

# Investigating Lignocellulose in Cornstalk Pretreated with *Trametes pubescens* Cui 7571 to Improve Enzymatic Saccharification

Xuejun Wu, Qi An, Yucheng Dai, and Jing Si \*

This study investigated the degradation and enzymatic saccharification of cornstalk by white-rot and brown rot fungi. The fungal strains *Trametes pubescens* Cui 7571, *Trametes velutina* Dai 10149, and *Antrodia wangii* Cui 7568 were analyzed in solid-state fermentation cultures. Various extracellular enzyme activities were assessed to determine biochemical changes during the degradation process. Fourier-transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) analysis were used to determine the structural changes. A correlation analysis between the chemical composition of cornstalk, extracellular enzyme activities, and structural changes indicated that *T. pubescens* Cui 7571 broke down lignin efficiently and subsequently degraded cellulose, and hemicellulose digestion was not affected by the lignin barrier. Enzymatic hydrolysis demonstrated that the *T. pubescens* Cui 7571-pretreated samples increased cellulose and hemicellulose conversion in cornstalk. Overall, *T. pubescens* Cui 7571 displayed excellent performance as a biological pretreatment agent, and lignin played a significant role in the enzymatic saccharification of cornstalk.

*Keywords:* White-rot fungi; Extracellular enzyme activity; Chemical composition; FTIR; XRD; Enzymatic hydrolysis

*Contact information:* Institute of Microbiology, P.O. Box 61, Beijing Forestry University, Beijing 100083, China; \*Corresponding author: samantha0128@163.com

## INTRODUCTION

Biological fuel is the most indispensable and renewable energy source in the 21<sup>st</sup> century, and it has received extensive research attention, owing to its environmentally friendly properties and recyclability. According to a report from the U.S. Department of Energy, biomass transportation fuels increased from 0.5% in 2001 to 4% in 2010; and it will keep increasing to 10% in 2020 and 20% in 2030 (Perlack *et al.* 2005). Potential sources for low-cost ethanol production are lignocellulosic materials, such as crop residues, grasses, sawdust, wood chips, and solid animal waste (Sun and Cheng 2002; Sánchez 2009). However, enzymatic hydrolysis of non-pretreated lignocelluloses is not particularly effective because of the high resistance of these materials to enzymatic or microbial treatments (Taherzadeh and Karimi 2008). Developing the potential of lignocellulosic biomass requires screening for a suitable decomposing organism, understanding the degradation process, clarifying the changes in chemical composition, and overcoming the high resistance to microbial or enzymatic degradation. To resolve these problems, researchers have attempted to improve the hydrolysis rate of lignocellulose complexes by pretreatment with a microorganism, hot fluid water, and acid (Fackler and Schwanninger 2012; Wang *et al.* 2013).

Wood-decomposing fungi degrade wood components more effectively than other microorganisms (Lee *et al.* 2008). Recently, pretreatment with white-rot fungi, which produces large amounts of lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases (Lac), has been extensively investigated. However, few studies have compared brown-rot fungi as an approach to explain the superior decomposition that has been achieved with white-rot fungi. Brown-rot fungi selectively degrade carbohydrates without affecting the surrounding lignin, which is required to prevent microbial attack (Kerem *et al.* 1999). Compared with white-rot fungi, brown-rot fungi have a stronger cellulase system and produce measurable amounts of endoglucanases and  $\beta$ -glucosidases, which cannot be generated by bacteria, *Actinomyces*, or other fungi (Kuhad *et al.* 1997). By adding brown-rot degradation as a comparison to the white-rot degrading process, the interactions among cellulase, hemicellulose, and laccase should be demonstrated more systematically.

The optimal conditions and enzyme activities for lignin-degrading peroxidase production by *Phanerochaete chrysosporium* have been established (Singh and Chen 2008). However, limited information is available on biodegradation by non-model organisms, which are typically more efficient in decomposing lignocellulose. To explain the degradation process and improve the enzymatic saccharification of cornstalk, the non-model organisms *Trametes pubescens* (Schumach.) Pilát, *Trametes velutina* (Pers.) G. Cunn, and *Antrodia wangii* Y. C. Dai & H. S. Yuan were selected from a number of wood-rot fungi. Various ligninolytic enzymes and cellulases were assessed to determine biochemical changes during degradation. Fourier-transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) experiments were conducted to determine structural changes. Chemical changes in cornstalk were analyzed with regard to extracellular enzyme activities and the input materials including cellulose, hemicellulose, and lignin. Enzymatic hydrolysis was also conducted to test the efficiency of wood-rot fungi in decomposing cornstalk and increasing cornstalk saccharification.

## EXPERIMENTAL

### Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, USA). The cornstalk was obtained from farmers in the countryside of Beijing, China. After cleaning, oven drying, and mill-grinding, particles with sizes of 40-mesh and 100-mesh were collected as the carbon source for lignin peroxidase and cellulase production.

All fungi used in the evaluation were obtained from the herbarium of the Institute of Microbiology, Beijing Forestry University (BJFC), Beijing, China. Ten white-rot fungal isolates were screened using guaiacol agar plates, and seven brown-rot fungi were grown on filter paper and CMCNa-Congo red media.

### Methods

#### *Fungal strains and inoculum preparation*

All strains were maintained on 2% (w/v) malt-extract agar (MEA) slants at 4 °C. The strains were activated in 100 mL of yeast extract medium (20 g/L glucose, 5 g/L yeast extract, 1 g/L  $\text{KH}_2\text{PO}_4$ , 0.5 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.01 g/L vitamin B<sub>1</sub>) and cultured on a rotary shaker at 28 °C and 150 rpm. After 5 days, mycelial pellets were harvested and

mixed with 100 mL of distilled water in a laboratory blender for 30 s at 5000 rpm. The resulting suspensions were selected as inocula.

