and paper industry, environmental concerns have motivated the replacement of conventional, polluting, chlorine-based delignification and bleaching procedures (Widsten and Kandelbauer 2008). Laccases act on phenolic substrates by catalyzing the oxidation of their phenolic hydroxyl groups into phenoxy radicals while

molecular oxygen (O_2) is reduced to water (Andreu and Vidal 2013). Laccases are more readily available and easier to manipulate than lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) (Karp *et al.* 2013). Researchers have indicated that laccases selectively delignify biomass without negatively impacting the cellulose fraction (Qiu and Chen 2012). Laccases have garnered interest in the pretreatment of lignocellulosic resources. Generally, fuel ethanol from lignocellulosic biomass can be produced in three integrated stages: pretreatment, hydrolysis, and fermentation. In this study, laccases from *Trametes versicolor* were used to treat FRs, and four enzymatic hydrolysis strategies were carried out, including washing the pretreated solids, keeping the activity of laccases and deactivating the laccases after 24 h pretreatment, as well as simultaneously enzymatic hydrolysis and pretreatment. In order to increase the degree of lignin removal, HBT was employed as a mediator during pretreatment. The effects of laccase and HBT loading on the composition of FRs were also investigated. Variations on the fiber surface were observed by an energy-dispersive X-ray analysis system attached to a scanning electron microscope. The functional group changes of the solid fractions were characterized by Fourier transform infrared spectroscopy (FT-IR).

EXPERIMENTAL

Raw Materials and Enzymes

Furfural residues (FRs) were supplied by the Chunlei Furfural Corporation of Hebei, China. Before pretreatment, the FRs were immersed in fresh water for 24 h and rinsed until a neutral pH was reached. The residues were screened with 40-mesh screens after being dried for 12 h at 50 °C. Laccases prepared from the fungus *T. versicolor* were provided by Sigma (St. Louis, MO, USA). Its activity was determined by measuring the oxidation of 5 mM 2-2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS, Sigma Co., St. Louis, MO, USA) buffered with sodium acetate (pH 4.8) at 25 °C (Moniruzzaman and Ono 2013). The formation of ABTS cation radicals was monitored at 420 nm ($\varepsilon_{420} = 3.6 \times 10^4$ mol/L cm^{-1}) using an ultraviolet spectrophotometer (UV) and re-analyzed every 30 s for the next 3 min. One activity unit (U) was defined as the amount of enzyme required to transform 1 mol ABTS/min.

Methods

FRs pretreated with laccase or laccase-mediator

The FRs were pretreated with laccases alone or with laccases in the presence of 1-hydroxybenzotriazole (HBT, Sigma Co., St. Louis, MO, USA) as a mediator. The pretreatment was carried out in a 500-mL conical flask in a shaking incubator operating at 150 revolutions per min (rpm) and 50 °C. Five-gram (dry weight) samples at 2.5% (w/v) consistency were used in an acetate buffer (pH 4.8). Three different variables were evaluated: (1) the loading of HBT (0 to 3% (w/w) of dry substrate); (2) the laccase dosage (0 to 200 U/g dry substrate); and (3) the incubation time (0 to 24 h). The solid yield was calculated using the following equation:

$$\text{Solid yield (\%)} = \frac{\text{mass of pretreated dry solid (g)}}{\text{mass of untreated dry solid (g)}} \times 100 \quad (1)$$

Enzymatic hydrolysis

The enzymatic hydrolysis was performed at 48 °C, at a pH of 4.8, with a substrate consistency of 2.5% (w/v) in a shaking incubator operating at 150 rpm for 96 h. The filter paper activity of the cellulases (Celluclast 1.5 L, Sigma Co., St. Louis, MO, USA) was 90 filter paper units (FPU/mL), and the cellobiase activity of Novozyme 188 (Sigma Co.) was 175 cellobiase units (CBU/mL). The enzyme loading for the substrate was 18 FPU/g-cellulose for cellulases and 27 CBU/g-cellulose for β-glucosidases.

Four enzymatic hydrolysis strategies were carried out in this study:

- Scheme (a): before enzymatic hydrolysis, the pretreated FRs were filtered with quantitative paper and washed with distilled water until a neutral pH was achieved.
- Scheme (b): the pretreated FRs were not filtered or washed before enzymatic hydrolysis.
- Scheme (c): the pretreated FRs was boiled at 121 °C for 20 min to deactivate laccases, and enzymatic hydrolysis was carried out after it cooled. The deactivation procedure was carried out in a pressurized bottle into which the pretreated slurry was transferred.
- Scheme (d): simultaneous laccase pretreatment and enzymatic hydrolysis were conducted in a conical flask.

