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Ammonia Fiber Expansion Combined with White Rot Fungi to Treat Lignocellulose for Cultivation of Mushrooms

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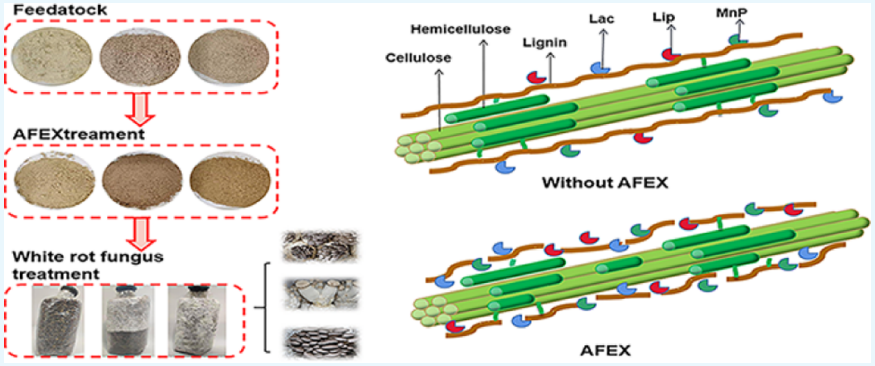

ABSTRACT: In order to improve the degradation efficiency of lignocellulose while increasing the yield of mushrooms, white rot fungi treatment (*Pleurotus ostreatus*, *Pleurotus eryngii*, and *Pleurotus geesteranus*) combined with ammonia fiber expansion was proposed as a method for treating lignocellulose (*Pennisetum sinese*, salix chips, and pine chips) for mushroom cultivation. Compared with treatment using either ammonia fiber expansion or white rot fungus, the combined treatment significantly improved lignocellulose degradation rate by 10−20% and reduced the time required significantly. Among them, *P. geesteranus* was the most effective bacterium for the combined treatment of lignocellulose. Ammonia fiber expansion-treated lignocellulose contributed to mycelial growth and increased the activity of three lignin hydrolase enzymes (laccase, manganese peroxidase, and lignin peroxidase) and mushroom yield. The mushroom yield was increased by 44.6%. The combined treatment method proposed in our study improves lignocellulose resource utilization and is therefore useful in the treatment of agricultural solid organic waste.

1. INTRODUCTION

China is a traditional agricultural country that produces more than 800 million tons of agricultural waste each year, accounting for a third of the global waste produced.[1] Most agricultural waste is returned to the fields or burned on the spot, whereas forestry waste (such as wood chips) is used as fuel, which results in pollution of the environment and wastage of resources. Agricultural and forestry wastes contain large amounts of lignocellulose, a valuable renewable resource that can replace fossil resources and is, thus, attracting increasing attention. Due to its complex structure, lignocellulose decomposition and utilization are difficult. Therefore, the effective use of lignocellulose has become an important problem.

Currently, physical, chemical, and biological methods are used for processing lignocellulose. Physical methods include dicing, crushing, ultrasound treatment, and radiation treatment. However, these methods are energy intensive and have relatively low rates of lignocellulose degradation.[2] Chemical methods (acidification, ammonification, organic solvent treatment, and others) can improve the degradation rate of lignocellulose. However, it causes reagent waste, equipment corrosion, environmental pollution, and other problems.[3] Biological treatment is also a promising method. Current biological treatment mainly involves lignocellulose degradation by white rot fungi. White rot fungi are one of the highly diverse groups of basidiomycetes, and the main genera include *Pleurotus ostreatus*, *Lentinula edodes*, *Hericium erinaceus*, *Pleurotus eryngii*, and *Festuca filiformis*. It effectively forms mycelial networks in the wood tissue to degrade the lignin by secreting the enzymes lignin peroxidase (Lip), manganese peroxidase (MnP), and laccase (Lac), free radicals, organic acids, and so forth. The advantages of biological treatment are low energy demand, mild environmental conditions, safety, less environmental pollution, and so forth. However, the long treatment time and the low treatment efficiency limit the further

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Table 1. Media Composition for Cultivation

ingredient	*P. ostreatus* medium		*P. eryngii* medium		*P. geesteranus* medium	
	control (g)	mixture (g)	control (g)	mixture (g)	control (g)	mixture (g)
lignocellulosic[a]	350		350		350	
lignocellulosic after AFEX[a]		350		350		350
cotton seed	50	50	50	50	50	50
sawdust						
wheat bran			50	50	95	95
corn flour	85	85	40	40		
KH_2PO_4			1	1		
gypsum powder			5	5		
lime powder	15	15	4	4	5	5
spawn[b]	10	10	10	10	10	10

[a]Lignocellulose included *P. sinese*, salix chips, and pine chips. [b]Spawn includes *P. ostreatus*, *P. eryngii*, and *P. geesteranus*.

development of this method.[4] Therefore, it is necessary to develop a low-cost, rapid, efficient, and environment friendly treatment method for lignocellulose degradation. Ammonia fiber expansion (AFEX) is a unique physical and chemical method that uses a high concentration of liquid ammonia to treat biomass under high temperature and high pressure,[5] with 70% biomass moisture content (by dry weight; dwb), temperature 130 °C, pressure 350 psi, residence time 15 min, and ammonia load on lignocellulose 1:1 (w/w). AFEX is a promising physicochemical pretreatment process. AFEX has four advantages: (1) AFEX-pretreated lignocellulose is readily hydrolyzable and fermentable with no detoxification or external nutrient supplementation necessary. (2) Nearly all of the ammonia can be recovered and reused while the remaining serves as a nitrogen source for microbes in downstream processes. (3) The lignocellulose after AFEX treatment is used as a culture medium, and there is no need to adjust the pH value. (4) Cellulose and hemicellulose are well preserved in the AFEX process, with little or no degradation. Compared with alkali treatment, AFEX pretreatment is a physical and chemical method. The combined chemical and physical effects of AFEX result in partial lignin solubilization and partial hemicellulose hydrolysis and increase the surface area of the substrate for microbial attachment. At the same time, ammonia can be recycled during AFEX pretreatment, reducing equipment corrosion and overall costs as well as the environmental impact. Compared with other physicochemical methods, such as steam blasting, CO_2 blasting, microwave and ultrasonic treatment, and so forth, it can save energy, reduce costs, and does not require large instruments and equipment, and pretreatment materials can be used directly for enzymatic digestion without washing, detoxification, and additional nutrients. The lignocellulose after AFEX treatment is easier to treat by white rot fungi, and the residual ammonia after AFEX treatment can provide a nitrogen source for the growth of white rot fungi. Unlike most acidic and alkaline pretreatments, the biomass pretreated by AFEX is easy to hydrolyze and ferment without detoxification or external nutritional supplements, and the ammonia used in the AFEX process can be recovered and reused.[6] However, AFEX treatment of lignocellulose releases phenols, lignin fragments, and many other unidentified compounds that inhibit enzymatic hydrolysis.[7] Due to the limitations of using a single treatment method, a series of joint treatment methods have been considered. Combined treatment of lignocellulose with AFEX and white rot fungus is thus a possible option. We hypothesized that AFEX may reduce treatment time by white rot fungi, and white rot fungi may degrade lignocellulose while utilizing the phenolic compounds produced by AFEX, thus promoting mycelial growth and reducing resource wastage and environmental pollution. After a period of incubation, white rot fungal hyphae emerge as various forms of fruiting bodies called mushrooms.[8] These are rich in high-quality protein, dietary fiber, vitamin B, trace elements, antioxidants, and anti-cancer active agents and are popular among consumers.[9]

