Utilization of Nematode-infected Pinewood for Mushroom Cultivation and Production of Reducing Sugar

ChenYan Huang,[a,1] Xuan Wu,[a,b,1] XiaoYi Dai,[a] Rebeca Carballar-Lejarazú,[c] YongXin Tao,[d] JianRong Wen,[e] Ren Chen,[f] FeiPing Zhang,[a,*] and SongQing Wu [a,*]

To improve the nematode-infected pinewood application value, an effective strategy was developed based on the cultivation of *Flammulina velutipes* to process the pinewood that was infected by the nematode *Bursaphelenchus xylophilus*. Different treatments were compared to determine the optimal method to obtain the highest yields of reducing sugars. The results showed that the cultivation of *F. velutipes* using the nematode-infected pine sawdust overcame the disadvantage of cultivation by using healthy pine sawdust, and whether fungal infection that happened in the period of pine wilt disease or during the cultivation of *F. velutipes* both can contribute on the degradation of the polysaccharide content. A high yield of reducing sugars can be obtained using 2% dilute sulfuric acid at 121 °C for 1 h to treat recyclable nematode-infected pine sawdust after the cultivation of *F. velutipes.* Additionally, the results showed that fungal infection that occurred in the period of the pine wilt disease and during the cultivation of *F. velutipes* in addition to acid hydrolysis effectively converted hemicellulose to reducing sugars.

Keywords: Nematode-infected pinewood; Flammulina velutipes cultivation; Acid hydrolysis; Reducing sugars

*Contact information: a: Key Laboratory of Integrated Pest Management in Ecological Forest, Fujian Province University, College of Forestry, Fujian Agricultural and Forestry University, Fuzhou, 350002, China; b: College of Environment and Ecology, Xiamen University, Xiamen, 361000, China; c: Department of Microbiology and Molecular Genetics, University of California Irvine, Irvine, CA 92697 USA; d: China National Engineering Research Center of JUNCAO Technology, Fujian Agriculture and Forestry University, Fuzhou, 350002, China; e: Longyan Customs, Longyan, 364000, China; f: Putian Entry-exit Inspection and Quarantine Bureau, Putian, 351100, China; 1: These authors contributed equally to this work; *Corresponding author: fpzhang1@163.com, dabinyang@126.com*

INTRODUCTION

The pine wilt disease is caused by the *Bursaphelenchus xylophilus* nematode. This devastating disease results in a large area of pine trees (*Pinus massoniana*) dying. There are more than one million trees of *P. massoniana* that have been infected since 1982 (Shi *et al.* 2008). Pine wilt disease has a strong transmission and destruction index, which leads to millions in economic losses. Currently, the main direct and effective strategy to control the pine wilt disease is to discover the infected pine trees in real-time that are felled immediately to prevent the dispersion of the disease to the surrounding healthy trees. However, the lack of proper treatment of the felled trees can cause a secondary transmission of the disease (Jiang *et al.* 2001). Therefore, it is important to find an effective method to deal with a large number of felled infected pine trees and prevent a secondary spreading.

Currently, the main remediation methods for the nematode-infected wood are fumigation, local incineration, heat, and microwave treatment (Song *et al.* 1994; Jiang *et al.* 2006; Gan *et al.* 2010). Although these remediation methods are effective and can reduce some losses, they also cause serious pollution effects on the environment and consume a lot of energy. In contrast, other methods are environmentally friendly, such as wood rot bacteria decomposition (Chen *et al.* 2008) and using the nematode-infected pinewood as a substrate to cultivate *Poria cocos* (Wu 2013). This last method suggests that the nematode-infected pinewood can be used for edible fungus cultivation. Moreover, sawdust from the nematode-infected pinewood is abundant in cellulose and hemicellulose and it can be used as a substrate to grow edible filamentous fungus (Lu *et al.* 2016). *Flammulina velutipes* is one of the main edible fungi with high nutritional and medicinal value (Yu *et al.* 2004). However, healthy pine sawdust cannot be directly used to cultivate the *F. velutipes* due to the effect the pine wood terpenoids (Chen *et al*. 2002). Alternatively, cultivation of *F. velutipes* by using nematode-infected pinewood is feasible because the terpenoids content of the nematode-infected pinewood is lower than in the healthy pinewood (Kuroda 1989). If the nematode-infected pine sawdust is used for the cultivation of *F. velutipes*, it can effectively prevent the spread of *B. xylophilus* and greatly improve the utilization rate of nematode-infected pinewood and help to reduce the cost of *F. velutipes* cultivation.

While *F. velutipes* cultivation can effectively improve the utilization rate of the nematode-infected pine sawdust, in contrast, the spent mushroom substrate will still have a certain impact on the environment. If the efficient recycling mechanism of nematode-infected pine sawdust can be established, it will be beneficial to overcome the disadvantages of traditional methods and gain new profits. It has been reported that reducing sugar can be obtained using the biomass conversion method to treat spent mushroom substrate (Wu *et al.* 2013). The method can degrade lignin or hemicellulose on the surface of the material to realize the conversion of cellulose into reducing sugar, thereby increasing the yield of reducing sugar. Therefore, finding the suitable conditions for the conversion of nematode-infected pine sawdust into reducing sugar will contribute in developing a sustainable method to treat nematode-infected pinewood based on the re-utilization of the infected materials.

In this study the authors used broad-leaved wood sawdust, healthy pine sawdust, and nematode-infected pine sawdust to cultivate *F. velutipes*, and the feasibility of growing *F. velutipes* was evaluated by comparing the yield of *F. velutipes.* The reducing sugar yield was analyzed by using different material sources (healthy pine sawdust and nematode-infected pine sawdust, healthy pine substrate, and nematode-infected pine substrate) under different treatments (acid, alkali, and water treatment). The healthy pine substrate and nematode-infected pine substrate refer to a type of spent mushroom substrate. The healthy pine and nematode-infected pine substrates are made from using the healthy pine sawdust and nematode-infected pine sawdust to cultivate *F. velutipes*, respectively. The treatment efficiency was determined using scanning electron microscopy (SEM), X-ray diffraction (XRD), Fourier transforming spectroscopy (FTIR), and X-ray photoelectron spectroscopy (XPS) to analyze the morphological, physical, and chemical structures of the sample surface under the same treatment conditions. The data provide insight into a more economical and environmentally friendly method and a high-efficiency recycling process for the use of the nematode-infected pinewood.