Analysis of substrate composition

The Klason lignin and carbohydrate contents of raw FRs and pretreated materials were determined according to the National Renewable Energy Laboratory's (NREL) lignin analysis method for biomass (Sluiter *et al.* 2008). The Klason lignin content was considered the ash-free residue present after acid hydrolysis. Before carbohydrate analysis, samples were centrifuged at 10000×g for 5 min. The supernatants were then filtered through 0.22-μm filters and quantified using a Waters liquid chromatographic system (Waters 2695e, USA) with an Aminex HPX-87P column (300×7.8 mm, Bio-Rad, USA) at 85 °C with a refractive index detector at 35 °C. The injection volume of the sample was 10 μL, and distilled water was used as the eluent with a flow rate of 0.6 mL/min. The glucose yield was calculated by assuming that 1 g of cellulose present in the liquid should theoretically render 1.11 g of glucose. Each data point generated was the average of duplicate experiments.

Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) analysis

The FRs treated with the laccase-mediator were washed with distilled water and dried in a vacuum-drying oven for 72 h before further testing. The dried samples were coated with gold-palladium in a sputter coater (E-1010, HITACHI, Japan). The morphological structure was observed by SEM (S-3400N, HITACHI, Japan). The surface chemical composition was determined with an energy-dispersive X-ray analysis system (EDX, GENESIS 2000, USA) attached to the electron microscope.

Fourier transform infrared spectroscopic analysis

The structural characteristics before and after pretreatment were also analyzed by Fourier transform infrared spectroscopy (FT-IR). The dried samples were mixed with Spectroscopic grade KBr and were pressed uniformly against the diamond surface using a spring-loaded anvil. FT-IR spectra were recorded between 4000 and 400 cm^{-1} with four scans recorded at a resolution of 4 cm^{-1}.

RESULTS AND DISCUSSION

Effect of Laccase or Laccase-Mediator Pretreatment on the Chemical Composition of FRs

The untreated FRs consisted of 38.6% glucan, 52.6% Klason lignin, and 7.3% ash (Table 1). Cellulose and lignin were the main components of the untreated FRs, accounting for more than 90% of their mass. The laccase pretreatment was carried out with a laccase loading of 100 U/g-dry substrate (DS) at 48 °C and a pH of 4.8. As shown in Table 1, laccase pretreatment dissolved only a small amount of lignin (2.4%). Small decreases in lignin content (of less than 5%) have also been observed following the laccase treatment of other lignocellulosic materials such as elephant grass, eucalyptus, and steam-pretreated spruce (Gutiérrez *et al.* 2012; Moilanen *et al.* 2011). Literature indicates that laccases can oxidize only phenolic lignin units (accounting for less than 20% of all lignin units in native biomass), because phenolic lignin units have lower reduction potential than non-phenolic lignin (C4-etherified) units (Bourbonnais and Paice 1990; Rodríguez *et al.* 2006). Furthermore, the lignin of FRs has been condensed, and the reactive groups at the α-position, such as hydroxyl groups and ethers, have been oxidized to form carbonyl groups or generate benzylic cations, which come from carbon-carbon bonds (*e.g.*, 5-5 or β-5) (Bu *et al.* 2012; Palonen and Viikari 2004). Because of these limitations, it is difficult for laccase alone to degrade the lignin of FRs. To improve lignin removal *via* laccases, a mediator, 1-hydroxybenzotriazole (HBT), was used in this study. The lignin content decreased gradually with increasing HBT loading (see Table 1). When 1% (w/w) HBT was added, approximately 10.5% of the present lignin was dissolved. No obvious lignin removal was obtained with further increases in the HBT dosage.

Table 1. Chemical Composition of Samples Pretreated with Laccase and Laccase-Mediator

HBT (% of dry substrate)	Component (%)			Yield (%)
	Klason Lignin	Glucan	Ash	
FRs	52.6±0.6	38.6±1.0	7.3±0.6	100±0.0
0	51.9±0.8	38.8±0.5	7.7±0.4	98.9±0.8
0.5	51.1±0.9	40.5±1.1	7.8±0.8	95.6±1.1
1	50.6±0.6	41.7±1.2	8.2±0.2	93.1±0.9
2	50.1±0.2	41.8±0.8	8.1±0.7	92.4±1.0
3	50.2±0.6	41.2±0.9	7.8±0.6	93.7±0.7

Effect of Laccase-Mediator Pretreatment Conditions on the Chemical Composition of FRs