#### *Fungal pretreatment of cornstalk*

The fungal pretreatment was carried out in a 250-mL Erlenmeyer flask with 5 g of air-dried corn stover and 12.5 mL of distilled water. Samples were sterilized in the autoclave for 30 min at 121 °C, followed by treatment with 1.5 mg/g ( $W_{\text{mycelia}} / W_{\text{cornstalk}}$ ) of inoculum. The cultures were incubated statically at 28 °C for 8 weeks in the incubator. The non-inoculated samples served as the controls. All experiments were performed in triplicate.

#### *Enzyme extraction and assays*

The sample in each flask was extracted with 100 mL of HAc-NaAc (pH 4.8) with stirring (150 rpm) at room temperature for 4 h and passed through fine filter paper. The resulting filtrate was used to measure enzyme activities by a Unico SpectroQuest 4802 UV-Vis double beam spectrophotometer (Dayton, USA).

Fungal crude enzymes were prepared by incubation on cornstalk as a carbon source. Endo- $\beta$ -1,4-glucanase (EC 3.2.1.4) levels were measured on azo dye carboxymethyl cellulose substrate and quantified as carboxymethyl cellulase (CMCase) units (CMCU) (Ghose 1987; Valášková and Baldrian 2006). To measure activity, sodium carboxymethyl cellulose solution served as substrate and 0.1 mol/L sodium acetate buffer (pH 4.6) was the reaction buffer. For total cellulase activity which were evaluated on crystalline filter paper and quantified as standard filter paper units (FPU) following the DOE-NREL standard (Adney and Baker 1996), filter paper was the substrate and also 0.1 mol/L sodium acetate (pH 4.6) was used as buffer. In terms of xylanase activity, we used xylan solution (1 g of xylan and 100 mL of 50 mM sodium dihydrogen phosphate-citric acid buffer (pH 5.0)) as substrate. All the three enzyme activities were determined by the 3,5-dinitrosalicylic acid (DNS) method. After specific incubation, the reaction was terminated by adding DNS, boiled for 5 min, and diluted with distilled water. Samples were then cooled to room temperature and measured. The amounts of additives and conditions in enzyme assays are shown in Table 1.

Laccase was assayed by monitoring the changes in 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Briefly, 1 mL of ABTS solution (0.05 mmol/L) was added to 500 mL of enzyme solution and 1 mL of 0.2 mol/L sodium dihydrogen phosphate-citric acid buffer (pH 4.0). Samples with a molar absorption coefficient of  $3.6 \times 10^4 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$  were measured at 420 nm in 1-min increments.

**Table 1.** Amounts of Additives and Conditions in Enzyme Assays

Type	Crude enzymes	Substrate	DNS	Time Temperature	Wavelength
Endo- $\beta$ -1,4-glucanase	1.9 mL	0.1 mL	1.5 mL	60 min 50 °C	520 nm
Total cellulose	1 mL	50 mg	3 mL	60 min 50 °C	520 nm
Xylanase	0.5 mL	1.5 mL	3 mL	10 min 40 °C	550 nm

### Enzymatic hydrolysis

To decompose the post-treatment samples, a commercial cellulase preparation (Celluclast, 1.5 L) and  $\beta$ -glucosidase were purchased from Sigma-Aldrich (St. Louis, USA). Enzymatic hydrolysis was conducted with 2% (w/v) substrate loading in 10 mL of 50 mM sodium acetate buffer (pH 4.8) supplemented with 40  $\mu$ L of tetracycline and 20  $\mu$ L of cycloheximide. The cellulase loading was 14 and 35 FPU/g substrate with 15 and 37.5 CBU/g, respectively, of  $\beta$ -glucosidase substrate. The mixture was incubated at 50 °C in a rotary shaker at 150 rpm. Samples were taken from the reaction mixture periodically, centrifuged for 10 min at 10000 rpm, and stored at 20 °C.

### Analytical methods

The cornstalk samples were prepared and characterized by the two-stage acid hydrolysis method described by Sluiter *et al.* (2008). The first hydrolysis step used 72% sulfuric acid at 30 °C for 1 h; immediately following the 1 h incubation, the samples were diluted to 4% and autoclaved for 1 h. The resulting solid residues were reported as acid-insoluble lignin (Klason lignin). The sugars in the aqueous phase were quantified with high performance anion exchange chromatography (HPAEC). The HPAEC system (ISC 3000, Dionex, Sunnyvale, CA, USA) was equipped with an amperometric detector, AS50 autosampler, a CarboPac™ PA20 column (4 × 250 mm, Dionex), and a guard PA20 column (3 × 30 mm, Dionex). Monosaccharides in the supernatant were also analyzed using HPAEC. Cellulose and hemicellulose conversion were calculated as follows:

$$\text{Cellulose conversion (\%)} = \frac{\text{amount of glucose in enzyme hydrolysate} \times 0.9 \times 100}{\text{amount of cellulose}} \quad (1)$$

$$\text{Hemicellulose conversion (\%)} = \frac{\text{amount of xylose in enzyme hydrolysate} \times 0.88 \times 100}{\text{amount of hemicellulose}} \quad (2)$$

### FTIR measurements

After incubation, fungal mycelia were removed from the cornstalk surface. The samples and KBr were dried for 48 h at 40 °C and 120 °C, respectively. After cooling to ambient temperature (25 °C), FTIR images were recorded in the transmission mode on a Spectrum Spotlight 400 FTIR microscope connected to a Spectrum 100 FTIR spectrometer (PerkinElmer Inc., Waltham, MA, USA) in the spectral region of 4000 to 400  $\text{cm}^{-1}$  with a 16 scan speed. Discs were prepared by first mixing 2-mg dried samples with 200 mg of KBr in an agate mortar. The resulting mixture was pressed at 10 MPa for 3 min using a YP-2 tablet press (Shanghai, China).