EXPERIMENTAL

Materials

Cultivation of F. velutipes

Infected pinewood and healthy pinewood were both collected from Minhou County, Fuzhou City (Fujian Province, China). All nematode-infected pinewood samples used in this research had been previously ground. The substrate ingredients for growing *F. velutipes* contain 58% cottonseed shell, 20% wood sawdust, 20% bran, 1% gypsum, and 1% sucrose. The dry material weight was 270 g for each sample (broad-leaved wood sawdust, healthy pine sawdust, and nematode-infected pine sawdust); 90 g of each material was put in bottles in triplicate. The bottles containing the materials were sterilized (121 °C, 4 h, 103.4 KPa) and then inoculated with *F. velutipes* FL19 and cultivated at 25 °C. After the mycelium grew and covered the material, it was kept in culture at 10 °C and at 80 to 90% humidity until the harvesting time (stipe length of 15 $\pm$ 2.5 cm and a cap diameter of 0.9 ± 0.2 cm). The fresh weight of *F. velutipes* was recorded immediately after harvest. The average mushroom yield of each bottle was recorded and analyzed. Finally, the spent mushroom substrate was recycled for later experiments.

Methods

Different hydrolysis treatment and composition detection

After air drying (60 °C, 72 h), each substrate (healthy pine sawdust, nematode-infected pine sawdust, healthy pine substrate, and nematode-infected pine substrate) was milled with a grinder to pass through a 40-mesh screen (425 um). Different treatment conditions were set using different chemicals, concentrations, and temperature to determine the optimal conditions that resulted in higher yields. The reaction conditions included acid hydrolysis (concentration 0.5 to 4% and 50 to 121 ℃, 1 h), alkaline hydrolysis (concentration 1%, 121 ℃, 1 h), and water treatment (121 ℃, 1 h) (Wu *et al.* 2013). Reactions were performed by adding 5 g of the substrate to each chemical at 1:10 ratio (solid : liquid) in a 250-mL conical flask. After the above reaction was finished, the mixed solution was allowed to cool, followed by the addition of the corresponding concentration of H_2SO_4 or NaOH to neutralize the mixture solution (pH = 7) and stop the reaction. The neutralized samples were washed four times with distilled water followed by centrifugation (8000 r/min, 4 °C, 5 min/time). The solid residues were collected and put into an oven at 45 °C to dry and seal the substrate surface for subsequent experiments. The supernatant was collected for chemical composition analysis and to determine the yield of reducing sugar. The moisture and ash contents of the samples were determined according to the People's Republic of China national standard GB/T 2677.2 (2011) and GB/T 742 (2008), respectively. The contents of cellulose, hemicellulose, acid-soluble lignin (ASL), and acid-insoluble lignin (AIL) were determined according to the National Renewable Energy Laboratory (NREL) analytical procedure (Sluiter *et al.* 2008). Each sample was performed in triplicate. The content of reducing sugar was determined by high-performance liquid chromatography (HPLC) (Agilent 1260; Agilent Technologies, Shanghai, China) with an Agilent Zorbax SB-C 18 column (4.6 mm × 250 mm, 5 μm). Chromatographic conditions were 16% acetonitrile to 84% 0.05 mol/L ammonium acetate solution and in a wavelength of 245 nm, with a column temperature of 30 °C and a flow rate of 1.5 mL/min.

SEM analysis

The morphology surface of the solid residues was analyzed by using SEM, field emission SU8010 (Hitachi High-Technologies, Tokyo, Japan). The dried solid powder was put on the clean silicon wafer, and then the metal (gold) spray was used to make the sample detectable (S. S. Aerosols Pvt. Ltd., Haryana, India). Finally, the image was scanned and saved using the SEM software (Microscope Control v5.2.2 build 2898, Hitachi High-Technologies, Tokyo, Japan).

XRD analysis

Structure surface changes of the samples were determined by X-ray diffraction. The detection instrument was a Rigaku Ultima IVX XRD (Tokyo, Japan). The sample was dried and added to a clean vessel and then placed on the instrument. The Cu Kα sample parameter was obtained at 40 kV and 40 mA from a Bragg angle (2θ) of 5° to 60° with a step size of 0.02° and scanning rate of 6°/min. The crystallinity index (CrI) of initial and treated samples was calculated according to the protocol already described. The CrI was determined using the following Eq. 1,

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \times 100 \quad (1)$$

where *CrI* is the cellulose crystallinity index (%), I_{002} is the adsorption peak intensity of peak 002 at 2θ of 22.3, and I_{am} is the minimum intensity height between peaks 002 and 101 at 2θ of 18°.

FTIR Analysis

Changes in the chemical composition of the sample surface were analyzed by FTIR. The dried samples were mixed with potassium bromide (mass ratio 1:100). The samples were ground into a powder and then pressurized, followed by analysis with a Nicolet iS10 (Thermo Fisher Scientific, San Diego, CA, USA) with a scanning spectrum range of 400 to 4000 cm^{-1}.

XPS Analysis

Changes in the content of the main components (polysaccharide, lignin, and surface extracts) were analyzed by XPS. The instrument used was an Escalab 250Xi (Thermo Fisher Scientific, San Diego, CA, USA). The analytical area of the sample was 500 μm × 500 μm, and the analytical depth was less than 10 nm. The vacuum degree of the analysis was 1×10^{-10} mbar. To weaken the effect of binding energy on the XPS test, the C1s peak was set to 284.8 eV to correct the charge. The raw data of the XPS test were processed using the XPSPEAK 4.1 and OriginPro 8 software (Microsoft Corporation, Redmond, WA, USA).