As previously described, the laccase-mediator pretreatment dissolved more lignin than the pretreatment with laccase alone. In this study, laccase-mediator pretreatments under different conditions were evaluated. Pretreatments with laccase loadings ranging from 0 to 200 U/g-DS and incubation times ranging from 0 to 24 h were performed. For each pretreatment, 1% (w/w) of HBT mediator was used. Table 2 shows the effect of laccase loading on the solid yield and chemical composition of pretreated samples at 48 °C and a pH of 4.8 after a pretreatment duration of 24 h. The solid yield decreased with increases in the laccase loading and was in the range of 95.2 to 92.1% under the

described conditions. It was possible to determine the percentage of each chemical constituent remaining in the solid fraction after the laccase-mediator pretreatment based on the solid yield and the baseline chemical composition, as shown in Fig. 1. As shown in Table 2, the lignin content in the FRs decreased gradually with increasing laccase dosages. Approximately 10.5% of the initial lignin was removed following laccase-mediator pretreatment with 100 U/g-DS laccases. However, the lignin removal rate rose only to 12.1% as the laccase loading was increased to 200 U/g-DS. Thus, laccase loading of 100 U/g-DS was judged to be the most appropriate for delignification.

The effect of incubation time on the chemical composition with laccase loading of 100 U/g-DS at 48 °C and a pH of 4.8 is shown in Table 3. Delignification improved with increasing incubation time. After 8 h of pretreatment, approximately 9.6% of the lignin was removed (Fig. 1B). When a longer pretreatment was carried out with the same laccase dosages, the lignin content did not obviously decrease (Fig. 1B). Thus, an 8-h laccase-mediator pretreatment of the FRs was adequate for lignin removal.

Table 2. Chemical Compositions of Solid Fractions before and after Pretreatment with Different Laccase Loading at 2.5% Consistency, 48 °C, pH 4.8, for 24 h, with 1% 1-Hydroxybenzotriazole

Laccase Dosage (U/g-DS)	Component (%)			Yield (%)
	Klason Lignin	Glucan	Ash	
0	52.6±0.6	38.6±1.0	7.3±0.6	100±0.0
50	50.7±0.5	39.2±0.9	7.6±1.1	97.3±0.7
80	50.3±0.6	39.7±0.4	8.1±0.4	95.8±0.3
100	50.6±0.1	41.6±0.6	7.8±0.6	93.1±0.8
150	50.3±0.2	41.5±0.1	8.3±0.4	93±0.7
200	50.2±0.6	41.9±0.2	8.1±0.7	92.1±1.0
DS: dry substrate.				

Table 3. Chemical Compositions of Solid Fractions before and after Pretreatment under Different Incubation Times at 2.5% Consistency, 48 °C, pH 4.8 with 100 U/G-DS Laccase Loading and 1% 1-Hydroxybenzotriazole

Time (h)	Component (%)			Yield (%)
	Klason Lignin	Glucan	Ash	
0	52.6±0.6	38.6±1.0	7.3±0.6	100±0.0
4	51.4±0.3	40.5±0.1	7.8±0.6	95.2±0.9
8	50.9±0.5	41.1±1.2	7.9±0.8	93.4±1.2
12	50.6±0.6	41.2±0.2	8.1±0.8	94.9±0.8
24	50.4±0.3	41.6±0.3	7.8±1.1	93.1±1.1
DS: dry substrate.				

Although more lignin (50%) could be removed using a chemical pretreatment, high cellulose loss (above 10%) is also observed (Yu *et al.* 2013). Laccase-mediator pretreatment allowed for retention of almost 100% of the initially present cellulose, as shown in Fig. 1. The glucan remaining in the solid fraction was in the range of 98.4 to 99.8% following laccase-mediator pretreatment under different laccase loadings and incubation times (Fig. 1). This indicates that cellulose was not significantly decomposed during the laccase-mediator pretreatment. This is a beneficial finding with respect to the commercial production of bioethanol. Similarly, the laccase-mediator pretreatment did not affect the amount of ash remaining in the solid fraction.