In this study, we propose a method to effectively treat lignocellulose with reduced treatment time, involving the combination of AFEX and white rot fungi. To the author's knowledge, there are no studies on AFEX combined with white rot fungi to treat lignocellulose for mushroom cultivation. A two-step method was used to process lignocellulose, with the AFEX treatment being used to break the lignocellulose structure, followed by treatment with white rot fungi for lignocellulose degradation and utilization. We then examined the effects of using AFEX in combination with white rot fungi on the degradation of different lignocelluloses and on the enzyme activity of lignin hydrolases, mycelial cell growth rate, and mushroom yield. The structural changes in untreated and treated lignocellulose were also examined by Fourier transform infrared (FTIR) spectroscopy, X-ray diffraction (XRD), and ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

2. MATERIALS AND METHODS

2.1. Strains and Materials. *P. ostreatus*, *P. eryngii*, and *Pleurotus geesteranus* were obtained from the Shandong Academy of Agricultural Sciences and stored at 4 °C. The strains were inoculated into a glucose potato medium and cultured on a shaker at 28 °C for 7 days. *Pennisetum sinese* (PS) and pine chips (P) were obtained from Haisheng Grass Co. Ltd. (Shandong, China). Salix chips (SL) were collected in August 2020 from local farms in Xinjiang, People's Republic of China. The samples were cut into small pieces, crushed, passed through a 40-mesh sieve, dried, and stored at 4 °C in a refrigerator until required.

2.2. Ammonia Fiber Expansion Treatment. The AFEX treatment was performed as described in a previous report, with slight modification.[10] For the AFEX pretreatment, the sample was saturated with deionized water until the water load (g water/g dry biomass) reached 0.7. The prewetted biomass was then put into a stainless steel pressure vessel and sealed. The wetted samples were then placed in an autoclave. Liquid ammonia was injected into the high-pressure reactor at a 1:1

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ammonia-biomass ratio (dry mass of biomass), and the temperature was rapidly increased to 130 °C and maintained for 15 min. The treated samples were then dried and stored at 4 °C until needed.

2.3. Medium Preparation and White Rot Fungus Cultivation. Untreated lignocellulose and that treated using AFEX were combined with other components to form a solid medium for the cultivation of white rot fungi, as indicated in Table 1. Among them, untreated lignocellulose was the control group, and AFEX-treated lignocellulose was the experimental group. PS, SL, and P contain large amounts of lignocellulose and can act as carbon sources for white rot fungi,[23] while lime powder (s) and gypsum powder (s) can be used to adjust the pH of the solid medium and to improve its physicochemical properties.[11] The water content of the solid medium was 65% and compacted into a vinyl bag (17 cm × 22 cm). The compacted bags were sterilized in a vertical autoclave at 121 °C for 1 h. After cooling to room temperature, the strains were inoculated into each bag, and the bags were placed in an incubator maintained at 25 °C. The experimental cycle spanned 1 month, with weekly observations and measurements of mycelial growth, as well as observation of the changes in lignocellulose degradation and enzyme activity. After 1 month of cultivation, the bags were transferred to the mushroom house for mushroom production. The experiment was conducted in triplicate.

2.4. Determination of Lignin Hydrolase Activity. Laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (Lip) activities were determined using a plate reader (Infinite 200 PRO, Tecan, Switzerland) with the Lac, MnP, and Lip activity assay kits (Beijing Boxbio Science & Technology Co., Ltd. Beijing, China), respectively.

Briefly, samples for the determination of enzyme activity were prepared as follows: To 0.1 g of sample tissue, 1 mL of extraction solution was added to treat the sample. This was homogenized in an ice bath and centrifuged at 4 °C and 10 000 rpm for 10 min, and the supernatant was taken on ice to be measured.

Lac can decompose the substrate 2,2′-azino-bis-3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) to produce ABTS free radicals, with a characteristic absorption peak at 420 nm, which can then be characterized by the change in absorbance. The volume of enzyme required to oxidize 1 nmol of ABTS per minute per gram of sample was defined as one unit of Lac enzyme activity. MnP can oxidize guaiacol to tetra-*o*-methoxyl phenol in the presence of Mn^{2+}, and the product has a characteristic absorption peak at 465 nm, which can be characterized by the change in absorbance. The amount of enzyme required to oxidize 1 nmol of guaiacol per gram of sample per minute was defined as one unit of MnP enzyme activity. Lip oxidizes veratryl alcohol to produce veratraldehyde with a characteristic absorption peak at 310 nm, which can be characterized by changes in absorbance. The volume of enzyme required to oxidize 1 nmol of veratryl alcohol per gram of sample per minute was defined as one unit of Lip enzyme activity.

2.5. Determination of Mycelial Growth Rate and Fruiting Body Yield. The incubation phase for mycelial growth was 1 month, after which the sample was moved to the mushroom house for fruiting body culture with regular observation and recording of mycelial growth.

$$\text{Growth rate of mycelium (mm·d}^{-1}) = \frac{\text{length of mycelium (mm)}}{\text{mycelial growth period (d)}} \quad (1)$$

Yields were determined by measuring the total weight of freshly harvested mature mushrooms and the average mushroom yield.