Statistical Analysis

After the sample treatment and analysis, the samples of each different treatment condition were performed in triplicate, and the data were expressed as mean ± standard deviation. The analysis of variance (ANOVA) analysis PRISIS 6 (GraphPad software, La Jolla, CA, USA) and SPSS statistical software (Version 19.0, IBM Corp., Armonk, NY, USA) were used to analyze the significance of the difference, and the result, $P < 0.05$, showed that there were significant differences corresponding to different experimental conditions.

RESULTS AND DISCUSSION

Comparative Analysis of *F. velutipes* Yield

To determine the feasibility of cultivating *F. velutipes* on nematode-infected pine sawdust, the yield of *F. velutipes* was compared and analyzed on three different kinds of wood sawdust (broad-leaf wood sawdust, healthy pine sawdust, and nematode-infected pine sawdust). Cultivation of *F. velutipes* on broad-leaf wood sawdust takes 27 days, and 31 days was taken when cultivated on healthy or nematode-infected pine sawdust (Table 1). However, the mycelial growth was sparse when *F. velutipes* was cultivated on healthy pine sawdust. Interestingly, *F. velutipes* yields were different among the substrates, the average yield per bottle was 39.7 g on broad-leaved wood sawdust, 38.5 g on nematode-infected pine sawdust, and 28.6 g on healthy pine sawdust (Table 1). The observed differences may be because the growth of hyphae can be affected by the number of terpenoids during its cultivation on healthy pine sawdust. In contrast, the yield of *F. velutipes* cultivated in nematode-infected pine sawdust was not significantly different from that cultivated on broad-leaved wood, because the terpenoids can be volatilized more easily after the pinewood was infected by nematode (Kuroda 1989). These results suggest that nematode-infected pine sawdust is suitable for the cultivation of *F. velutipes* and can help to improve the economic value of nematode-infected pinewood.

Table 1. Mycelium Growth and Mushroom Emergence Period on Various Substrate Materials

Cultivation Materials	Number of Cultivation Days	Growth Situation	Number of Harvest Days	Average Yield Per Bottle (g)
Broad-leaf Wood Sawdust	27	White, thick	21	39.7 ± 0.67 [a]
Healthy Pine Sawdust	31	White, sparse	17	28.6 ± 0.72 [b]
Nematode-infected Pine Sawdust	31	White, thick	17	38.5 ± 0.63 [a]

[a, b, c] Values statistically significant different ($P < 0.05$)

Chemical Composition Analysis

The chemical components of the different substrates under different treatment conditions were determined and compared with some of the most common agricultural wastes. The percentage of polysaccharide (including cellulose and hemicellulose) content in healthy pine sawdust was 54.2%; however, it was lower in nematode-infected pine sawdust (51.5%), healthy pine substrate (48.4%), and nematode-infected pine substrate (50.2%). In addition, the percentage of lignin content of nematode-infected pine sawdust (29.8%) was also lower than that of healthy pine sawdust (34.1%). It can be inferred from the results that the differences were due to the hydrolysis of some cellulose and hemicellulose that occurred during the period of infection by the pinewood nematode and the cultivation of *F. velutipes*, and the hydrolysis may have been caused by the fungal infection. According to Lara *et al.* (2010) the pine bark composition can be easily digested by fungus. Moreover, some other studies reported that blue-stain fungi often can be found in the nematode-infected pinewood during the period of the pine wilt disease (Mamiya 1983; Song 1993). In contrast, *F. velutipes* is a fungus, and its cultivation on wood to some degree means that the wood is infected by fungi (Zhao *et al.* 2017).

Table 2. Chemical Composition of Pine Wood Before and After Treatment, and Comparison with Some Agricultural Wastes

Item	Cellulose (%)	Hemi-cellulose (%)	Acid Soluble Lignin (%)	Acid Insoluble Lignin (%)	Water Content (%)	Ash Content (%)
Healthy Pine Sawdust [a]	46.1 ± 2.77	8.1 ± 0.72	2.0 ± 0.07	32.1 ± 1.96	6.7 ± 0.58	0.6 ± 0.03
2% H_2SO_4-treated Healthy Pine Sawdust [a]	40.2 ± 3.14	3.9 ± 0.22	1.2 ± 0.02	30.5 ± 0.02	7.0 ± 0.40	ND [d]
1% NaOH-treated Healthy Pine Sawdust [a]	30.8 ± 1.69	7.3 ± 0.57	1.3 ± 0.01	26.2 ± 0.26	4.5 ± 0.24	ND
Water-treated Healthy Pine Sawdust [a]	33.9 ± 2.34	7.6 ± 0.45	1.4 ± 0.03	30.8 ± 0.30	4.8 ± 0.18	ND
Nematode-infected Pine Sawdust [a]	44.1 ± 3.98	7.4 ± 0.61	1.3 ± 0.39	28.5 ± 2.82	6.2 ± 0.04	0.7 ± 0.01
2% H_2SO_4-treated Nematode-infected Pine Sawdust [a]	36.4 ± 1.35	3.0 ± 0.21	1.3 ± 0.12	22.9 ± 1.85	7.3 ± 0.45	ND
1% NaOH-treated Nematode-infected Pine Sawdust [a]	41.5 ± 1.48	1.7 ± 0.16	1.4 ± 0.04	22.9 ± 2.14	8.0 ± 0.44	ND
Water-treated Nematode-infected Pine Sawdust [a]	33.8 ± 3.37	6.7 ± 0.67	1.8 ± 0.08	25.6 ± 0.43	8.2 ± 0.16	ND
Healthy Pine Substrate [a]	30.7 ± 1.89	17.7 ± 1.45	1.6 ± 0.08	35.3 ± 1.63	1.8 ± 0.12	4.0 ± 0.17
2% H_2SO_4-treated Healthy Pine Substrate [a]	26.1 ± 0.92	8.1 ± 0.78	1.1 ± 0.10	27.3 ± 0.75	3.5 ± 0.30	ND
1% NaOH-treated Healthy Pine Substrate [a]	21.9 ± 2.01	15.4 ± 1.33	0.9 ± 0.02	13.5 ± 1.30	5.9 ± 0.56	ND
Water-treated Healthy Pine Substrate [a]	25.1 ± 1.78	17.0 ± 1.52	1.5 ± 0.04	28.6 ± 2.42	4.4 ± 0.37	ND
Nematode-infected Pine Substrate [a]	33.7 ± 2.56	16.5 ± 1.36	1.6 ± 0.06	35.6 ± 0.77	1.9 ± 0.12	3.6 ± 0.20
2% H_2SO_4-treated Nematode-infected Pine Substrate [a]	31.5 ± 2.97	6.5 ± 0.61	0.9 ± 0.02	28.5 ± 0.33	6.0 ± 0.59	ND
1% NaOH-treated Nematode-infected Pine Substrate [a]	17.9 ± 1.23	14.3 ± 1.12	0.9 ± 0.05	15.8 ±0.33	5.3 ± 0.11	ND
Water-treated Nematode-infected Pine Substrate [a]	25.3 ± 2.04	16.1 ± 1.28	1.4 ± 0.07	31.5 ± 0.29	6.4 ± 0.61	ND
Raw Distiller's Grains [b]	28.5 ± 0.39	13.1 ± 0.17	4.2 ± 0.20	-	5.1 ± 0.12	14.5 ± 0.44
Corn Stover [c]	37.7	-	-	16.4	-	-