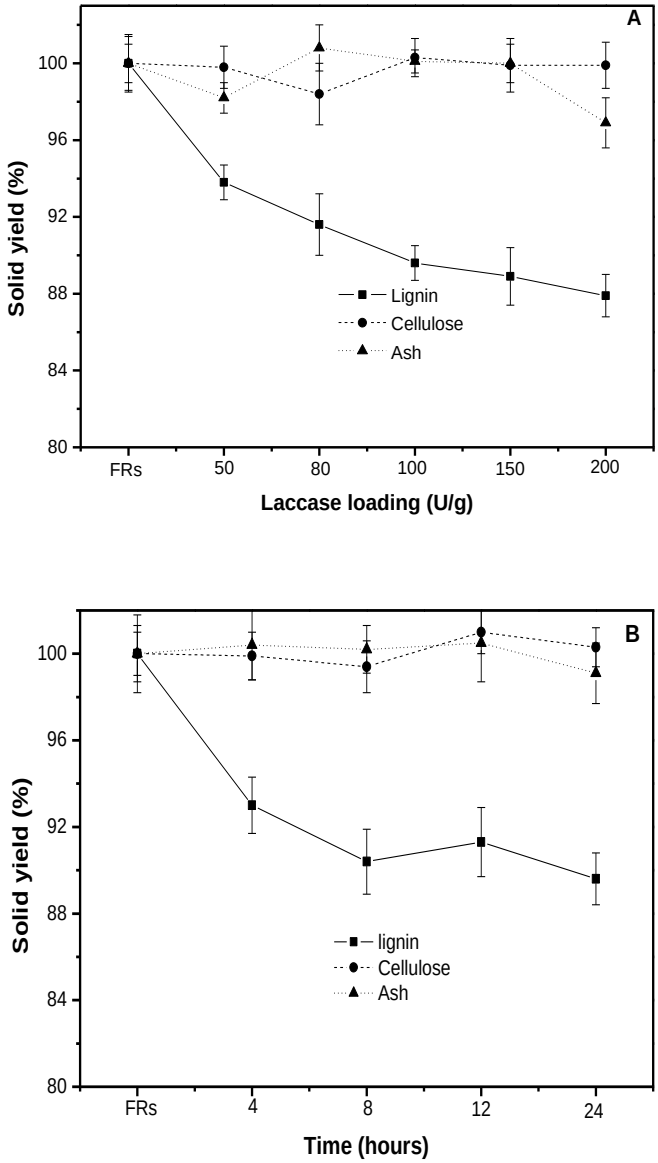

Fig. 1. Percentage of each component remaining in the solid fraction after laccase-mediator pretreatment under different conditions compared to the original amounts of these components present in the raw material. A, furfural residues were pretreated with different laccase loading. B, furfural residues were pretreated with different incubation times.

Effect of Laccase and Laccase-Mediator Pretreatment on Enzymatic Hydrolysis in Different Strategies

Before enzymatic hydrolysis, FRs were treated for 24 h to obtain the highest possible degree of lignin removal. Figure 2 shows the glucose yield from substrates pretreated with laccases using four enzymatic hydrolysis strategies. As shown in Fig. 2, the glucose yield increased rapidly during the initial stage but the growth began to level out after just 24 h. The highest conversion of cellulose was obtained from samples washed with distilled water (Fig. 2 (a) series). Approximately 66.9% of the glucose yield was attained within 96 h of enzymatic hydrolysis, which was higher than that of the untreated FRs (61.2%). It is well known that lignin is a major inhibitor of enzymatic hydrolysis. However, laccase pretreatment did not notably improve lignin removal from the FRs (Table 1). Improvement of enzymatic hydrolysis can be attributed to the modification of lignin, reducing its inhibiting effect on enzymatic hydrolysis. At the end of the 24-h laccase pretreatment, the enzymatic hydrolysis was carried out without filtration or washing. The effects of activated (Fig. 2b) and deactivated laccases (Fig. 2 (c) series) on enzymatic hydrolysis were investigated. Compared to untreated FRs, nearly the same glucose yield (61.9%) was observed from the slurry that was not deactivated of laccases after pretreatment with laccase alone (Fig. 2 (b) series). The glucose yield increased to 63.5% when laccases were deactivated at 121 °C for 20 min (Fig. 2 (c) series). This indicates that the laccase inhibited enzymatic hydrolysis. Although the glucose yield was increased by deactivating laccases, it was still lower than the yield obtained from the substrate washed with distilled water. Some of the compounds generated *via* lignin degradation caused an observed decrease in glucose yield.