2.6. Lignocellulose Sample Analysis. Sampling was performed on days 0, 7, 14, 21, and 28 of the cycle, and 0.1 g of the sample was used to measure enzyme activity (with operation at low temperature throughout the process). The remaining samples were dried at 60 °C and analyzed chemically. Cellulose, hemicellulose, and lignin contents were determined according to Scheme 1 using two-stage acid hydrolysis, described in the NREL study.[12] Lignin, cellulose, and hemicelluloses of the samples were determined by the protocol described in the NREL report (NREL/TP-510-42618) using two-stage acid hydrolysis. Cellulose and hemicellulose in the samples were hydrolyzed by acid to monosaccharides. Monomeric sugars were measured by an HPLC instrument (Agilent 1200 series, MN, USA) equipped with an Amnex HPX-87H column and a refractive index detector. The filtered sample was dried in a blast drying oven at 105 °C and weighed (m_1). After drying, it was transferred to a muffle furnace and burnt at 575 °C for 4 h. After cooling to room temperature, it was taken out and weighed (m_2). The data in the above process use the following formula to calculate the required value.

$$\text{Cellulose (\%)} = \frac{C_{glu} \times 87 \times 10^{-3} L \times 0.9}{m} \times 100\% \quad (2)$$

Here, C_{glu} is the glucose concentration, g/L, 0.9 is the conversion coefficient of monosaccharides and glycans, V is the total volume of the filtrate 0.087 L, and m is the mass of the acid-hydrolyzed sample, g

$$\text{Hemicellulose (\%)} = \frac{(C_{xyl} + C_{ara}) \times 87 \times 10^{-3} L \times 0.88}{m} \times 100\% \quad (3)$$

Here, C_{xyl} is the xylose concentration, g/L, C_{ara} is the arabinose concentration, g/L, 0.88 is the conversion coefficient of xylose and xylan, V is the total volume of the filtrate 0.087 L, and m is the mass of the acid-hydrolyzed sample, g

$$\text{Lignin (\%)} = \frac{m_1 - m_2}{m} \times 100\% \quad (4)$$

where m_1 is the mass of the sand core funnel containing the filter residue after drying, g, m_2 is the mass of the sand core funnel after burning, g, and m is the mass of the acid-hydrolyzed sample, g.

Dry mass loss was calculated as the percentage of total solids lost after the treatment to remove mycelial mass.[13] All analyses were conducted in triplicate.

2.7. Characterization of Samples. FTIR spectra of lignocellulose were obtained using a Cary 600 series FTIR spectrometer (IRAffinity-1S, Agilent technology). The sample was mixed with KBr powder (1:100 ratio) and crushed uniformly and then compressed into pellets by pressing for 5 min in a tablet press with a pressure of 2 ton. The spectrum was obtained in a wavelength range of 4000−400 cm^{-1} with 32 scans.

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Table 2. Lignocellulosic Component of the Medium[a]

sample	cellulose (%)	hemicellulose (%)	lignin (%)	Cel/Lig
PS	44.58 ± 0.44a	23.34 ± 0.47a	24.35 ± 0.99d	1.83 ± 0.08a
AFEXPS	42.84 ± 0.67b	21.33 ± 1.25b	24.31 ± 0.67d	1.76 ± 0.03a
SL	39.84 ± 1.34c	21.63 ± 0.47c	26.84 ± 1.66c	1.48 ± 0.04b
AFEXSL	39.81 ± 0.41c	20.60 ± 0.82d	26.87 ± 0.31c	1.48 ± 0.03b
P	42.82 ± 0.67b	20.62 ± 0.41d	29.10 ± 0.55a	1.47 ± 0.09b
AFEXP	42.79 ± 0.83b	20.57 ± 0.47d	28.65 ± 0.30b	1.49 ± 0.06b

[a]Values are shown as mean ± standard deviation. Lowercase letters indicate significant differences ($P < 0.05$).