[a] Reaction at 121 °C for 1 h, [b] Zheng *et al.* 2015, [c] Zhao *et al.* 2017, [d] ND: No detection

The fungal hydrolysis that happened in the process of *F. velutipes* cultivation could lead to a lower percentage of polysaccharide content in the healthy pine substrate (48.4%) when compared with the healthy pine sawdust (54.2%). The lower lignin content percentage is helpful to reduce the blocking effect of the dense structure on the surface of the material and improve the conversion rate of the polysaccharide content (Zheng *et al.* 2018). However, the percentage of lignin content in the healthy pine substrate (37.2%) was higher than that of healthy pine sawdust (34.1%) due to the partial digestion of the polysaccharide content during the cultivation of *F. velutipes*, which indirectly increased the proportion of lignin content in the material. In addition, the percentage of polysaccharide content in the four kinds of materials (54.2%, 51.4%, 48.4%, and 50.2%) was higher than that of the common agricultural wastes such as corn straw (37.7%) (Chen *et al.* 2016) and distiller's grains (41.6%) (Zheng *et al.* 2015). Therefore, all four materials might have great potential to produce reducing sugar.

Comparison of Reducing Sugar Production

To find the optimal treatment conditions for reducing sugar conversion, this study used HPLC to analyze the yield of reducing sugar under different treatment conditions. As shown in Table 3, the alkali-treated reducing sugar content was not detectable. The reason may be that sodium hydroxide reacts chemically with the monosaccharides hydrolyzed by cellulose, resulting in a decrease in the yield of reducing sugars under high-temperature conditions (Zheng *et al.* 2015). The yield of reducing sugar after hot water treatment was also unsatisfactory. Only healthy pine sawdust (4.8 g/kg) and nematode-infected pine sawdust (13.5 g/kg) produced a small amount of reducing sugar. This may be due to the degradation of a small portion of the surface hemicellulose or cellulose (Pérez *et al.* 2008). Therefore, although water treatment has the advantages of low cost and no corrosion, it is not efficient enough for transforming woody cellulose.

Among the treatments, acid treatment showed a higher yield of reducing sugar due to the degradation of most of the hemicellulose; these results are similar to those in *Salvinia molesta* (Syaichurrozi *et al.* 2018). The yield of reducing sugar in all the substrates increased with the reaction temperature and acid concentration, especially at 121 °C with 4% H_2SO_4 for 1 h. The highest reducing sugar yield was obtained for healthy pine sawdust, nematode-infected pine sawdust, and healthy and nematode-infected pine substrate with 111.9 g/kg, 236.8 g/kg, 101.3 g/kg, and 137.2 g/kg, respectively. However, considering the cost of the reagents, instruments, and wastewater treatment costs, the most economical and effective treatment was 121 °C with 2% H_2SO_4 for 1 h. In this condition, it was found that the yield of reducing sugar from healthy (97.7g/kg) and nematode-infected pine substrate (126.3 g/kg) were not higher than that of nematode-infected pine sawdust (202.2 g/kg), probably because the polysaccharide content of nematode-infected pine substrate was digested by the fungi during the period of the infection by pinewood nematode. In addition, the polysaccharide content of healthy and nematode-infected pine substrate was digested by *F. velutipes*, resulting in a decrease in the yield of reducing sugar. However, the yield of reducing sugar from the nematode-infected pine substrate (126.3 g/kg) was still higher than that obtained from the healthy pine sawdust (98.6 g/kg) after acid hydrolysis. The results show that the nematode-infected pine substrate still has a great utilization value due to its capacity to be recycled to produce reducing sugar. Moreover, the obtained reducing sugar can be used as a cost-effective carbon source for cultivating microorganisms being of great benefit not only for the biopesticide manufacture but also for reducing agricultural waste (Wu *et al.* 2013; Zheng *et al.* 2015, 2018).

Table 3. Yield of Reducing Sugar Under Different Treatment

Hydrolysis Method	Parameter			Yield of Reducing Sugar (g/kg)			
	Temp. (°C)	Concen-tration (%)	Pres-sure (MPa)	After Treat. of Healthy Pine Sawdust	After Treat. of Healthy Pine Substrate	After Treat. of Nematode-infected Pine Sawdust	After Treat. of Nematode-infected Pine Substrate
Acid Hydrolysis	121	0.5	0.5	36.5 ± 3.17^{e}	48.8 ± 3.79^{c}	159.4 ± 4.56^{d}	42.3 ± 4.15^{d}
	121	1	0.5	78.5 ± 3.24^{c}	71.2 ± 4.58^{b}	184.6 ± 10.20^{c}	113.0 ± 10.63^{b}
	121	2	0.5	98.6 ± 6.67^{b}	97.7 ± 9.71^{a}	202.2 ± 15.34^{b}	126.3 ± 5.85^{a}
	121	4	0.5	111.9 ± 9.12^{a}	101.3 ± 9.36^{a}	236.8 ± 9.96^{a}	137.2 ± 10.51^{a}
	50	4	0.1	ND*	ND	16.8 ± 0.64^{e}	ND
	100	4	0.1	52.3 ± 4.45^{d}	81.2 ± 6.53^{b}	65.1 ± 4.70^{e}	97.1 ± 9.52^{c}
	100	2	0.1	-	25.3 ± 1.37^{d}	-	20.4 ± 1.71^{e}
	100	1	0.1	-	9.0 ± 0.75^{e}	-	9.0 ± 0.32^{e}
Alkaline Hydrolysis	121	1	0.5	ND	ND	ND	ND
Water	121	-	0.5	4.8 ± 0.22^{f}	ND	13.5 ± 0.92^{f}	ND