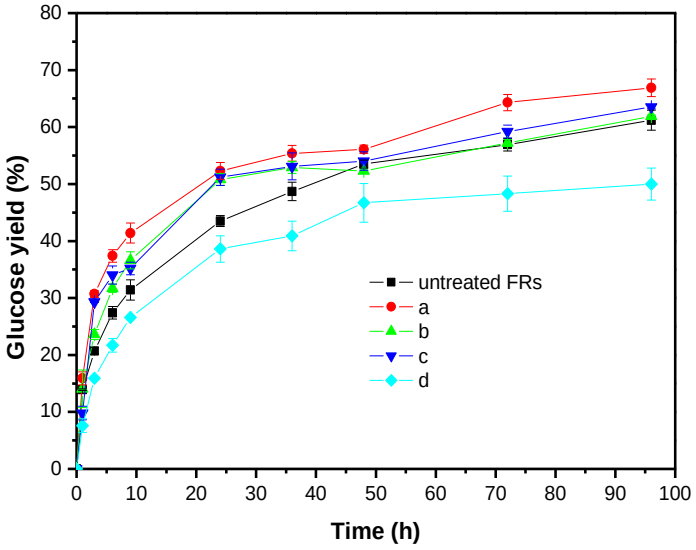

Fig. 2. Glucose yield from substrates pretreated with laccase alone and hydrolyzed with FPU of cellulases and 27 CBU of β-glucosidase/g of cellulose using four enzymatic hydrolysis schemes: (a), before enzymatic hydrolysis, pretreated furfural residues were filtered and washed with distilled water; (b), after pretreatment, the pretreated slurry was used for enzymatic hydrolysis without washing or deactivation of laccases; (c), after pretreatment, laccases were deactivated at 121 °C for 20 min and after it cooled, enzymatic hydrolysis was carried out; and (d) simultaneous laccase pretreatment and enzymatic hydrolysis. The pretreatment was carried out at 48 °C for 24 h with 100 U/g-DS laccases, at 2.5% (w/v) substrate consistency in an acetate buffer (pH 4.8).

A significant decrease in the glucose yield, from 61.9 to 50%, occurred when simultaneous laccase pretreatment and enzymatic hydrolysis were carried out. This shows that the high activity of laccases in the early stages of the hydrolysis seriously inhibited enzymatic hydrolysis.

The effect of the laccase-mediator pretreatment on the glucose yield with 1% (w/w) added HBT and 100 U/g-DS laccase loading with different enzymatic hydrolysis schemes is shown in Fig. 3. As shown in Fig. 3 (a) series, the 96-h enzymatic hydrolysis yield from the substrate washed with distilled water increased from 61.2% (for the untreated FRs) to 74.5%, greater than that of laccase-only pretreatment *via* the same enzymatic hydrolysis strategy. The greater lignin removal by the laccase-mediator pretreatment (Table 1) could account for this improvement in the enzymatic hydrolysis. The highest glucose yield, 80.9%, was obtained after 96 h of enzymatic hydrolysis when laccases were deactivated for 20 min at 121 °C after laccase-mediator pretreatment with 1% (w/w) HBT (Fig. 3 (c) series). This result was different from that of the laccase-only pretreatment, in which the highest glucose yield was obtained from the washed samples (Fig. 2 (a) series). It is possible that the presence of HBT improved the enzymatic hydrolysis. In order to verify this conclusion, 1% (w/w) HBT was used to hydrolyze untreated FRs with 18 FPU/g-cellulose for cellulases and 27 CBU/g-cellulose for β-glucosidase. According to Fig. 4, the 96-h glucose yield of the FRs increased from 61.2 to 65.1% following the addition of 1% (w/w) HBT. Thus, HBT had a defined, positive effect on enzymatic hydrolysis.

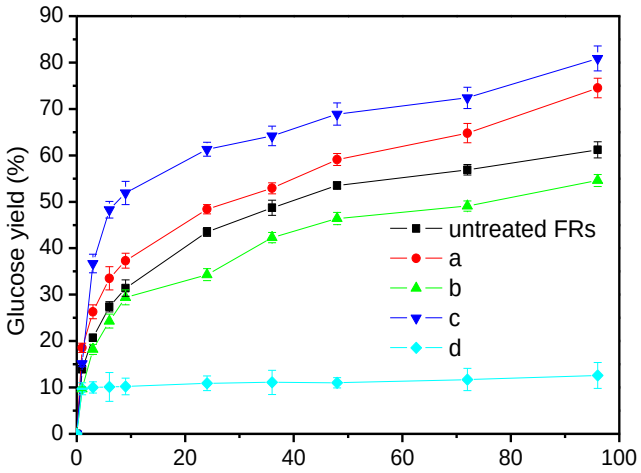

Fig. 3. Glucose yield from substrates pretreated with laccase-mediator and hydrolyzed with 18 FPU of cellulases and 27 CBU of β-glucosidase/g of cellulose using four enzymatic hydrolysis schemes: (a), before enzymatic hydrolysis, pretreated furfural residues were filtered and washed with distilled water; (b), after pretreatment, the pretreated slurry was used for enzymatic hydrolysis without washing or deactivation of laccases; (c), after pretreatment, laccases were deactivated at 121 °C for 20 min and after it cooled the enzymatic hydrolysis process was carried out; and (d) simultaneous laccase-mediator pretreatment and enzymatic hydrolysis. The pretreatment was carried out at 48 °C for 24 h with 100 U/g-DS laccases, 2.5% (w/v) substrate consistency in an acetate buffer (pH 4.8).