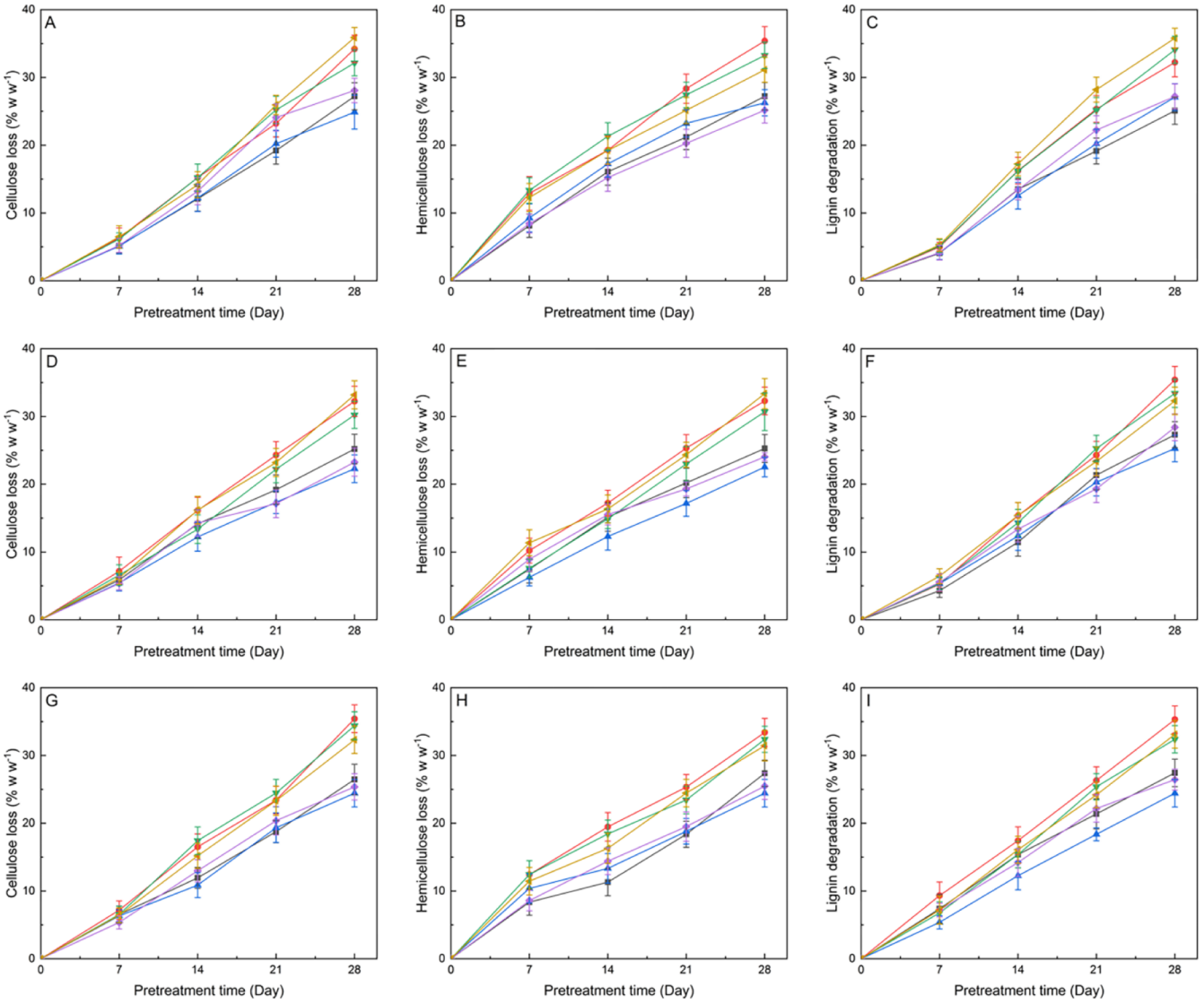

Figure 1. Loss rates of cellulose and hemicellulose and the lignin degradation rate during cultivation of three kinds of white rot fungi. (A) Cellulose loss rate of *P. ostreatus* during culture, (B) hemicellulose loss rate of *P. ostreatus* during culture, (C) lignin degradation rate of *P. ostreatus* during culture, (D) cellulose loss rate of *P. eryngii* during culture, (E) hemicellulose loss rate of *P. eryngii* during culture, (F) lignin degradation rate of *P. eryngii* during culture, (G) cellulose loss rate of *P. geesteranus* during culture, (H) hemicellulose loss rate of *P. geesteranus* during culture, and (I) lignin degradation rate of *P. geesteranus* during culture. (-■-) *P. sinese*; (-●-) AFEX *P. sinese*; (-▲-) salix chips; (-▼-) AFEX salix chips; (-◆-) pine chips; (-◀-) AFEX pine chips.

The crystallinity index of lignocellulose before and after treatment was determined using an X-ray diffractometer (D8-ADVANCE) with the following formula

$$\mathrm{CrI} = \frac{I_{002} - I_{\mathrm{am}}}{I_{002}} \times 100\% \quad (5)$$

where I_{002} is the intensity of the peak at 2θ about 22.4 and I_{am} is the intensity at 2θ about 18.4.[14,15]

NMR spectra of the lignocellulose samples were obtained using a BRUKER AVANCE III HD 400 MHz instrument (Bruker, Switzerland). The samples were loaded into a small rotor and tested under high-speed rotation conditions. The parameters were as follows: probe (4 mm MAS BB/1H

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H12138/0067), number of scans (1024), receiver gain (124.5), relaxation delay (3.0000), and pulse width (4.0000).

3. RESULTS AND DISCUSSION

3.1. Degradation of Lignocellulose. The composition of cellulose and hemicellulose of the medium was obtained by taking three replicate experiments, and the data are in Tables S1, S3, and S5. The composition of lignin was also taken by three replicate experiments, and the data are in Tables S2, S4, and S62. Table 2 and Figure 1 show the degradation of lignocellulosic components and lignocellulosic materials in the initial medium, respectively. After AFEX treatment of *P. sinese*, the loss rates of cellulose and hemicellulose and the lignin degradation rate were determined to be 3.8, 2, and 0.8%, respectively. In SL treated with AFEX, these values were determined to be 0.02, 6.4, and 2.2%, respectively. In P treated with AFEX, the loss rates of cellulose and hemicellulose and the lignin degradation rate were determined to be 1.8, 5.9, and 1.4%, respectively. Overall, AFEX alone had a limited effect on lignocellulose.

PS: *P. sinese*; SL: salix chips; P: pine chips; AFEXPS: *P. sinese* after AFEX treatment; AFEXSL: salix chips after AFEX treatment; AFEXP: pine chips after AFEX treatment.

The various lignocellulosic components of the medium were significantly different between various periods ($P < 0.05$). During mycelial growth, the average loss of hemicellulose was between 22.20 and 35.38%; the differences in these values depended on the differences in the substrates and strains. Cellulose consumption was also high (22.28−35.42%) because of the large amount of nutrients needed for mycelial growth. Thus, cellulose and hemicellulose may be utilized as nutritional sources for the growth of white rot fungi. The solid media preparations were classified into two different groups (lignocellulose without and with AFEX). The media with untreated lignocellulose showed limited lignocellulose degradation and slow mycelial growth. The media containing lignocellulose after AFEX treatment exhibited significant degradation of lignocellulose and rapid mycelial growth.

Of the three white rot fungi, *P. geesteranus* showed the best ability to degrade lignin, followed by *P. ostreatus*, while *P. eryngii* showed the weakest ability. Lignin degradation was facilitated after AFEX treatment, with a 10% higher degradation rate than that of lignin without AFEX. AFEX pretreated biomass has also been reported to be easily hydrolyzed and fermented.[16] Lignin degrades slowly in the early stages of mycelial growth. As the mycelium grew, the lignin content of the substrate was seen to become lower than the initial lignin content, indicating that the enzymes necessary for lignin degradation had started to secrete. In the initial stage of solid culture, the lignin degradation rate of the mycelia was slow, but it increased suddenly after 7 days, possibly because of the activity of the lignin hydrolase secreted by the fungus. After 40 days of incubation, *Ganoderma lobatum* could degrade 50.3% of the lignin.[17] Further, lignin is reported to be degraded more rapidly in the later stages of culture. The highest lignin degradation rate of *P. geesteranus* was observed in the medium with AFEX-treated *P. sinese* (AFEXPS); the degradation rate was 35.56 ± 0.75% by the end of cultivation. After 35 days of fermentation of corn stover inoculated with *L. edodes*, the lignin degradation rate was 29.88%.[2] In contrast, the lignin content degraded more slowly in PS, SL, and P media than in AFEX media. The use of white rot fungi combined with AFEX treatment of lignin achieved the same effect and reduced the degradation time by a quarter.

The ability of the three white rot fungi to degrade hemicellulose was the best in *P. ostreatus* followed by *P. geesteranus*, with the weakest being *P. eryngii*. Among these, compared with the loss rate of hemicellulose without AFEX, the loss rate of hemicellulose after AFEX treatment was 6−10% higher. With rapid mycelial growth, the hemicellulose content in PS, SL, and P media was also decreased significantly. Due to the structural characteristics and physicochemical properties of lignocellulose, hemicellulose is more susceptible to degradation by white rot fungi than lignin and cellulose.[18] Degradation of hemicellulose by white rot fungi has been reported to reach 24.4−34.9% after 30 days of treatment.[19] This is similar to the results of the present experiments with *P. eryngii* and *P. geesteranus*. However, the mycelium needs many nutrients in the early stage of growth. After AFEX treatment of lignocellulose, hemicellulose is more easily absorbed and degraded by the mycelium because of its structural characteristics. *P. ostreatus* presented the maximum hemicellulose loss rate in the AFEX-treated substrate. Further, the hemicellulose loss rate of the AFEXPS substrate reached 35.38 ± 1.15% at 28 days. Previously, 35.0 ± 1.3% w w^{-1} hemicellulose removal has been reported, wherein sweet sorghum bagasse was treated with *C. versicolor* in synergy with three supplements.[13] In this study, *P. eryngii* showed the worst degradation of hemicellulose on SL medium, with a loss of 21.43% at 28 days of treatment. This may be due to the specific strain used as among the three fungi, *P. eryngii* had the slowest mycelial growth rate.