The data with different letter values are considered to have significant differences ($P < 0.05$); ND: No detection

SEM Analysis

It is well known that the nematode-infected pinewood can be infected also by fungi during the pine wilt disease (Mamiya 1983; Song 1993), and while the pinewood sawdust was used to cultivate *F. velutipes*, it will also be infected by fungi. To determine the effect of fungal infection during the period of pine wilt disease and its impact on mushroom cultivation and on the cell wall structure of the substrate, the morphology of the raw and the treated materials were analyzed by SEM. The surface structure of the raw healthy pine sawdust was smooth and compact; however, a rough and disordered surface appeared in the raw nematode-infected pine sawdust, as wells as the healthy and the nematode-infected pine substrate (Fig. 1). It can be observed that the fungus infection during the period of pine wilt disease and mushroom cultivation all can promote the hydrolysis of polysaccharide and lignin and destroy the original dense structure of the healthy pinewood under the optimal acid treatment (121 °C, 2% H_2SO_4, and 1 h). The surface of the four materials was disintegrated and contained a large number of holes and deep cracks as a result of a large amount of hydrolyzed hemicellulose. Chen *et al.* (2016) determined that the acid treatment of maize straw produced similar results. In addition, the structure of nematode-infected pine sawdust, healthy pine substrate, and nematode-infected pine substrate were more damaged when compared with healthy pine sawdust, probably due to the fact that the surface structure of healthy and nematode-infected pine substrate is pre-digested as a result of the infection, which makes them more sensitive to acid hydrolysis (Alexandropoulou *et al.* 2016).

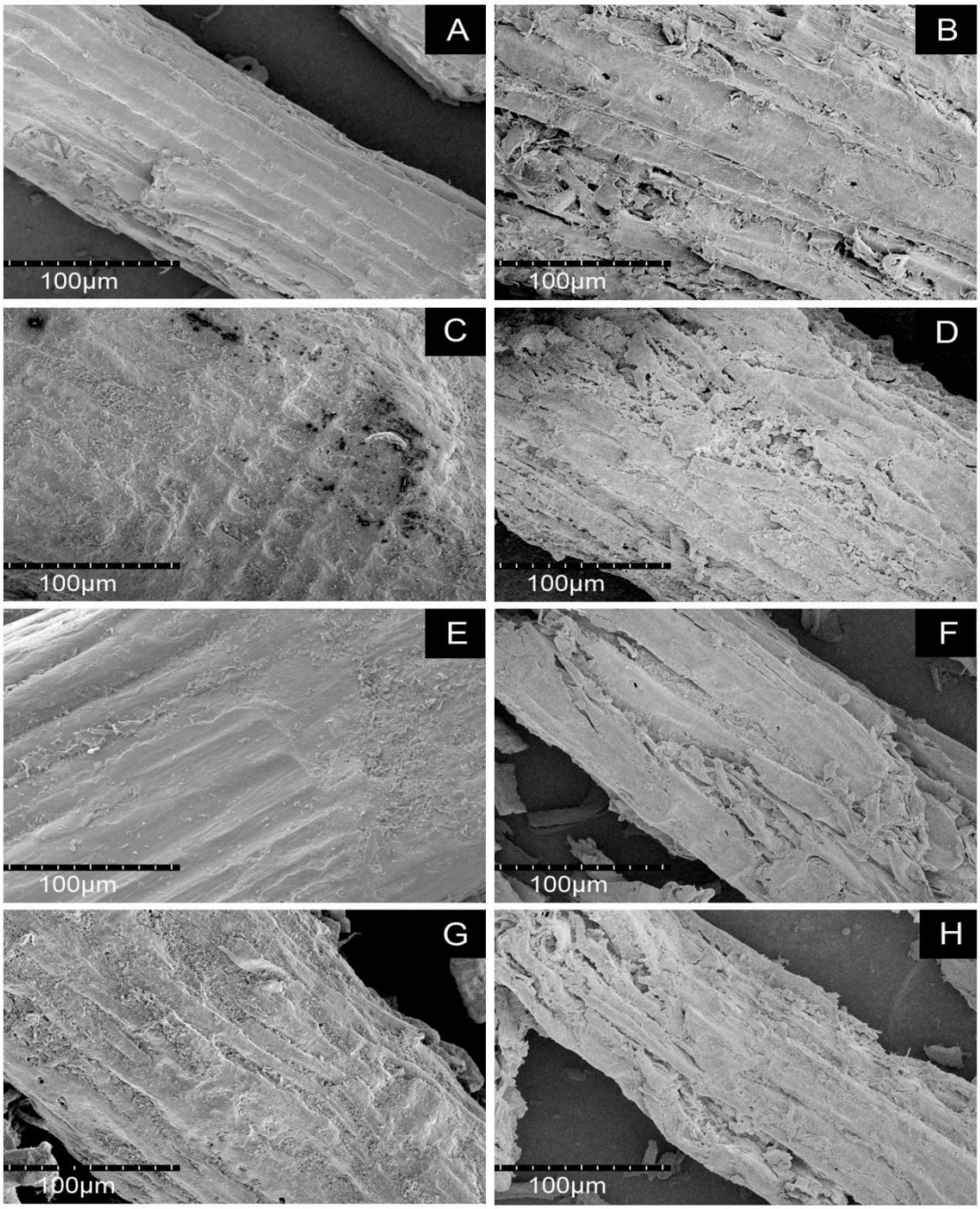

Fig. 1. SEM images of raw and treated materials: A: raw healthy pine sawdust, B: healthy pine sawdust with acid treatment, C: raw healthy pine substrate, D: healthy pine substrate with acid treatment, E: raw nematode-infected pine sawdust, F: nematode-infected pine with acid treatment, G: raw nematode-infected pine, H: nematode-infected pine substrate with acid treatment; acid treatment: 121 °C, 2% H_2SO_4, and 1 h