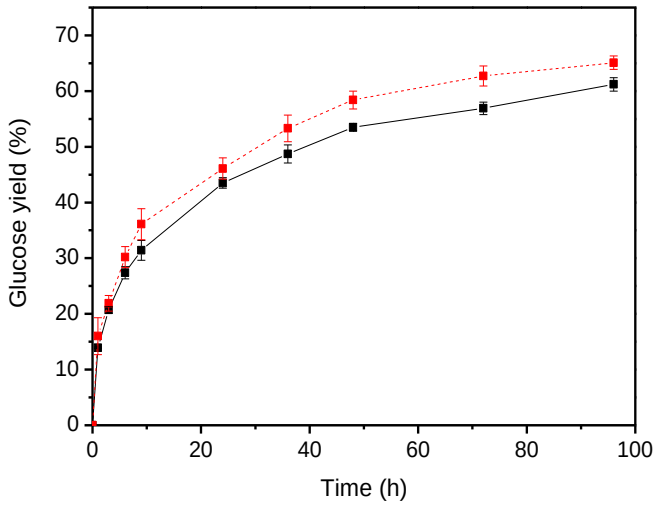

Fig. 4. Effect of 1-hydroxybenzotriazole on the glucose yield of furfural residues hydrolyzed with 18 FPU of cellulases and 27 CBU of β-glucosidase/g of cellulose

A remarkable decrease in the glucose yield, from 61.2 to 12.6%, was observed when the laccase-mediator pretreatment and enzymatic hydrolysis were carried out simultaneously (Fig. 3 (d) series). This yield was far less than that of the samples pretreated with laccase alone. It is well known that HBT acts as an electron transfer mediator within the laccase-mediator system. Generally, the HBT was oxidized to its nitroxide radical by laccases, which can oxidize a variety of aromatic compounds (Srebotnik and Hammel 2000). Research has demonstrated that the N-hydroxy-N-phenylacetamide (NHA) mediator oxidized by laccases inhibits cellulase activity (Palonen and Viikari 2004). Thus, the oxidized HBT also restrains the conversion of cellulose during enzymatic hydrolysis. When laccases were deactivated, HBT radical inhibition of cellulases did not occur. In contrast, the non-oxidized HBT will be adsorbed onto the surface of the lignin, which could prevent lignin from inhibiting cellulases. As such, the enzymatic hydrolysis was improved.

Compared to the simultaneous laccase-mediator pretreatment, the enzymatic hydrolysis was improved after 24 h of laccase-mediator pretreatment without the deactivation of laccases. As shown in Fig. 3c, the glucose yield increased from 12.6 to 54.6%. It is likely that the inhibiting effect of oxidized HBT was weakened after 24 h of pretreatment following a decrease in laccase activity. In addition, the 96-h enzymatic hydrolysis yield of the laccase-mediator pretreatment (54.6%) was greater than those of pretreatment with laccase alone (50%) under the same hydrolysis scheme. It is clear that the non-oxidized HBT caused the observed variation.

Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDX) Analysis

Figure 5 shows SEM images of FRs before and after laccase-mediator pretreatment. The surface of the untreated FRs was covered with small particles (Fig. 5A), which are thought to be formed by the melting of lignin at high temperatures and subsequent condensation during furfural production. The pores on the fiber surface were blocked by small lignin particles (Fig. 5B, in the red ring), which prevented cellulases

from entering into the fiber. These morphological characteristics resulted in the low glucose yields from untreated FRs during enzymatic hydrolysis. After 24 h of pretreatment with the laccase-mediator system, the fiber surface became smooth, and the lignin bound to the surface was removed, as well as that trapped inside pores (Figs. 5A and B). These changes decreased the lignin's inhibition of cellulases, consequently improving the enzymatic hydrolysis efficiency.