The ability of the three white rot fungi to degrade cellulose was the best in *P. geesteranus*, followed by *P. ostreatus*, with the weakest being *P. eryngii*. Among these, the cellulose loss rate after AFEX treatment was 10% higher than that without AFEX. The cellulose content of PS, SL, and P media changed slowly in the early and middle stages of mycelial growth but decreased drastically in the late stage of mycelial growth and in the fruiting body stage. As more nutrients are needed during mycelial growth, it is possible that hemicellulose is consumed during the mycelial growth phase and more cellulose is exposed to be degraded and consumed by the mycelium. Similarly, *G. lobatum CCCT16.03* and *Gloeophyllum trabeum CCCT16.04* were reported to demonstrate limited cellulose processing during the earlier stages of wheat straw degradation, indicating that these fungi consume more lignin and hemicellulose before 14 days.[17] *P. sinese* showed a cellulose loss rate of 36.65% when fermented with *P. geesteranus* for 28 days. After 14 days, cellulose begins to be degraded rapidly, possibly because of a large amount of hemicellulose being degraded, which destroys the stable lignocellulose structure, enabling a large amount of cellulose to be accessible to mycelium and resulting in cellulose degradation. However, in this experiment, the same white rot fungi showed different degradation rates for each lignocellulose component in different media. The lignocellulosic degradation rate was also found to be different with *P. ostreatus* treatment in different solid media.[20]

3.2. Cultivation of White Rot Fungi and Mushroom Yield. Significant differences were found in the yield, mycelial growth rate, and mycelial bag filling time between different media ($P < 0.05$) (Table 3). *P. geesteranus* showed the fastest mycelial growth rate, followed by *P. ostreatus* and, finally, *P. eryngii*. Mycelial growth was faster in AFEX-treated lignocellu-

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Table 3. Mycelial Growth Rate, Bag Filling Time, and Mushroom Yield of Three White Rot Fungi[a]

fungus	media	mycelial growth rate/(mm·d^{-1})	bag full time/d	yield (raw) (g/bag)
PO	PS	4.875c	30.5 ± 0.90c	98 ± 6.76
	AFEXPS	5.616b	24.1 ± 0.76d	149 ± 7.89
	SL	5.420b	29.7 ± 0.45c	98 ± 5.79
	AFEXSL	6.370a	24.8 ± 0.68d	176 ± 4.82
	P	5.670b	31.0 ± 0.50c	119.5 ± 8.98
	AFEXP	5.955ab	25.9 ± 0.70d	142.5 ± 6.42
PE	PS	3.170d	41.1 ± 0.48a	138 ± 13.22
	AFEXPS	3.589d	35.3 ± 0.34b	249.25 ± 31.60
	SL	3.652d	41.4 ± 0.86a	128 ± 5.10
	AFEXSL	4.063cd	35.3 ± 0.71b	166 ± 7.76
	P	3.741d	42.0 ± 0.48a	132.5 ± 9.21
	AFEXP	5.027bc	36.8 ± 0.39b	180.5 ± 4.15
PG	PS	5.125b	30.5 ± 0.76c	100.5 ± 4.30
	AFEXPS	5.714b	25.5 ± 0.58d	163.5 ± 10.64
	SL	5.607b	29.4 ± 0.79c	105.5 ± 4.97
	AFEXSL	6.554a	24.2 ± 0.33d	139.5 ± 23.44
	P	6.089ab	30.7 ± 0.48c	107 ± 7.07
	AFEXP	6.714a	24.5 ± 0.78d	172 ± 24.40

[a]Values are shown as mean ± standard deviation. Lowercase letters indicate significant differences ($P < 0.05$). Values followed by the same letters in each column are not significantly different ($P < 0.05$). PS: *P. sinese*; SL: salix chips; P: pine chips; AFEXPS: *P. sinese* after AFEX; AFEXSL: salix chips after AFEX; AFEXP: pine chips after AFEX; PO: *P. ostreatus*; PE: *P. eryngii*; PG: *P. geesteranus*.

lose than in lignocellulose without AFEX. Further, all three white rot fungi filled the bag faster in the AFEX-treated medium (in 24.1 ± 0.76 to 36.8 ± 0.39 days) compared with the medium without AFEX. The growth of *P. eryngii* was slower, filling the bag about a week later than the other two white rot fungi, possibly because of differences between strain types. Therefore, using lignocellulose after AFEX treatment reduced the time taken to fill a vinyl bag with mycelial growth to 6 days compared with that by using lignocellulose without AFEX. This is because lignocellulose after AFEX treatment is more easily consumed by the mycelium. Moreover, most of the ammonia in the AFEX process can be recovered, and residual ammonia enriches the nutrient content of the AFEX-pretreated biomass.[21] Nitrogen fertilizers are known to greatly affect the growth and development of fungi, and nitrogen is also a major component of proteins. Shortening the mycelial growth time also helps eliminate the risk of infection with other fungi during the cultivation stage.

Table 3 shows the mushroom yield for each group. Table 4 shows the comparison of mushroom yield in this study with

Table 4. Mushroom Yield Compared with Other Literature

	this study	this study	22	23
incubation time	46 days	39 days	48 days	49days
mushroom yields	138 g	249 g	90 g	164 g
cultivation method	No AFEX	AFEX	No AFEX	No AFEX

other literature. The harvest time of each mushroom may be different depending on the differences between strains and the composition of the medium. Of the strains in our study, the growth of *P. ostreatus* and *P. geesteranus* was the most obvious. The total fresh mushroom yield varied from 98 ± 5.79 to 249.25 ± 31.60 g. In the AFEXPS substrate, *P. eryngii* showed a high yield of 249.25 ± 31.60 g. The yield of mushrooms without AFEX substrate was only 138 ± 13.22 g. Analysis of the growth of white rot fungi on six different media showed that the AFEX-treated media had higher mushroom yields. Thus, the use of AFEX-treated lignocellulose as a substrate for white rot fungal cultures not only accelerates mycelial growth but also increases mushroom production.

3.3. Enzyme Activity. White rot fungi are the only class of microorganisms known to be effective in converting lignin to CO_2 and H_2O in pure lineage cultures. They can secrete extracellular oxidases which form free radicals in the catalytic process and thus degrade the total lignin structure. Among these, Lac, Lip, and MnP are the three main enzymes that degrade lignin. Previous studies have shown that *P. ostreatus*, *P. eryngii*, and *P. geesteranus* can secrete these three lignin hydrolases.