Studies have shown that highly loose and chaotic surfaces contribute to the degradation of more quantities of cellulose and hemicellulose (Auxenfans *et al.* 2017). However, although the destruction degree of the surface structure of the nematode-infected pine substrate was higher than that observed from the nematode-infected pine sawdust, the

yield of reducing sugar was the opposite due to a pre-degradation of the cellulose and hemicellulose during the cultivation of edible fungi which is consistent with previous studies (Chen *et al.* 2002).

XRD Analysis

Crystallinity is an important factor to demonstrate the compact structure of the material surface. In this study, the crystallinity of the four substrates was analyzed by XRD before and after the treatment. The strengths of the crystalline region (002) and the non-crystallized region (am) of the sample after fungal infection and acid hydrolysis were different (Fig. 2). The percentage of crystallinity in the healthy pine sawdust, healthy pine substrate, nematode-infected pine sawdust, and nematode-infected pine substrate was 51.5%, 49.6%, 48.1%, and 50.1%, respectively (Table 4). The crystallinity from the other three materials decreased slightly when compared with the crystallinity from the raw healthy pine sawdust. A possible explanation is that some cellulose degradation may occur in some crystalline regions during fungal infection (Sánchez 2009). After the acid hydrolysis (121 ℃, 2% H_2SO_4, and 1 h), the percentage of crystallinity in the healthy pine sawdust, healthy pine substrate, nematode-infected pine sawdust, and nematode-infected pine substrate increased to 62.1%, 60.5%, 56.8%, and 58.1%, respectively (Table 4). These results show that acid hydrolysis can effectively remove the xylan from the materials and then destroy the surface structure of the hemicellulose and lignin promoting more cellulose to be exposed to the acidic solution and finally converted into reducing sugar (Li *et al.* 2016). However, the increase of crystallinity from the nematode-infected pine sawdust and the nematode-infected pine substrate was smaller after acid hydrolysis, which may result from the fact that some of the biodegradable non-crystalline cellulose is digested during fungal infection.

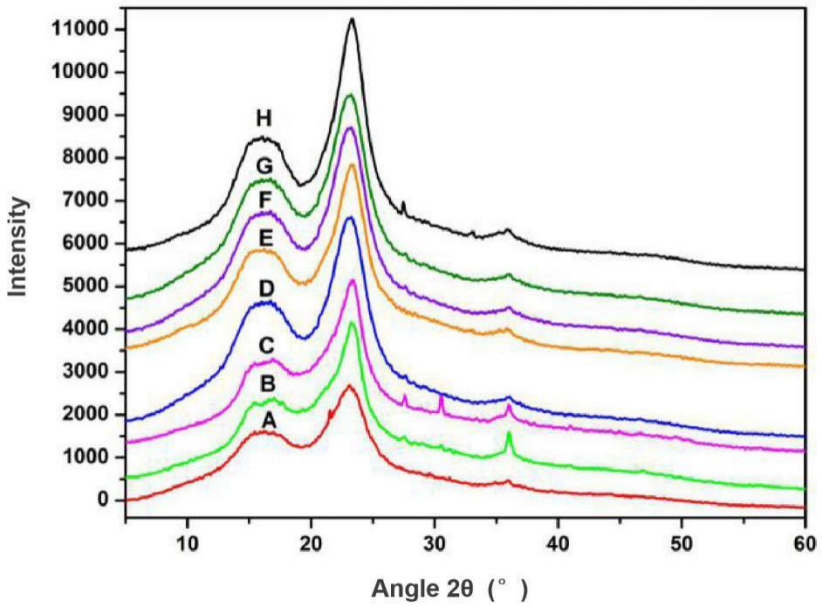

Fig. 2. XRD curves of raw and treated materials: A: raw nematode-infected pine sawdust, B: raw healthy pine substrate, C: raw nematode-infected pine substrate, D: raw healthy pine sawdust, E: nematode-infected pine with acid treatment, F: nematode-infected pine substrate with acid treatment, G: healthy pine substrate with acid treatment, H: healthy pine sawdust with acid treatment; acid treatment: 121 °C, 2% H_2SO_4, and 1 h

Table 4. Crystallinity of Raw and Acid Treated Substrates

Sample	Treatment	CrI (%)
Healthy Pine Sawdust	No treatment	51.5 ± 0.77
	121 °C, 2% H_2SO_4, 1 h	62.1 ± 0.95
Healthy Pine Substrate	No treatment	49.6 ± 1.86
	121 °C, 2% H_2SO_4, 1 h	60.5 ± 1.36
Nematode-infected Pine Sawdust	No treatment	48.1 ± 0.76
	121 °C, 2% H_2SO_4, 1 h	56.8 ± 0.64
Nematode-infected Pine Substrate	No treatment	50.1 ± 0.87
	121 °C, 2% H_2SO_4, 1 h	58.1 ± 0.55

FTIR Analysis

To determine the changes in the chemical structure of the substrates, FTIR was used to analyze the characteristic functional groups of the component surface (cellulose, lignin, and hemicellulose) before and after the sample treatment. The absorption spectra are shown in Fig. 3. The change in the absorption peak strength after the treatment is related to the change of the corresponding lignocellulose composition.

The absorption at 1458 cm^{-1} is related to the deformation of CH_3 in the lignin, and the stretching vibrations of C = C in the lignin are indicated by an absorptive peak 1509 cm^{-1}. The absorption peak intensity from the healthy pine substrate, nematode-infected pine substrate, and nematode-infected pine sawdust at 1458 cm^{-1} and 1509 cm^{-1} was lower than in the healthy pine sawdust before the acid hydrolysis (Fig. 3), indicating that the fungal infection caused by pine wilt disease or the edible fungus could hydrolyze lignin content. Whereas acid hydrolysis can further degrade lignin content (Jordan *et al.* 2008), the absorption peak intensity of all samples decreased in the treated materials when compared with the untreated materials.