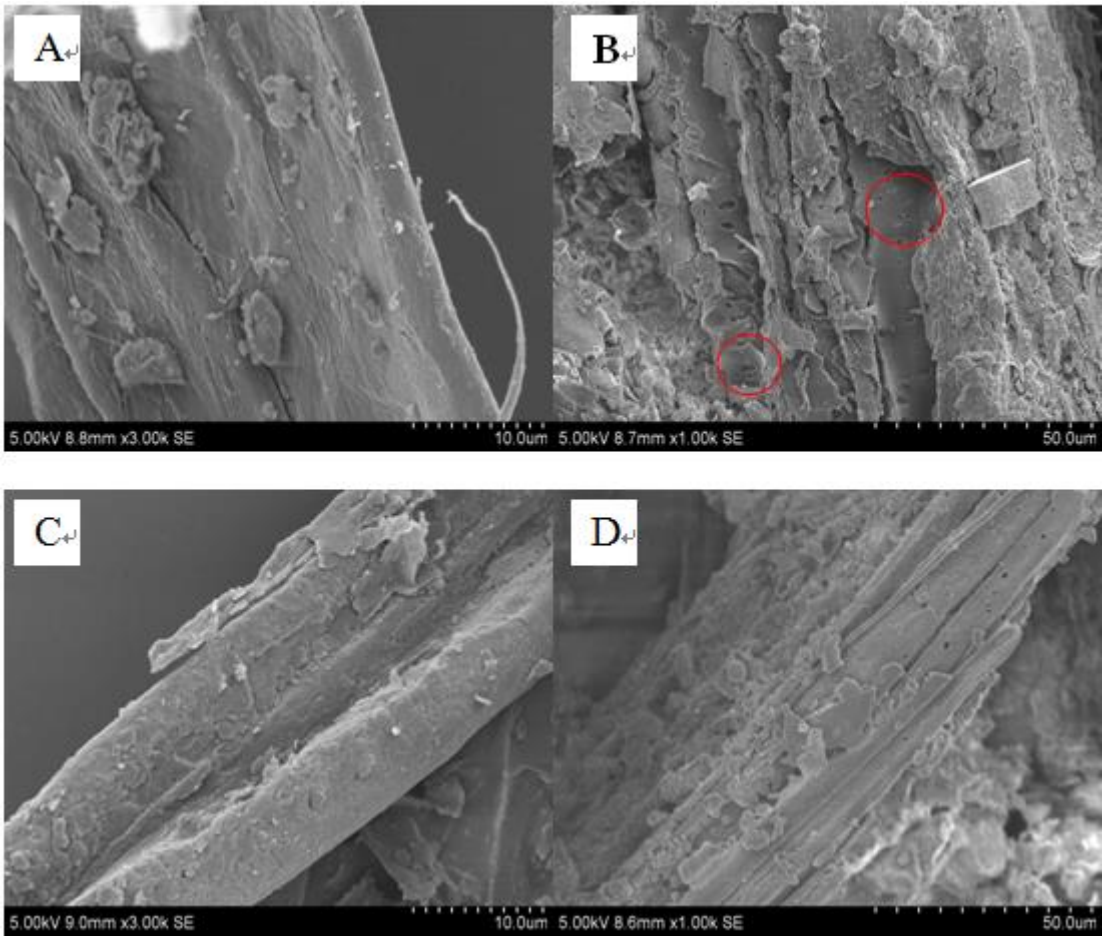

Fig. 5. Scanning electron microscopy (SEM) images of different samples: A and B: untreated furfural residues (FRs); C and D FRs treated with laccase-mediator for 24 h. A and C taken at 3000× magnification. B and D taken at 1000× magnification.

Energy dispersive spectroscopy (EDX) analysis revealed a change in the elemental composition on the surface of the FRs following pretreatment. The O/C value expresses the surface lignin content of the substrate. Theoretically, the O/C values of cellulose and lignin are 0.83 and 0.33, respectively (Laine *et al.* 1994; Pang *et al.* 2012). Therefore, lower lignin content corresponds to a higher O/C value. As shown in Table 4, the O/C value increased from 0.38 to 0.45 following laccase-mediator pretreatment, indicating that the surface lignin was removed. This agrees with the morphological structure analysis. In addition, a small amount of metallic elements such as potassium (K) and calcium (Ca) were observed on the fiber surface (Table 4). The potassium content decreased significantly in the laccase-mediator treated sample, whereas an increase in calcium content was noted in the reference sample. This could be due to the formation of

carboxylic acid during the oxidative treatment by laccase-mediator, and the consequent binding of calcium as a deposit adsorbed onto the fiber surface. The potassium carboxylates were easily removed because they were water-soluble.

Table 4. Atomic Composition (%) on the Solid Surface of Furfural Residues before and after Laccase-Mediator Pretreatment

	O	C	Mg	Ca	O/C
Untreated FRs	27.2±1.9	70.7±2.7	0.19±0.01	0.16±0.05	0.38
Laccase-mediator treated FRs	30.9±0.4	68.5±0.7	0.11±0.01	0.19±0.05	0.45