### X-ray diffraction

The relative crystallinity of the decayed cellulose sample was analyzed with an XRD-6000 X-ray diffractometer (model D/max-B, Shimadzu, Kyoto Japan), using CuK $\alpha$  radiation ( $\lambda = 0.15418$  nm) operating at a voltage of 40 kV, a current of 40 mA, and a scan speed of 5 °C/min from 5 °C to 70 °C. MDI Jade 5.0 software (<http://mdi-jade.software.informer.com/5.0/>) was employed for data analysis. Plots were normalized to a unit area. Peaks were assigned according to the monoclinic cellulose I. unit cell, as described by Sugiyama *et al.* (1991). The crystallinity index ( $C_rI$ ) was calculated using the Segal method (Segal *et al.* 1959) based on Eq. 3,

$$CrI (\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (3)$$

where  $I_{002}$  is the intensity of diffraction from the (002) plane typically located in the range  $2\theta = 21^\circ$  to  $23^\circ$ , representing both crystalline and amorphous material, and  $I_{am}$  is the intensity of the background scatter measured at  $2\theta = 18^\circ$ , representing only amorphous material.

#### Statistical analysis

All experiments were performed in triplicate, with data expressed as mean values. An analysis of variance (ANOVA) was employed to determine the significant differences among isolates or other factors. Statistical analysis was performed using SPSS System for Windows 20.0 (IBM, Armonk, USA). In cases where the overall F ratio was statistically significant ( $\alpha = 0.05$ ;  $P < 0.05$ ), multiple comparison *t* tests (Fisher's protected least significant difference (LSD)) were conducted within each group to compare each treatment mean with the control for that respective group. A correlation analysis was also conducted.

## RESULTS AND DISCUSSION

### Selection of Fungal Strains

The hyphal and chromogenic diameters ( $d_1$  and  $d_2$ ) are listed in Table 2. Four cultures formed chromogenic rings, and eight grew well on guaiacol agar plates. A higher chromogenic diameter signifies a relatively stronger lignin degradation capacity during biological pretreatment. A  $d_1/d_2$  ratio close to one represents a greater amount of lignin decomposition. Although *Trametes orientalis* Cui 6320 was the most prominent fungus in lignin decomposition ( $d_1/d_2 = 0.83$ ) among all the tested fungi after the 10-day cultivation period, the strain was not suitable for cornstalk pretreatment because of its slow growth ( $d_1 = 3.5$ ). Hyphal diameter would be a good index to evaluate the growth ability of fungi. *Trametes velutina* Dai 10149 was the most stable with fast growth, and it had the largest hyphal diameter (4.4 cm) among all the strains (10 day). Accordingly, the white-rot fungus *T. velutina* Dai 10149 was selected for comparison with *Trametes pubescens* Cui 7571 in terms of its utility in cornstalk degradation.

The weight loss of the filter, the hydrolysis diameter, and the hyphal diameter are listed in Table 2. A higher value signifies stronger cellulase activity and can therefore be applied to screen brown-rot fungi suitable for biological pretreatment purposes. Except for *Fomitopsis pinicola* Dai 8195, all cultures grew well on the filter paper and CMCNa-Congo red media. The *Antrodia wangii* Cui 7568 isolate was selected from seven brown-rot strains for pretreatment because of its higher cellulase activity.

The weight loss of filter paper was greater with increasing cellulase activity of the isolate. After a 7-day cultivation on the filter paper medium, *Gloeophyllum trabeum* Dai 10267 displayed a significant difference in weight loss relative to the other strains ( $P < 0.05$ ), while the differences among the six other strains were not distinguishable. However, the hydrolysis diameter/hyphal diameter ratio obtained from CMCNa-Congo red medium differed from the results of the selection with a filter paper medium. Ultimately, *Antrodia wangii* Cui 7568 was selected for further study owing to its higher yield and cellulase activity (value = 1.53).

**Table 2.** Locality, Host, and Screening Data of 18 Wood-Decomposing Fungal Strains

Strain	Host	Locality	Selected Results			
			Hyphal Diameter, d <sub>1</sub> (cm)	Chromogenic Diameter d <sub>2</sub> (cm)	Value (d <sub>1</sub> /d <sub>2</sub> )	
White-Rot Fungi						
<i>Lenzites betulinus</i> Cui 5617	<i>Acer</i>	Tianhua Mts., Liaoning	0.7	3.1	0.23	
<i>Bjerkandera adusta</i> Dai 8222	<i>Populus</i>	Changbai Mts., Jilin	1.9	-----*		
<i>Trametes ochracea</i> Cui 6888	<i>Populus</i>	Lingshan, Hebei	3.0	4.8	0.63	
<i>Corioloopsis polyzona</i> Z 3	Angiosperm	Nonggang, Guangxi	-----*	-----*		
<i>Lentinula edodes</i> Dai 10131	<i>Quercus</i>	Changbai Mts., Jilin	0.6	-----*		
<i>Trametes orientalis</i> Cui 6320	Angiosperm	Changjiang, Hainan	3.5	4.2	0.83	
<i>Fomitiporia torreyae</i> Dai 8180	<i>Cryptomeria</i>	Hengshan, Hunan	0.8	-----*		
<i>Perenniporia japonica</i> Dai 9232	<i>Platyclusus</i>	Xiangshan, Beijing	-----*	-----*		
<i>Trichaptum pargamentum</i> Dai 5896	<i>Betula</i>	Shennongjia, Hubei	0.7	-----*		
<i>Trametes velutina</i> Dai 10149	<i>Betula</i>	Changbai Mts., Jilin	4.4	5.7	0.77	
Brown-Rot Fungi			Weight Loss (%)	Hydrolysis Diameter, d <sub>3</sub> (cm)	Hyphal Diameter d <sub>4</sub> (cm)	Value (d <sub>3</sub> /d <sub>4</sub> )
<i>Gloeophyllum trabeum</i> Dai 10267	<i>Larix</i>	Shidong, Liaoning	67.48	4.3	4.1	1.05
<i>Fomitopsis pinicola</i> Dai 8195	<i>Picea</i>	Changbai Mts., Jilin	36.07	-----*	-----*	
<i>Gloeophyllum sepiarium</i> Cui 5578	Gymnosperm	Tianhua Mts., Liaoning	36.32	3.9	3.0	1.30
<i>Fomitopsis palustris</i> Cui 7597	Angiosperm	Chebaling, Guangdong	35.49	2.4	2.5	0.96
<i>Fomitopsis cajanderi</i> Cui 3674	<i>Abies</i>	Changbai Mts., Jilin	35.51	2.0	2.0	1.00
<i>Laetiporus sulphurens</i> Cui 3504	<i>Quercus</i>	Changbai Mts., Jilin	35.26	3.0	2.8	1.07
<i>Antrodia wangii</i> Cui 7568	<i>Pinus</i>	Chebaling, Guangdong	38.44	4.9	3.2	1.53
* No growth or change						

