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Biodegradation of cotton stalk by three white rot fungi and the effects on composition and structure changes of cotton stalk

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In recent years, fungal pretreatment has received renewed attention for biorefinery of lignocellulosic feedstock. The present research was aimed to examine the biodegradation effects of three white rot fungi on cotton stalk. The results showed that three white rot fungi could degrade the lignocellulose of cotton stalk effectively at a certain degradation stage, the forming ratio of hyphal protein had significant negative correlation at the 0.01 level with hemicellulose and cellulose contents in biodegradation process; the degradation period was determined as 15 days, and *Phanerochaete chrysosporium* was chosen as the main strain for cotton stalk biodegradation. The analysis of FTIR (Fourier transform infrared spectroscopy) and SEM (Scanning electron microscopy) revealed that the intensity of absorption peaks of lignin, cellulose and hemicellulose decreased in 15-day degraded samples compared to original sample, especially the lignin related groups were degraded strongly, which demonstrated that all the three white rot fungi degraded the cotton stalk lignin effectively; correspondingly, the hypha of white rot fungi entered inside the lignocellulose of cotton stalk, and destroyed the structure, forming many cracks and holes, which exhibited good breaking and extending capability.

Keywords: biodegradation, cotton stalk, white rot fungi, lignocellulose, hyphal protein.

Introduction

In recent years, researchers have paid more and more attentions to transform lignocellulosic materials to biofuels, an effective pretreatment should be introduced in the biorefinery process to deal with lignin exist in lignocellulosic feedstocks[1]. Lignin is a very complex molecule constructed of phenylpropane units linked in a three-dimensional structure which is particularly difficult to degrade. Fungal pretreatment has been proved to be the best method to degrade lignin, and previously explored to degrade lignocellulosic materials for feed and paper applications[2,3]. Recently, this environment friendly approach has received renewed attention as a pretreatment technique for enhancing saccharification and fermentation of lignocellulosic biomass to ethanol[4-7]. Several white rot fungi such as *Phanerochaete chrysosporium*, *Ceriporiopsis subvermispora*, *Trametes versicolor* and *Pleurotus ostreatus*, which were thought as the most effective lignin-degrading microorganisms, have been examined on different lignocellulosic biomass to evaluate their delignification efficiencies[8,9]. It has been highlighted that fungal pretreatment shows potential advantages over the prevailing physical and chemical pretreatment technologies due to reduced energy and material costs, relatively simple equipment, and use of biological methods[8]. However, the feasibility of pretreatment by white rot fungi is still questioned, mainly due to the extremely long treatment time as well as the difficulty in selectively degrading lignin[9]. Some white rot fungi could simultaneously degrade cellulose and lignin, resulting in a low cellulose recovery[10,11], some species degrade lignin firstly, and hemicellulose secondly, leaving most cellulose residue[12], which maybe show the selectivity and order time law of lignocellulosic degradation.Cotton stalk is the most important feedstock in Xinjiang province, China, and produces in large amounts every year[13]. In recent years, high value utilization of cotton stalk has attracted many attentions, however, this feedstock contains high lignin content of about 44.0% (w/w)[14], which appreciably impacts the hydrolysis of cellulose, so an effective pretreatment is considered to be the key step for hydrolysis and further saccharification of cotton stalk. Several pretreatments, such as alkali, laccase, dilute acid hydrolysis[15-17] have been introduced to deal with the lignin in cotton stalk,

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yet some problems, like microbial hydrolysis period was too long (6-9 days), saccharification ratio hadn't been high enough, were still in existence. Shi *et al*. (2009) studied the effect of microbial pretreatment on enzymatic hydrolysis, and found that white rot fungus could effectively degrade lignin in cotton stalk, while the continuous hydrolysis rate was too low for ethanol production[11]. That is to say, up to the present, there haven't got effective pretreatment to deal with lignin in cotton stalk. The present work was carried out to examine the effects of three white rot fungi on cotton stalk degradation with the goals for understanding the dynamics of lignocellulose degrading ratios by different white rot fungi, and grasping the degradation mechanism of cotton stalk by white rot fungi.