Fourier Transform Infrared Spectroscopic (FT-IR) Analysis

FT-IR analysis was carried out to detect any changes in functional groups potentially caused by pretreatment. Figure 6 shows the FT-IR spectra of the untreated FRs (Fig. 6a) and of the FRs pretreated with the laccase-mediator system (Fig. 6b). The broad absorption peak at 3420 to 3450 cm^{-1} is related to O-H stretching of hydrogen bonds. As shown in Fig. 6, the hydrogen bands of laccase-mediator-pretreated FRs arose at high wave numbers and their band intensity decreased. This indicates that the structure of laccase-mediator-pretreated FRs was looser than that of untreated FRs due to decreases in intramolecular hydrogen bonding. For both samples, peaks attributable to the aromatic skeletal vibrations (at 1509 cm^{-1}) of lignin were observed (Fig. 6). As shown in Fig. 6, laccase-mediator pretreatment decreased the intensity of the band at 1509 cm^{-1}. Moreover, the bands corresponding to non-conjugated C=O stretching at 1701 cm^{-1} and conjugated C=O at 1636 cm^{-1} decreased in intensity after 24 h of laccase-mediator pretreatment. Generally, C=O bonds exist in the lignin and hemicelluloses of biomass materials. In FRs, they are found only in lignin due to the thorough removal of hemicelluloses. Thus, decreases in aromatic skeletal vibrations and C=O vibrations indicate that lignin was removed during the laccase-mediator pretreatment.

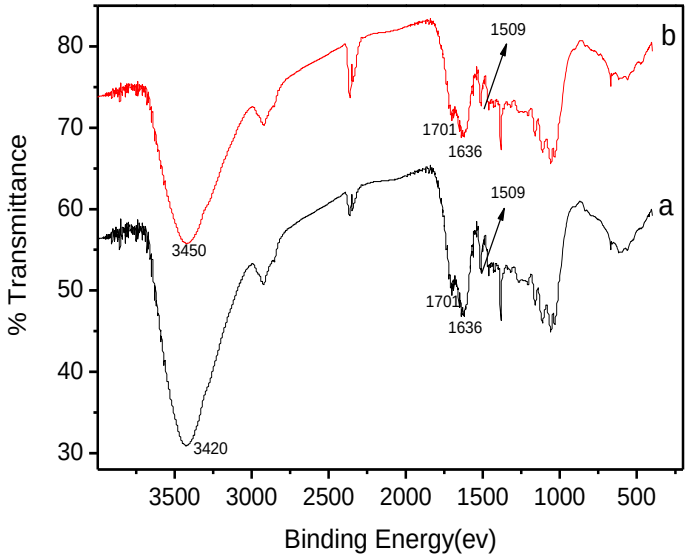

Fig. 6. Fourier transform infrared spectra of (a) untreated and (b) laccase-mediator pretreated furfural residues.

It was reported in a previous work that the C=O bond plays an important role in the binding of enzymes to lignin (Yu *et al.* 2013). Hence, the decrease in C=O bonding could reduce the unproductive adsorption of cellulases onto lignin.

CONCLUSIONS

1. Laccase pretreatment did not effectively dissolve the lignin of FRs. Only about 2.4% of the initially present lignin was removed. Delignification was improved when a laccase-mediator pretreatment was performed. Under optimized conditions (100 U/g-DS laccases, 1% (w/w) HBT, pH of 4.8, incubation time of 24 h, 48 °C), approximately 10.5% lignin removal was obtained.
2. Different enzymatic hydrolysis strategies were carried out, including washing the pretreated solids, keeping the activity of laccases, deactivating laccases after 24 h of pretreatment, and simultaneously performing the pretreatment and enzymatic hydrolysis. Due to inhibitions by active laccases and oxidized HBT, the strategies of maintaining the activity of laccases after pretreatment and employing simultaneously pretreatment and enzymatic hydrolysis were not appropriate for enzymatic hydrolysis.
3. For pretreatment with laccase alone, the highest glucose yield was obtained from substrates washed with distilled water.
4. The best hydrolysis scheme for the laccase-mediator pretreatment was that in which laccases were deactivated at 121 °C for 20 min. In this strategy, the glucose yield increased from 61.2 (in untreated FRs) to 80.9%, which was higher than that of substrates washed with distilled water (74.5%). This was due to the positive effect of HBT on enzymatic hydrolysis.
5. The laccase-mediator pretreatment could be well-coupled into a sequential process with enzymatic hydrolysis for producing fermentable sugar without a washing step.
6. SEM images, EDX analysis, and FT-IR spectra showed that the laccase-mediator pretreatment removed lignin particles that had adsorbed onto the surface of fibers or into pores and modified the lignin's structure.

ACKNOWLEDGMENTS

The authors are grateful for financial support from the Fundamental Research Funds for the Central Universities (BLYJ201415), the China Ministry of Science and Technology (2014DFG32550), and the Guangxi Key Laboratory of Chemistry and Engineering of Forest Products (GXFC13-03).

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Article submitted: April 2, 2014; Peer review completed: June 2, 2014; Revised version received: June 12, 2014; Accepted: June 14, 2014; Published: June 17, 2014.