Lignin hydrolase content and white rot fungus culture time had a significant influence on the activities of Lac ($P < 0.05$), MnP ($P < 0.05$), and Lip ($P < 0.05$). The lignin hydrolase enzyme activities of the three different white rot fungi during the culture are presented in Figure 2. The Lac activity of *P. eryngii* was significantly higher than those of the other two strains, reaching a maximum of 2195.67 U/g. Further, of the three lignin degrading enzymes, Lac activity was much higher than those of Lip and MnP. Lac gradually developed in the mycelial stage and showed high activity until the end of the culture, helped the mycelium to settle on the wood, and degraded lignin as well as the phenolic compounds in wood interestingly. The Lac activity in *P. ostreatus* was relatively reduced on the 14th day and increased again to reach the maximum, possibly because of insufficient nutrition or due to species differences. *Tricholoma giganteum AGHP* has been reported to produce large amounts of Lac in the early stages of wheat straw treatment, which decreased in the later stages due to nutrient exhaustion.[24] As shown in Figure 2, of the three types of lignocellulose sources used, the white rot fungus on *P. sinese* showed the highest Lac secretion. This may be because the nutrients in *P. sinese* are richer or easier to obtain. However, the MnP of *P. ostreatus* and *P. eryngii* showed a tendency to level out by 14 days and rapidly increase thereafter, maintaining a relatively high enzyme activity until the end of the culture. *P. ostreatus* was the best MnP-producing strain, reaching its maximum activity (258.538 U/g) after 21 days of incubation, followed by a decrease. The decreased levels of nutrients in the medium may be responsible for the decrease in enzyme activity.

P. geesteranus was the best Lip-producing strain (maximum activity of 91.18 U/g at 28 days in SL media). Lip activity increased with increasing incubation time, reaching a peak at 28 days, except in *P. ostreatus*. *P. ostreatus* produced less Lip, and its activity remained almost unchanged during the fermentation process. *P. eryngii* and *P. ostreatus* reached peak Lip activity by day 28 (89.63 and 8.86 U/g, respectively). In lignin degradation, Lac and MnP appeared to be the major operational Lignin degrading enzymes due to their higher secretion and stability. Although Lip activity was low, its role in the enzymatic hydrolysis system cannot be denied.

3.4. Lignocellulosic Structure before and after Degradation. The results showed that the highest lignocellulose degradation rate was achieved with *P. geesteranus* treatment. Therefore, samples of *P. geesteranus*-treated lignocellulose were selected as the representative samples.

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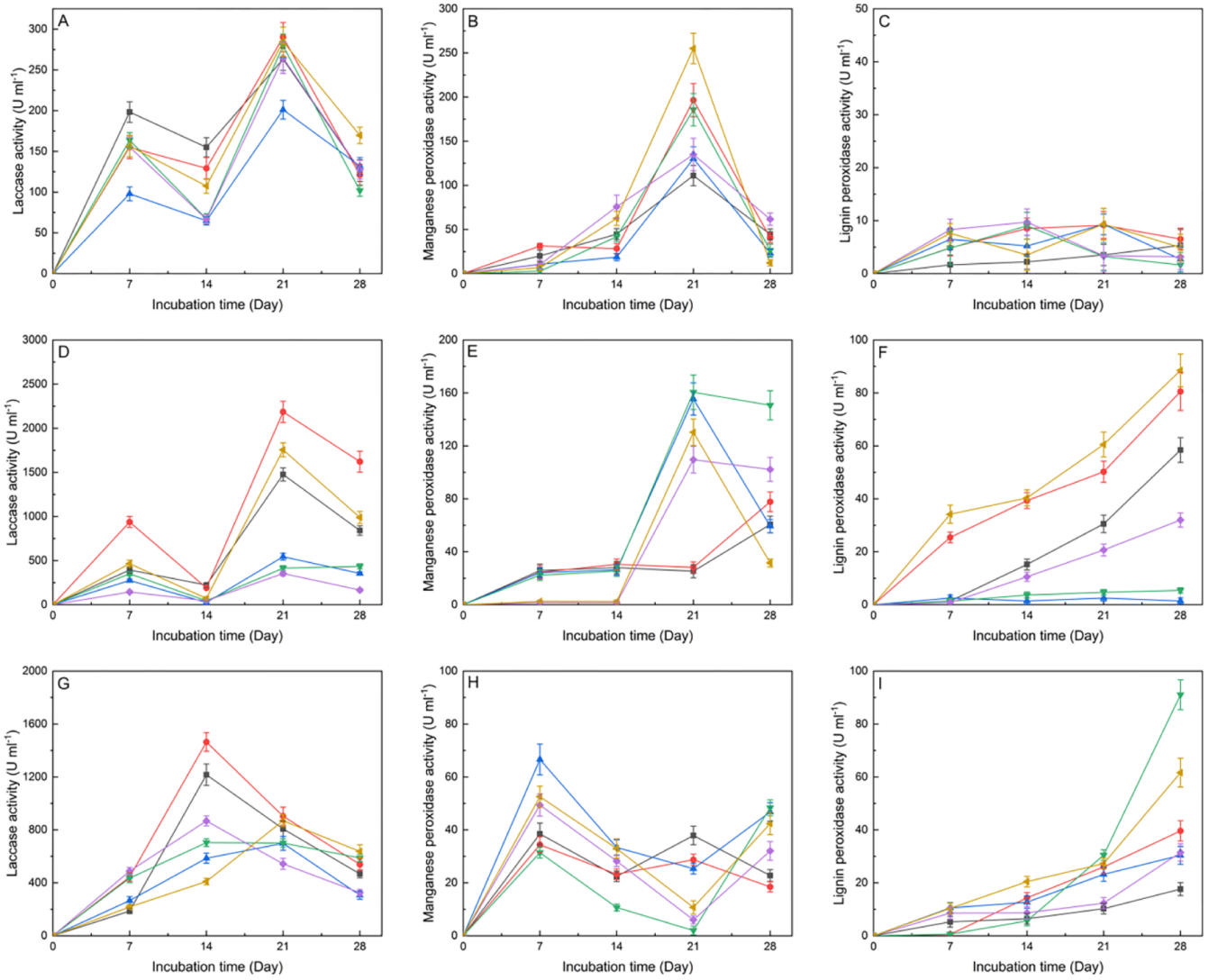

Figure 2. Lignin hydrolase enzyme activity in white rot fungal culture stages. (A) *P. ostreatus* degrades lignocellulose to produce Lac, (B) *P. ostreatus* degrades lignocellulose to produce MnP, (C) *P. ostreatus* degrades lignocellulose to produce Lip, (D) *P. eryngii* degrades lignocellulose to produce Lac, (E) *P. eryngii* degrades lignocellulose to produce MnP, (F) *P. eryngii* degrades lignocellulose to produce Lip, (G) *P. geesteranus* degrades lignocellulose to produce Lac, (H) *P. geesteranus* degrades lignocellulose to produce MnP, and (I) *P. geesteranus* degrades lignocellulose to produce Lip. (-■-) *P. sinese*; (-●-) AFEX *P. sinese*; (-▲-) Salix chips; (-▼-) AFEX Salix chips; (-◆-) Pine chips; (-◀-) AFEX Pine chips.