The absorptions at 1245 cm^{-1} and 1737 cm^{-1} are related to the alkyl ester of the acetyl group and non-conjugate stretching of C = O in the hemicellulose, respectively. It can be seen that the peaks significantly decreased after fungal infection or acid hydrolysis compared with the peaks of raw healthy pine sawdust and raw healthy pine substrate (Fig. 3), suggesting that both fungal infection and acid treatment can effectively hydrolyze hemicellulose. It is worth noting that the absorption peak intensity of nematode-infected pine substrate and healthy pine substrate after acid hydrolysis was lower than that of nematode-infected pine sawdust after acid hydrolysis (Fig. 3), which indicates that hemicellulose in the sample of fungal infection combined with acid hydrolysis can be used more effectively.

The broad peak at 3000 cm^{-1} to 3500 cm^{-1} and the absorbance at 898 cm^{-1} represents the O-H stretching and aliphatic or aromatic C-H bonds, respectively. Compared with the raw healthy pine sawdust, the peak intensity of the nematode-infected pine sawdust, the healthy, and the nematode-infected pine substrate was decreased (Fig. 3), suggesting that the cellulose content of the digestible part of the fungal infection was consistent with the results of the previous component analysis. The peak strength of the four materials after acid hydrolysis decreased slightly at 3000 cm^{-1} to 3500 cm^{-1} and at 898 cm^{-1} (Fig. 3), suggesting that some cellulose contents were hydrolyzed during acid treatment, but the ability of acid to degrade cellulose was limited.

The absorptions at 2850 cm^{-1} and 2920 cm^{-1} are related to the stretching of the CH_2 group on the surface of fatty extracts. The peak intensity of nematode-infected and healthy

pine substrate was higher than corresponding raw materials (Fig. 3), which may have been because fungal infection decreased the proportion of surface extracts indirectly by decreasing the lignocellulose components. However, the peak strength of all samples after acid hydrolysis did not change significantly (Fig. 3), indicating that acid hydrolysis cannot remove fat extracts.

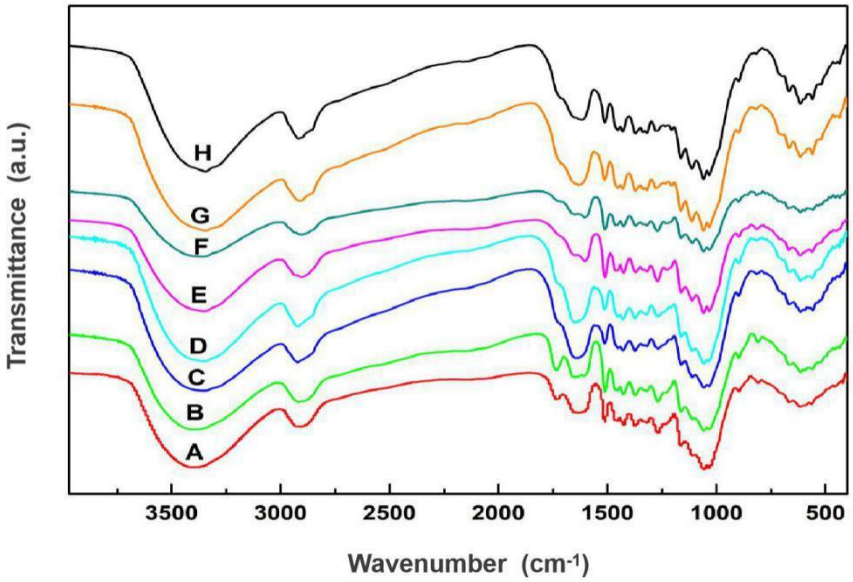

Fig. 3. FTIR curves of raw and treated substrates: A: raw nematode-infected pine sawdust, B: raw healthy pine sawdust, C: raw nematode-infected pine substrate, D: raw healthy pine substrate, E: nematode-infected pine with acid treatment, F: healthy pine substrate with acid treatment, G: nematode-infected pine substrate with acid treatment, H: healthy pine sawdust with acid treatment; acid treatment: 121 °C, 2% H_2SO_4, and 1 h

XPS Analysis

The XPS analysis can be used to obtain the characteristic information about polysaccharide, lignin, and extract on the sample surface. The O/C ratio of the sample can reflect the composition ratio of the surface extract, lignin, and polysaccharide. Some studies have shown that the theoretical O/C ratio of surface extracts, lignin, hemicellulose, and cellulose are 0.03 to 0.11, 0.33, 0.81, and 0.83, respectively (Laine *et al.* 1994). The peaks of C1, C2, and C3 in the C1s core level spectra are related to the three components of lignocellulose, respectively. The C1 peak consists mainly of the characteristic signals generated by 50% lignin content and most surface extract content. The characteristic signals generated by the C2 peak are mainly approximately 80% hemicellulose and 85% cellulose content. The C3 peak signal mainly comes from 20% hemicellulose and 15% cellulose content.

The O/C value of raw healthy and nematode-infected pine substrate and nematode-infected pine sawdust were lower than raw healthy pine sawdust (Table 5). Moreover, the corresponding C1 signature was increased in healthy and nematode-infected pine substrate and nematode-infected pine sawdust, when compared with raw healthy pine sawdust (Fig. 4) probably due to the effect of surface extracts (including fatty acids and waxes) in contributing to lower O/C ratios. This indicates that some cellulose or hemicellulose content of healthy and nematode-infected pine substrate and nematode-infected pine sawdust is digested during the process of fungal infection, resulting in an increase in the

proportion of surface lignin and extract (Ohgren *et al.* 2007). After acid hydrolysis, the O/C ratio in healthy pine sawdust, healthy pine, and nematode-infected pine substrate increased to 0.5, 0.4, and 0.42, respectively, as a result of a small lignin degradation content after the acid hydrolysis (Samuel *et al.* 2010). While the O/C ratio of the acid-treated nematode-infected pine substrate (0.37) was slightly lower than that of the raw nematode-infected pine substrate (0.38), the result was contradictory with the effective degradation of the lignin content of the nematode-infected pine substrate by the acid treatment. However, considering the FTIR results, it can be hypothesized that the fungal infection can benefit the acid degradation of hemicellulose content, thus the proportion of lignin content in the material will increase. In addition, one of the contents of C2 and C3 in nematode-infected pine sawdust and healthy and nematode-infected pine substrate after acid treatment was decreased. However, compared with that in raw material, the contents of C2 and C3 in healthy pinewood were all increased after acid treatment, which also indicated that the content of hemicellulose of the material infected by fungus could be more effectively hydrolyzed in acid treatment.