## Correlation between Chemical Compositions of Cornstalk and Extracellular Enzyme Activities

The lignin, cellulose, and hemicellulose contents of cornstalk during an 8-week solid-state fermentation with the three fungal isolates (*Trametes velutina* Dai 10149, *Trametes pubescens* Cui 7571, and *Antrodia wangii* Cui 7568) are presented in Table 3. In the pretreated samples, lignin and hemicellulose were partially degraded, as noted in previous reports (Yu *et al.* 2009). Lignin degradation varied widely among the different experimental isolates and incubation times, ranging from 0% to 19.88%. *T. velutina* Dai 10149 and *T. pubescens* Cui 7571 greatly reduced lignin content to 21.06% and 19.51%, respectively, at week 8. Additionally, delignification was mainly attributed to the removal of acid insoluble lignin (AIL) because large quantities of AIL were removed at each treatment level, compared to the limited changes in the acid soluble lignin (ASL). The lignin content was more significantly affected by *T. pubescens* Cui 7571 ( $P < 0.05$ ) than the other two isolates. The cellulose content in substrates varied from 34.72% to 50.72%, and the hemicellulose content ranged from 22.48% to 25.02% (Table 3). The highest cellulose content was observed on week 8 following pretreatment with *Antrodia wangii* Cui 7568; this strain could not break down lignin barrier or digest cellulose. However, hemicellulose was effectively digested by *Antrodia wangii* Cui 7568. In general, hemicellulose was more easily utilized than cellulose (Isikhuemhen and Mikiashvili 2009). It is possible that hemicellulose digestion was not affected by the lignin content. Furthermore, hemicellulose degradation was markedly influenced by the brown-rot fungus ( $P < 0.05$ ), while there was no evidence of significant differences in degradation by the two white-rot fungi.

Enzyme activities during cultivation on cornstalk are shown in Fig. 1. The highest endo- $\beta$ -1,4-glucanase, total cellulase, and xylanase activities were observed in *A. wangii* at week 2 (6.98, 0.96, and 56.71 U/mg, respectively). Among the three fungi, xylanase activity varied from 25.83 to 56.71 U/mg during the 8-week period. The recorded xylanase activities were consistent with the findings of Ferraz *et al.* (2000), who reported that various organisms use hemicellulose as a carbon source without breaking lignin. *Trametes pubescens* Cui 7571 showed significant laccase activity from week 6 onwards, which was the highest on week 8 (0.17 U/mg). One possible explanation for this finding is that the fungus secretes metabolites that activate the expression of specific genes related to laccase generation (Edens *et al.* 1999; Lin *et al.* 2012). *T. pubescens* Cui 7571 secretes more laccase other microorganisms including actinomycetes *Streptomyces sp.* SB086 (Fernandes *et al.* 2014) and basidiomycetes *T. velutina* Dai 10149.