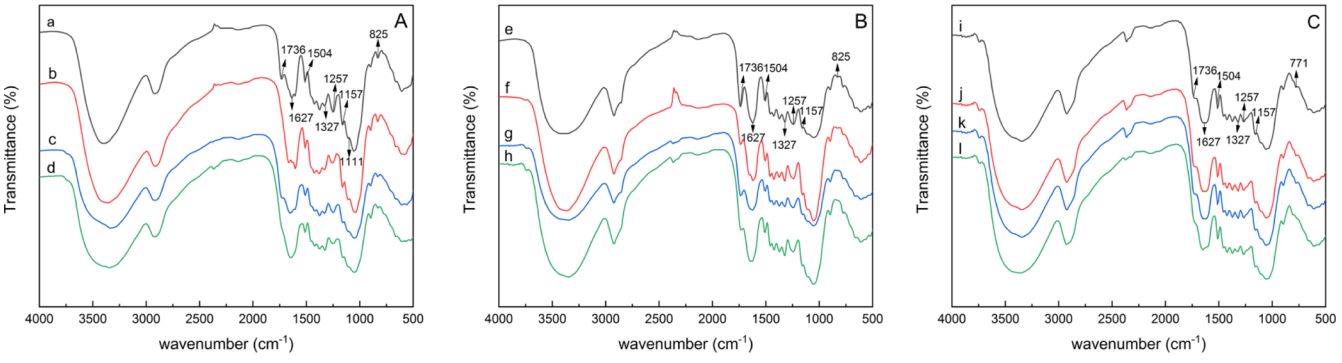

Figure 3. FTIR spectra of lignocellulose samples. A: *P. ostreatus*; B: *P. eryngii*; C: *P. geesteranus*. a: *P. sinese*; b: *P. sinese* after AFEX treatment; c: *P. sinese* after *P. geesteranus* treatment; d: *P. sinese* after AFEX combined with *P. geesteranus* treatment; e: salix chips; f: salix chips after AFEX treatment; g: salix chips after *P. geesteranus* treatment; h: salix chips after AFEX combined with *P. geesteranus* treatment; i: pine chips; j: pine chips after AFEX treatment; k: pine chips after *P. geesteranus* treatment; l: pine chips after AFEX combined with *P. geesteranus* treatment.

The effect of *P. geesteranus* treatment on *P. sinese* was examined using ^{13}C CP/MAS solid-state NMR spectroscopy.

FTIR spectroscopy was used to measure the intensity change of lignocellulose before and after degradation and

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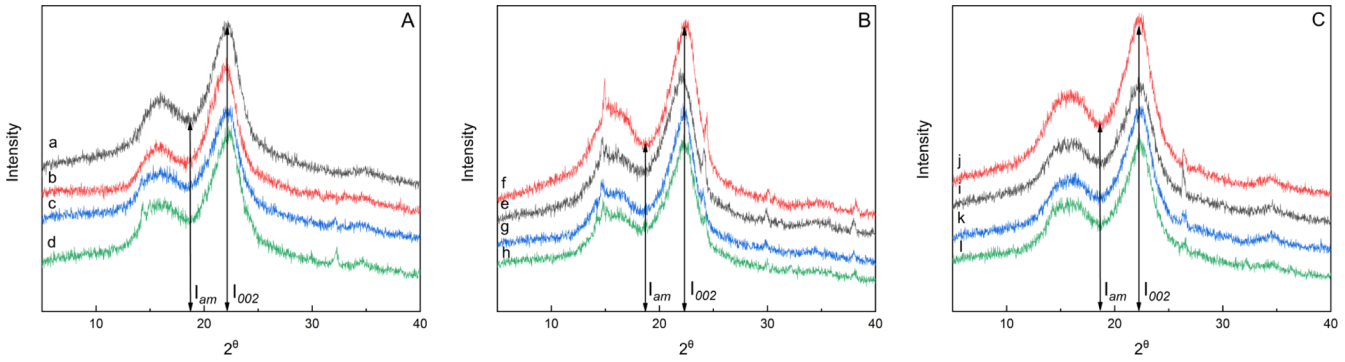

Figure 4. XRD pattern of lignocellulosic samples. A: *P. ostreatus*; B: *P. eryngii*; C: *P. geesteranus*. a: *P. sinese*; b: *P. sinese* after AFEX treatment; c: *P. sinese* after *P. geesteranus* treatment; d: *P. sinese* after AFEX combined with *P. geesteranus* treatment; e: salix chips; f: salix chips after AFEX treatment; g: salix chips after *P. geesteranus* treatment; h: salix chips after AFEX combined with *P. geesteranus* treatment; i: pine chips; j: pine chips after AFEX treatment; k: pine chips after *P. geesteranus* treatment; l: pine chips after AFEX combined with *P. geesteranus* treatment.

indicated whether the lignocellulose was degraded.[25] Figure 3 shows the FTIR spectra of untreated and treated lignocellulosic samples. The intensities of the peaks at 1736 cm^{-1}, which are indicative of unconjugated C═O (hemicellulose), disappeared in the AFEX-treated samples, indicating that AFEX degrades some of the hemicellulose in the sample. In samples treated with AFEX in combination with *P. geesteranus*, the peaks at 1627 cm^{-1}, 1257, and 1111 cm^{-1}, which indicate lignin, were shifted compared with those in the original sample, indicating that lignin was decomposed by the combination treatment. The intensity of the peak at 1157 cm^{-1} indicated partial cellulose and hemicellulose and had disappeared in the AFEX combined with *P. geesteranus*-treated samples, which suggests the destruction of cellulose and hemicellulose by the combined treatment. The bonds between 1300 cm^{-1} and 1000 cm^{-1} represent the presence of different amounts of C−C, C═O, and C−O groups in the lignocellulosic samples.[26] Figure 3 shows the peak descriptions of the wavelengths corresponding to the guaiacyl unit (G) and the syringyl unit (S) (1327 and 1257 cm^{-1}, respectively). The relative strength results showed that compared with those of the untreated AFEX lignocellulose, the peaks of cellulose, hemicellulose, and lignin treated with *P. geesteranus* in combination with AFEX were either shifted or had disappeared, indicating that combining AFEX with white rot fungus is a more effective lignocellulose treatment (compared with AFEX treatment or white rot fungus treatment alone).