Materials and Methods

Cotton stalks and preparation

Cotton stalks (variety *Gossypium hirsutum Linn* Zhong 35), were harvested in early November 2010 from the cotton field in Xinjiang Alaer, China. The stalk materials were air-dried, comminuted and sifted in 20-mesh sieve, stored in air tight containers at room temperature until using for degradation by white rot fungi.

White rot fungi and culture conditions

Three white rot fungi were used in the research, the strains, *P. chrysosporium* (CICC 40719) and *T. versicolor* (CICC 14001) were purchased from China Center of Industrial Culture Collection (Beijing, China), the strain F11 was obtained from Xinjiang Production & Construction Corps Key Laboratory of Protection and Utilization of Biological Resources in Tarim Basin (Xinjiang, China).The strains were propagated on potato dextrose agar (PDA) medium for 5 days. Spore suspensions of *P. chrysosporium* were prepared by washing the agar surface with 10 mL of sterilized physiological saline. Spore counts were determined with a hemacytometer, and the final spore concentration was adjusted to $1.0\text{-}6.0\times10^8$ spores mL^{-1} with inoculation quantity of 1% (v/w). The hyphal agars of *T. versicolor* and strain F11were used as inoculum, which were divided into identical agar pieces with diameter of 2.0 cm by a punching bear, three identical pieces were chosen and embedded into each solid state fermentation bottle. Solid state fermentation was conducted using Kirk medium described previously[18] as liquid basal medium, adding cotton stalk powder as solid substrate, adjusting moisture content as 60%, pH4.5.

Examination of lignocellulose content and protein forming content

Lignocellulose content was examined by the method found by Van Soest[19], the total and ligninocellulose (lignin, cellulose, hemicellulose) degrading ratios were calculated using Equations (1), and (2):

$$\textit{Total degrading ratio} = (W_i - W_f) \times 100/W_i \quad \ldots (1)$$

$$\textit{Lignin (Cellulose,Hemicellulose) degrading ratio} = (W_i \times L_i (C_i, H_i) - W_f \times L_f (C_f, H_f)) \times 100 / (W_i \times L_i (C_i, H_i)) \quad \ldots (2)$$

Where W_i = initial weight of cotton stalks (g), W_f = final weight of cotton stalks (g), L_i = initial content of lignin (%, w/w), L_f = final content of lignin (%, w/w), C_i = initial content of cellulose (%, w/w), C_f = final content of cellulose (%, w/w), H_i = initial content of hemicellulose (%, w/w) and H_f = final content of hemicellulose (%, w/w).

Protein content was examined by kjeldahl method[20]. The protein forming ratio was calculated using Equations (3):

$$\textit{Protein forming ratio} = (W_f \times P_f - W_i \times P_i) \times 100/(W_i \times P_i) \quad \ldots (3)$$

Where P_i= initial content of protein (%, w/w), P_f= final content of protein (%, w/w)

Fourier transform infrared spectroscopy and Scanning electron microscopy

The degraded cotton stalks were dried under the temperature of 80°C, and milled into 100-mesh sieve for fourier transform infrared spectroscopy. Taken little cotton stalk powders, mixed with KBr powder, milled and pressed into disc, analyzed by fourier transform infrared spectrometer Nicolet 380, with scanning times 64, resolution ratio 2 and data point interval 0.964 cm^{-1}.The dried cotton stalks were also used for scanning electron microscopy. The specimens were coated with Au/Pd at 0.4 torr pressure and 20 mA for 3 min. And finally they were examined under a Jeol JSM-6490LV SEM at 15e 3.0 kV.