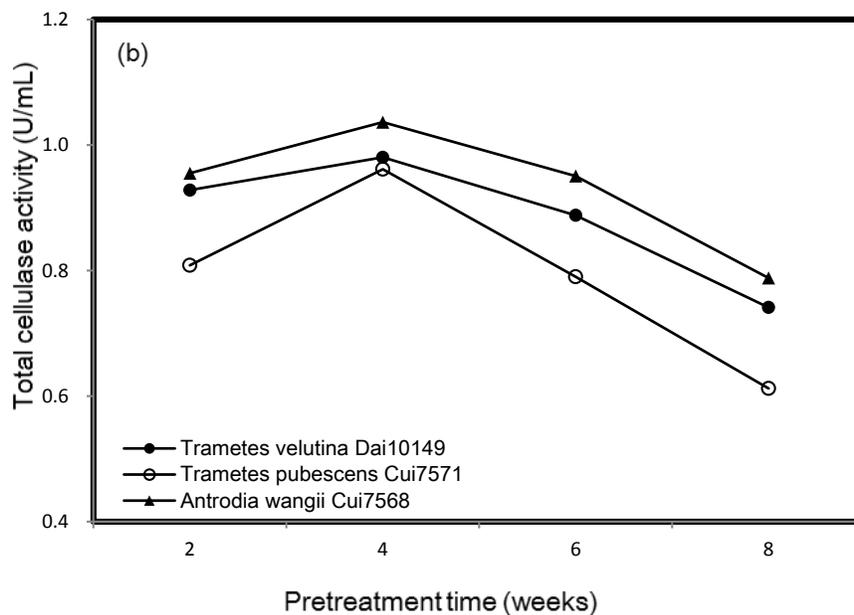
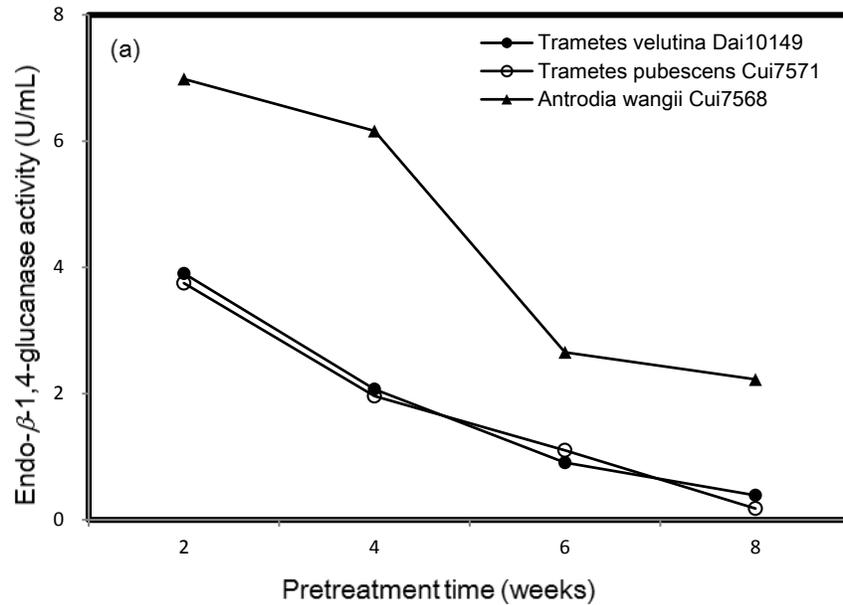
There was no correlation between CMCase activity and cellulose content in cornstalk treated with *T. velutina* Dai 10149 or *A. wangii* Cui 7568. However, a negative correlation was detected for *T. pubescens* Cui 7571, indicating that the strain broke down lignin efficiently and subsequently degraded cellulose. Similarly, a slight negative association between xylanase and hemicellulose was observed, which was particularly prevalent for *Antrodia wangii* Cui 7568. Xylanase activity was an important index to determine the digestion of pentose which was primary constituent of hemicellulose. Therefore the secrete of xylanase activity would reflect the degradation of hemicellulose and the digestion of lignocellulose. It is possible that hemicellulose digestion is not affected by the lignin content. Moreover, xylanase activity was significantly correlated with CMCU and FPU, which were determined as 0.861 and 0.985, respectively ( $P < 0.01$ ).

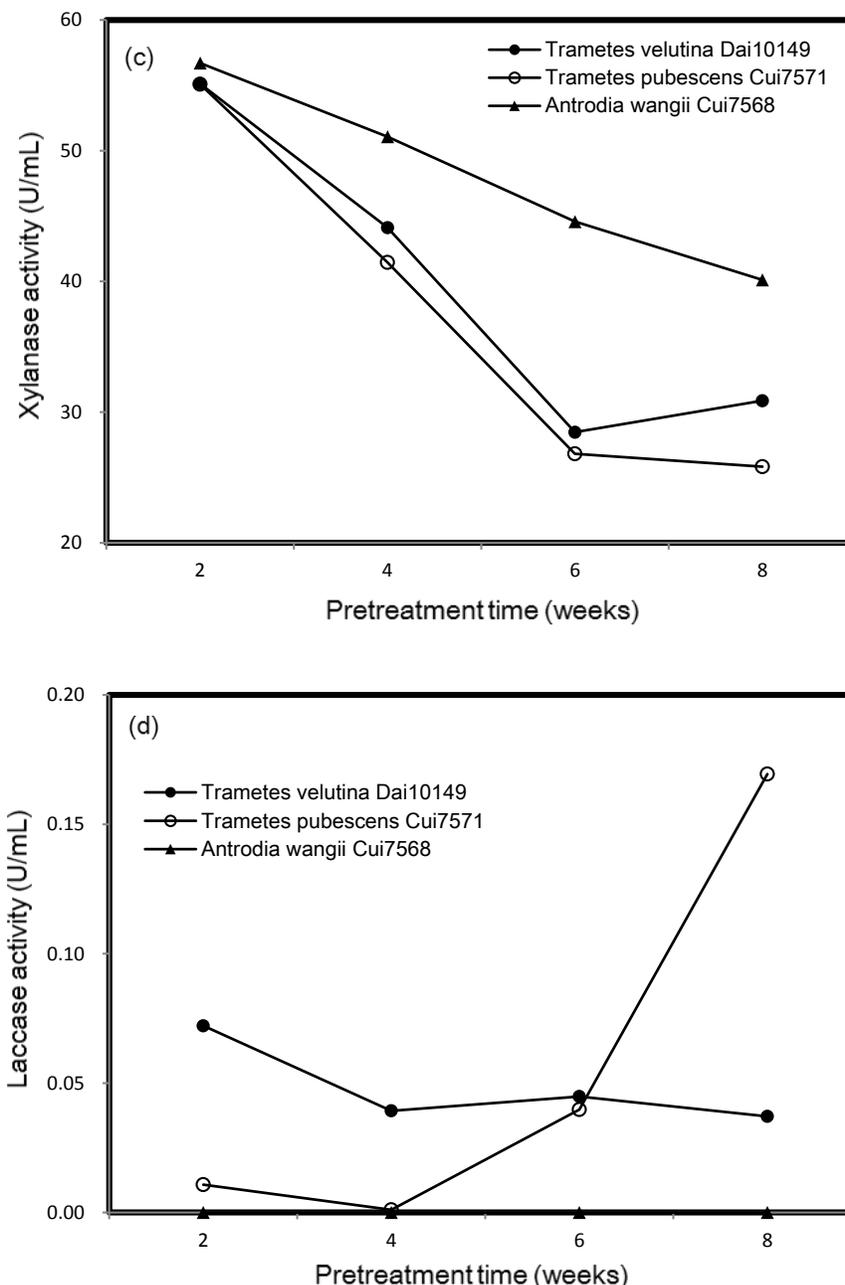
**Table 2.** Changes in the Chemical Components of Cornstalk after Bio-Pretreatment