The XRD patterns obtained from different lignocelluloses are shown in Figure 4, with two peaks at 22.4 and 18.4° representing the crystalline and noncrystalline regions, respectively. As seen in Table 5, the crystallinity analysis of the treated lignocellulose showed that the relative crystallinity of AFEX-treated lignocelluloses was increased and that it decreased with the combined treatment of AFEX with *P. geesteranus*. The increase in crystallinity occurs due to the removal of some hemicellulose and lignin during AFEX treatment, causing the amorphous region to decrease.[27] After the combined treatment with *P. geesteranus* and AFEX, the crystallinity index began to decrease, possibly because the cellulose degraded by *P. geesteranus* was absorbed and utilized as a nutrient source, resulting in a decrease in the strength of the crystalline zone. The crystallinity will be reduced to a reduction in intermolecular interactions in the cellulose, and correspondingly, the cellulose content will decrease.

Table 5. XRD Analysis of the Lignocellulose Crystallinity Index before and after Different Treatments[a]

	2θ scale		
sample	$I_{22.4}$	$I_{18.4}$	CrI (%)
PS	22400	11700	47.77
AFEXPS	20166	10066	50.08
PSPG	19233	9450	50.87
AFEXPSPG	20283	11333	44.13
SL	25383	12983	48.85
AFEXSL	30600	14633	52.18
SLPG	23983	11183	53.37
AFEXSPG	21816	11600	46.83
P	22983	12216	46.85
AFEXP	28333	14600	4847
PPG	22950	11686	49.08
AFEXPPG	20966	12050	42.53

[a]PS: *P. sinese*; SL: salix; P: pine; AFEXPS: *P. sinese* after AFEX; AFEXSL: salix chips after AFEX; AFEXP: pine chips after AFEX; PSPG: *P. sinese* after *P. geesteranus* treatment; SLPG: salix chips after *P. geesteranus* treatment; PPG: pine chips after *P. geesteranus* treatment; AFEXPSPG: *P. sinese* after AFEX combined with *P. geesteranus* treatment; AFEXSLPG: salix chips after AFEX combined with *P. geesteranus* treatment; AFEXPPG: pine chips after AFEX combined with *P. geesteranus* treatment.

Analysis of the chemical structure assignment in lignocellulosic samples by using NMR spectroscopy was performed based on the data reported in the literature.[28,29] As seen in Figure 5, all untreated and treated samples have four significant signals at 63.6, 73.4, 82.9, and 104.6 pp and two insignificant signals at 21.1 and 172.4 ppm. The signals at 63.6 ppm and 104.6 ppm represent partial chemical structures in hemicellulose and cellulose, whereas the signals at 73.4 and 82.9 ppm represent partial chemical structures in hemicellulose, cellulose, and lignin.[30] The signal at 21.1 ppm represents the CH_3 groups in the acetyl group of hemicellulose.[31] Thus, AFEX treatment resulted in the deconstruction of the CH_3 group. The signal at 172.4 ppm indicates the ester group in carbohydrates, which showed varying degrees of reduction in all treated samples, implying that the ester groups of carbohydrates in the samples were all susceptible to attack by NH_3 and *P. geesteranus*. The signal at 55.7 ppm represents the aryl methoxy carbon of lignin, and its intensity was slightly reduced by AFEX combined with *P. geesteranus* treatment. The signals at 115.8, 134.0, 146.7, and 152.8 ppm mainly represent

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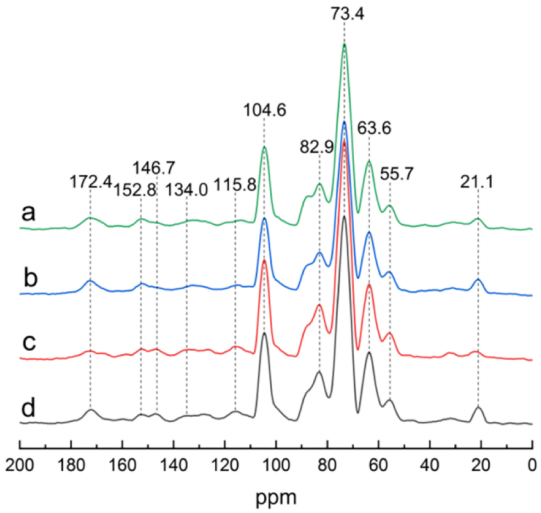

Figure 5. ^{13}C CP/MAS solid-state NMR spectra of lignocellulosic samples. (a) *P. sinese* after AFEX combined with *P. geesteranus*; (b) *P. sinese* after *P. geesteranus* treatment; (c) *P. sinese* after AFEX treatment; (d) *P. sinese*.

the aromatic hydrocarbons and phenolics in lignin.[31] After combined treatment with AFEX and *P. geesteranus*, the signals at 115.8, 134.0, and 146.7 ppm vanished, whereas those at 152.8 ppm, representing aromatic carbons, become weaker, indicative of lignin oxidation. After combined treatment of *P. sinese* with AFEX and *P. geesteranus*, most of the lignin was removed.

4. CONCLUSIONS

The combined use of white rot fungus with AFEX treatment of lignocellulose resulted in a reduced treatment time and an improved lignocellulose degradation efficiency. AFEX combined with *P. geesteranus* has the best effect when degrading *P. sinese*. The degradation rate of cellulose is 35.56%, hemicellulose is 33.40%, and lignin is 36.65%. The enzyme activities of the three lignin degrading enzymes also increased significantly. The Lac activity of *P. eryngii* was the highest, reaching 2195.67 U/g at 21 days of culture, and the MnP activity of *P. ostreatus* was the highest, reaching 258.538 U/g at 21 days of culture, and the Lip activity of *P. geesteranus* was the highest, reaching 91.18 U/g at 28 days of culture. Moreover, when white rot fungi were cultured with AFEX-treated lignocellulose as the medium, the total number of days required for mycelial growth was reduced by a quarter, and the yield was increased by 44.6%. Overall, this research provides a new method for lignocellulose treatment and utilization.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04388.

Details of the cellulose and hemicellulose components of lignocellulose and details of the lignin component of lignocellulose (PDF)

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Notes

The authors declare no competing financial interest.

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