Results and Discussion

Lignocellulose degradation and protein formation in biodegradation process of cotton stalk

The cotton stalk degradation samples were collected every five days, the dynamic curves of total degrading ratio, and lignin, cellulose, hemicellulose degrading ratios were built to reveal the lignocellulose

degradation rule. The curves of lignocellulose degrading ratios (Fig. 1(a, b and c)) showed that from the fifth day, cotton stalk degraded obviously by the three fungi, especially strain *P. chrysosporium* exhibited the strongest degrading capacity to degrade lignocellulose with total degrading ratio of 16.14% at the fifth day (Fig. 1(a)). A rule for cotton stalk lignocellulose degradation was then obtained that the degrading ratios of lignin, cellulose, hemicellulose were increased with culture time, the lignin degrading ratio was the highest, hemicellulose degrading ratio the second, and cellulose degrading ratio was the lowest. In addition, it was also found that the three white rot fungi degraded cotton stalks selectively, showing that lignin degraded first, hemicellulose second and cellulose the last. This selectivity was similar to other lignocellulose materials degraded by white rot fungi in previous reports[21-23]. The cellulose degrading curves showed that, strain F11 exhibited the highest cellulose degrading ratio (Fig. 1 (c)), especially higher than hemicellulose degrading ratio during 15-25 days; while strain *P. chrysosporium* maintained the highest lignin degrading ratio and the lowest cellulose degrading ratio (Fig. 1 (a)). Considering the degradation of lignin and the following utilization of cellulose, strain *P. chrysosporium* could be chosen as mainly strain for degradation.The lignocellulose degrading curves of strain *P. chrysosporium*, exhibited that the lignin degrading ratio increased rapidly at 5-15 days after inoculation, then stable in the stage of 16-30 days, while cellulose degrading ratio increased rapidly after 15 days (Fig. 1 (a)), so the degradation period should be controlled within 15 days, and the degradation period thus determined as 15 days. The time determination was agreed with the result got by Pang *et al*. (2008)[24].The protein forming ratio in the biodegradation process (Fig. 1 (d)) showed that the protein forming ratio had achieved high to above 20% since fifth day, indicating that the hyphal protein synthesized in the early stage of cotton stalks biodegradation, which was in accordance with the theory of lignocellulose biodegradation founded by Kirk *et al*. (1978)[18]. Further, the correlation analysis was carried on to explain the relationship between protein forming ratio and hemicellulose and cellulose contents. The correlation coefficients (Table 1) revealed the protein forming ratio had high significant negative correlation at the 0.01 level with hemicellulose and

Table 1—Correlation between protein forming ratio and hemicellulose and cellulose contents

Strain	Strain F11	*P. chrysosporium*	*T. versicolor*
With cellulose content	-0.9967**	-0.9260**	-0.9940**
With hemicellulose content	-0.8976**	-0.9367**	-0.9978**