Strains	Time (weeks)	Contents (%)					
		Cellulose	Hemicellulose	AIL	ASL	Lignin	Others
Untreated	-----	34.72	25.02	21.65	2.70	24.35	15.91
<i>T. velutina</i> Dai 10149 (WR)	2	36.26	25.67	21.60	2.57	24.17	13.90
<i>T. velutina</i> Dai 10149 (WR)	4	39.94	24.81	19.30	2.54	21.84	13.41
<i>T. velutina</i> Dai 10149 (WR)	6	48.52	24.64	18.73	2.51	21.24	5.60
<i>T. velutina</i> Dai 10149 (WR)	8	44.55	25.60	18.53	2.53	21.06	8.79
<i>T. pubescens</i> Cui 7571 (WR)	2	36.57	24.33	18.85	2.75	21.60	17.50
<i>T. pubescens</i> Cui 7571 (WR)	4	43.85	23.74	17.90	2.34	20.24	12.17
<i>T. pubescens</i> Cui 7571 (WR)	6	42.56	24.13	17.20	2.63	19.83	13.48
<i>T. pubescens</i> Cui 7571 (WR)	8	43.29	25.10	16.70	2.81	19.51	12.10
<i>A. wangii</i> Cui 7568 (BR)	2	37.96	24.05	20.75	2.57	23.32	14.67
<i>A. wangii</i> Cui 7568 (BR)	4	41.39	22.48	22.00	2.36	24.36	11.77
<i>A. wangii</i> Cui 7568 (BR)	6	49.78	23.23	20.25	2.81	23.06	3.93
<i>A. wangii</i> Cui 7568 (BR)	8	50.72	22.78	20.70	2.52	23.22	3.28

WR, white-rot; BR, brown-rot; AIL, acid insoluble lignin; ASL, acid soluble lignin

In general, the efficient degradation of lignocellulosic biomass requires the cooperative action of three enzymes (cellobiohydrolase, endoglucanase, and  $\beta$ -glucosidase) (Woodward 1991), although laccase also plays an important role in this process. Therefore, brown-rot fungi may be effective in hydrolyzing lignocellulosic biomass.



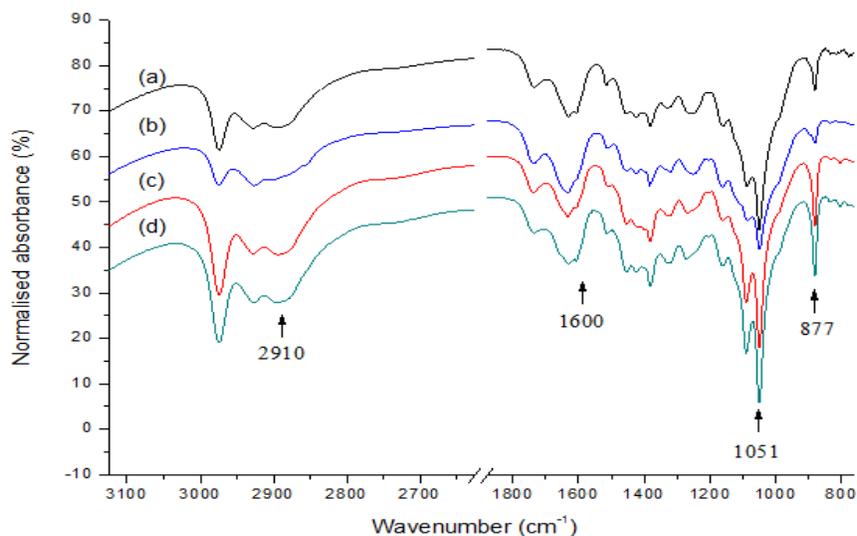


**Fig. 1.** Enzyme activities of three wood-decomposing fungi using cornstalk as a carbon source (a) instead of endo- $\beta$ -1,4-glucanase, CMCU; (b) instead of total cellulase, FPU; (c) instead of xylanase; and (d) instead of laccase. The values are the average of triplicate experiments.

### Effects of *Trametes pubescens* Cui 7571 on the Structure of Cornstalk

Each fungal strain was able to colonize and degrade cornstalk, although the brown-rot fungus preferably acted on coniferous wood, leading to lower lignin loss (Goodell 2003). Corresponding residues from all three groups on week 6 were assessed by FTIR (Fig. 2). Major changes in band intensities were observed near  $2910\text{ cm}^{-1}$ ,  $1600\text{ cm}^{-1}$ ,  $1051\text{ cm}^{-1}$ , and  $877\text{ cm}^{-1}$ ; these spectral regions are assigned to C-H stretching in methyl and methylene groups, C=O stretching in conjugated aryl ketones of lignin carbonyl groups, C-O deforming in secondary alcohols and aliphatic ethers, and cellulose  $\beta$ -chains and C-H

stretching out of plane in aromatic rings, respectively (Fackler *et al.* 2006, 2007, 2011). The spectra were plotted to highlight differences (Fig. 2). Lower level absorbance of C-H stretching in methyl and methylene groups of residue pretreated with *Trametes pubescens* Cui 7571 resulted in minimums near  $2910\text{ cm}^{-1}$  and  $1600\text{ cm}^{-1}$ , which meant it was efficient in delignification. Additionally, *T. velutina* Dai 10149 and *A. wangii* Cui 7568 showed the expected spectral images with a relative increase in the lignin content near  $1051\text{ cm}^{-1}$ , indicating almost exclusive decomposition of wood polysaccharides. *T. pubescens* Cui 7571 showed a minimum absorbance at  $877\text{ cm}^{-1}$ , which indicated that it was the most successful in attacking lignin and decomposing cellulose.