**denoting the correlation was high significant (P<0.01)

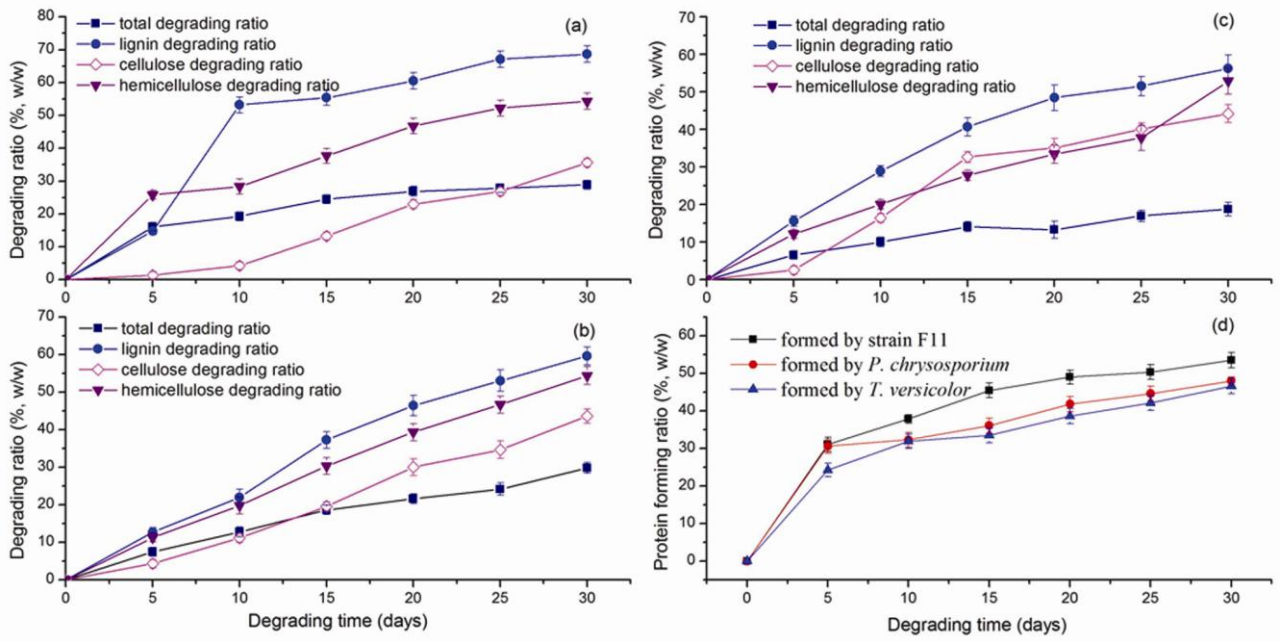

Fig. 1— Dynamic curves of cotton stalks degraded by white rot fungi and protein forming ratios in biodegradation process. (a) degraded by *P. chrysosporium*; (b) degraded by *T. versicolor*; (c) degraded by strain F11; (d) protein formation of three white rot fungi.

cellulose contents in biodegradation process. A conclusion could be achieved that degradation of cotton stalk by white rot fungi should be controlled in certain stage, to avoid cellulose degrading excessively.

Analysis of FTIR and SEM of cotton stalks degrading by white rot fungi

The structures of cotton stalk lignocellulose degraded at 15th day by white rot fungi were analyzed by FTIR spectroscopy. Compared to original sample, the intensity of absorption peaks of lignin, cellulose and hemicellulose decreased in degraded samples, especially the absorption bands of lignin and aromatic ring at about 1500 cm^{-1}~1650 cm^{-1} decreased significantly; the intensity of absorption peaks around 1421 cm^{-1}, 1117-1124 cm^{-1} and 911-915 cm^{-1} decreased to some extent, the guaiacyl absorption peaks at 1265 cm^{-1} had nearly disappeared, which demonstrated that all the three white rot fungi degraded lignin of cotton stalk strongly. Around 1049 cm^{-1}, representing C-O stretch in cellulose and hemicellulose, the peaks turned smoothly, and the intensity of absorption peaks around 898 cm^{-1} decreased little, which demonstrated the cellulose had been degraded partially. The SEM photographs revealed changes of microstructure of samples, the surface structure of original cotton stalk sample exhibited compact and smooth characteristics, the lignocellulose arranged tightly and regularly, while the cotton stalk degraded by white rot fungi, the surface structure was destroyed, the lignocellulose emerged from inner side, some structures even destroyed badly, with many cracks and holes appeared, and the hypha of white rot fungi also existed. The hypha of white rot fungi entered inside the lignocellulose of cotton stalk, and destroyed the structure, which exhibited good breaking and extending capability. In sample degraded by strain F11, massive hypha bound with the lignocellulose tightly, showing excessive growth of hypha; while in samples degraded by *P. chrysosporium* and *T. versicolor*, hypha hadn't grown excessively; the cracks and holes were clearly visible.

Conclusions

This study reveals the degradation rule of cotton stalk by three white rot fungi, and provides the technical reference for lignin degradation of cotton stalk. In general, three fungi showed strong lignin degrading capacity and lignocellulose degrading selectivity to degrade lignin first, and their hyphal protein forming ratio exhibited high significant negative correlation at the 0.01 level with hemicellulose and cellulose contents in biodegradation process, showing that the excessive growth of hypha should be avoided during degrading time. The FTIR and SEM analysis further proved three white rot fungi could degrade lignin related groups effectively, destroy the structure of lignocellulose and form many cracks and holes, and the hypha still can be seen bind tightly with lignocellulose if hypha grew excessively. So combined the results of lignocellulose degrading ratios and FTIR and SEM results, *P. chrysosporium* should be chosen as mainly strain for cotton stalks degradation within 15 days.

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