**Fig. 2.** FTIR spectra obtained from extracted milled cornstalk after six weeks of exposure to wood-decomposing fungi. (a) Non-degraded cornstalk; (b) *Trametes pubescens* Cui 7571; (c) *Trametes velutina* Dai 10149; and (d) *Antrodia wangii* Cui 7568

X-ray diffraction intensities of decayed cellulose samples from cornstalk are shown in Fig. 3. The crystallinity values of the samples pretreated with *Trametes pubescens* Cui 7571 at 4 and 8 weeks were 34.71% and 33.35%, respectively; the crystallinity values of the samples pretreated with *Antrodia wangii* Cui 7568 at 4 and 8 weeks were 37.11% and 36.55%, respectively. *T. pubescens* Cui 7571 clearly induced a greater decrease in cellulose crystallinity due to its highly efficient delignification.

### Influence of Lignin Removal on Cellulose Conversion and Hemicellulose Conversion

The pretreated residues (8 weeks) and untreated controls were submitted to enzymatic hydrolysis for 72 h (Fig. 4). Regardless of the enzyme loading, pretreatments substantially enhanced the efficiency of hydrolysis. After a 72-h saccharification in the *Trametes pubescens* Cui 7571 pretreated samples, 24.2% and 40.4% cellulose conversion was obtained by enzyme loadings of 14 and 35 FPU/g substrate, respectively. Similarly, in the *Trametes pubescens* Cui 7571 pretreated samples, 11.2% and 19.9% hemicellulose conversion was obtained after a 72-h saccharification with 14 and 35 FPU/g.

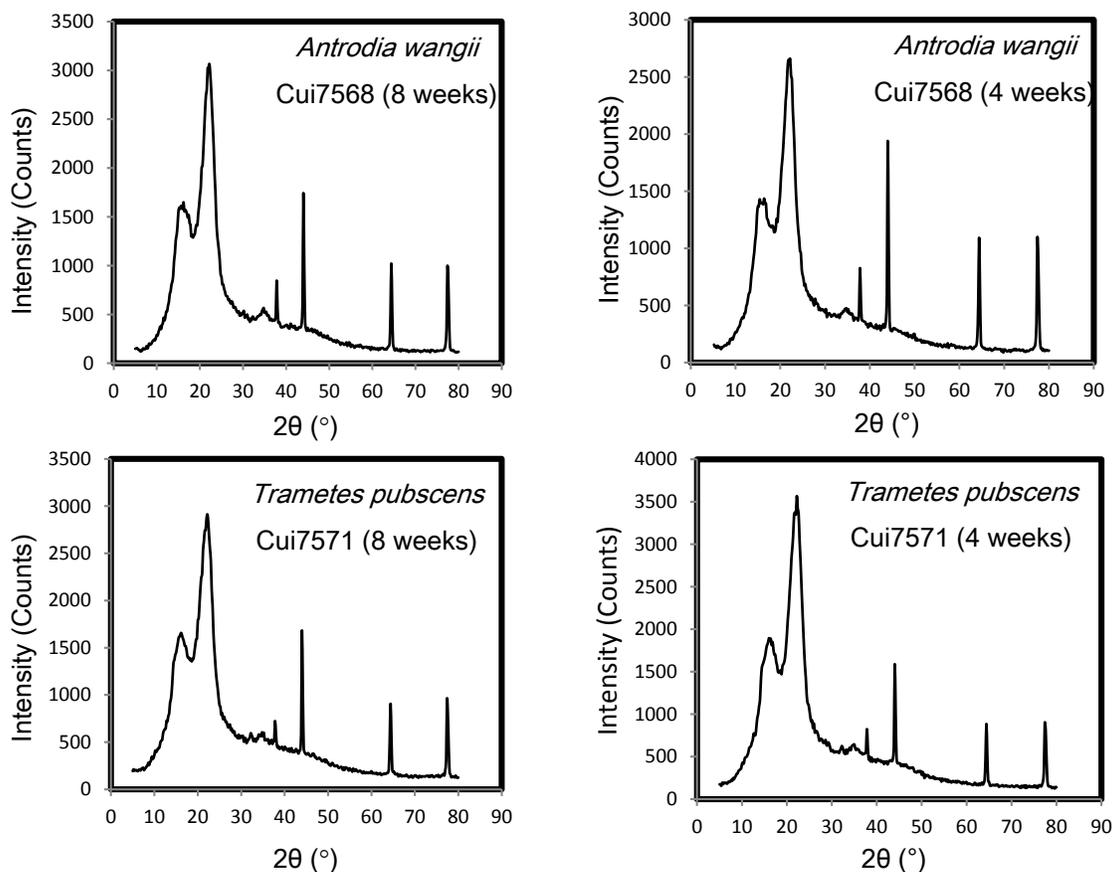
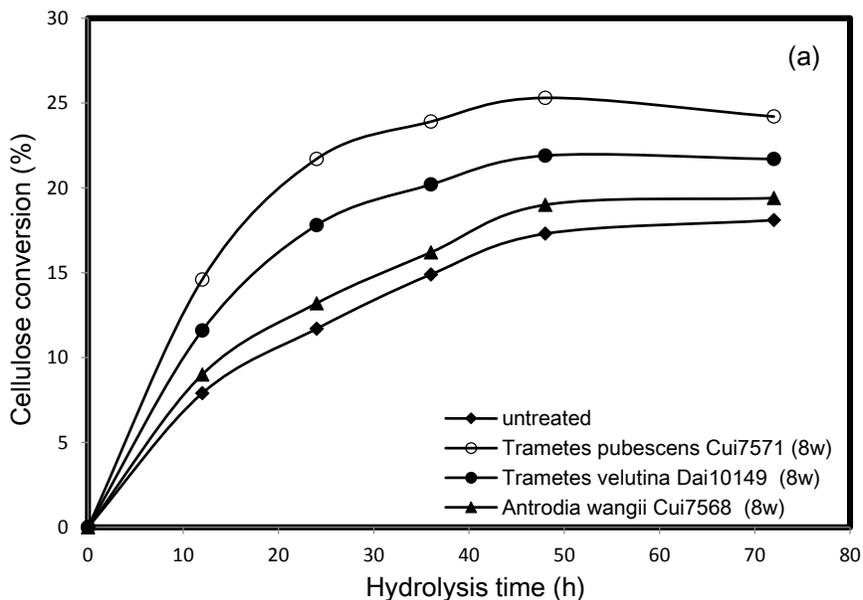
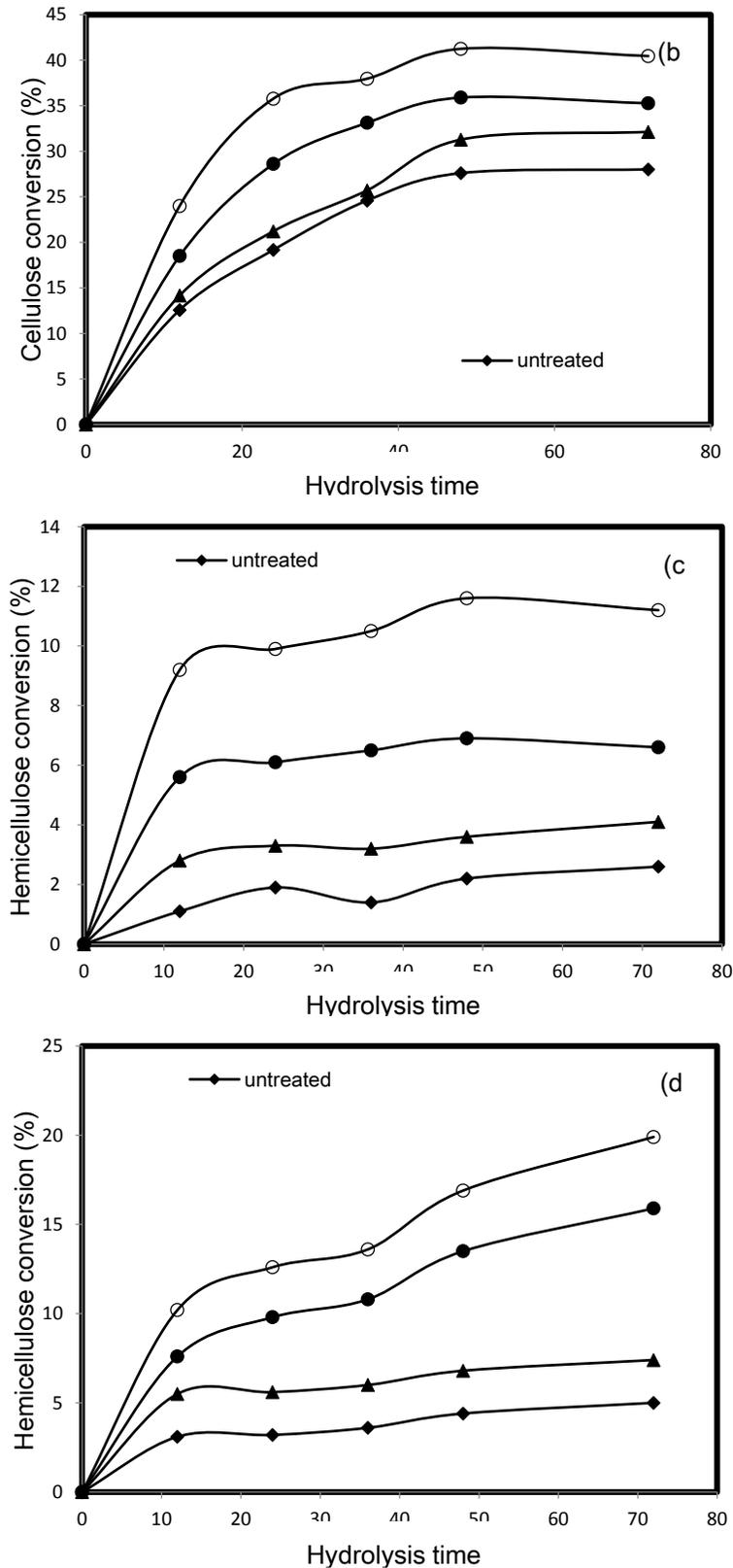


Fig. 3. XRD of cellulose from decayed cornstalk

As a whole, more delignification resulted in more cellulose and hemicellulose conversion.





**Fig. 4.** Pretreated poplar wood was subjected to 72-h saccharification and tested for (a, b) cellulose or (c, d) hemicellulose conversion using enzyme loadings of (a, c) 14 FPU cellulase/g substrate or (b, d) 35 FPU cellulase/g substrate.

These results demonstrated that lignin removal rendered xylan more accessible to xylanase, which in turn rendered cellulose more accessible to cellulase, as noted previously (Kumar and Wyman 2009). Interestingly, the brown-rot fungus *Antrodia wangii* Cui 7568 did not remove lignin but interfered with lignocellulose decaying to polysaccharides (Wu *et al.* 2007). It also contributed to cellulose and hemicellulose conversion, demonstrating that delignification was not the only way to accelerate conversion rates.

## CONCLUSIONS

1. The results collectively demonstrate that *Trametes pubescens* Cui 7571 displays excellent performance as a biological pretreatment agent.
2. Laccase is not the only factor affecting lignin content. High cellulase activity does not greatly impact cellulose degradation because it does not remove the lignin barrier. Hemicellulase affects hemicellulose content *via* a mechanism that bypasses lignin degradation.
3. Enzymatic hydrolysis experiments showed that lignin played a significant role in cellulose and hemicellulose conversion.

## ACKNOWLEDGEMENTS

This study was supported by the State Key Basic Research Development Program of China (No. 2014CB138301).

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Article submitted: November 6, 2015; Peer review completed: January 14, 2016; Revised version received: January 20, 2016; Accepted: January 21, 2016; Published: February 1, 2016.

DOI: 10.15376/biores.11.1.2768-2783