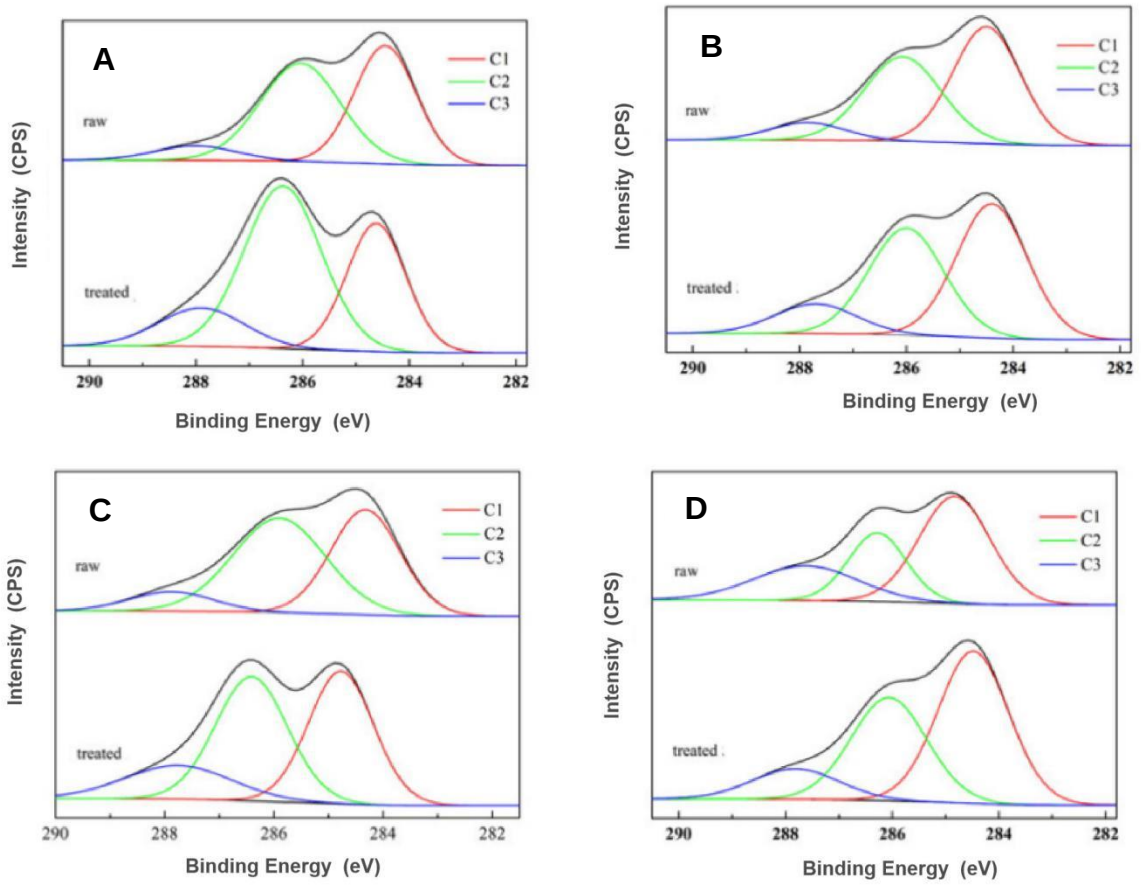

Fig. 4. C1s core level spectra of raw and treated materials: A: raw healthy pine sawdust and healthy pine sawdust with acid treatment, B: raw healthy pine substrate and healthy pine substrate with acid treatment, C: raw nematode-infected pine sawdust and nematode-infected pine sawdust with acid treatment, D: raw nematode-infected pine substrate and nematode-infected pine substrate with acid treatment; acid treatment: 121 °C, 2% H_2SO_4, and 1 h

Table 4. XPS Analysis of Untreated and Different Treatments Samples

Simple	Treatment	O/C Ratio	C1s Signature		
			C1	C2	C3
Healthy Pine Sawdust	Raw	0.40	44.6	48.3	7.1
	121 °C, 2% H_2SO_4, 1 h	0.50	32.4	53.6	14.0
Healthy Pine Substrate	Raw	0.39	50.2	41.5	8.3
	121 °C, 2% H_2SO_4, 1 h	0.40	47.6	40.2	12.2
Nematode-infected Pine Sawdust	Raw	0.40	46.8	44.0	9.2
	121 °C, 2% H_2SO_4, 1 h	0.42	40.8	42.2	17.0
Nematode-infected Pine Substrate	Raw	0.38	50.3	26.1	23.6
	121 °C, 2% H_2SO_4, 1 h	0.37	50.9	36.7	12.4

CONCLUSIONS

1. This study showed that the nematode-infected pine sawdust is a good substrate for the cultivation of *Flammulina velutipes*, producing 126.3 g/kg of reducing sugar under acid treatment (2% H_2SO_4 at 121 ℃ for 1 h).
2. The study can conclude that the fungal infections that happened in the period of pine wilt disease and during the cultivation of *F. velutipes* both can promote acid treatment to degrade hemicellulose content by comparing the yield of reducing sugar and the change of surface structure characteristics of nematode-infected and healthy pine sawdust, nematode-infected, and healthy pine substrate.
3. While fungal infection that happened in the period of pine wilt disease can also contribute to the degradation of the polysaccharide content, it was demonstrated that the degradation of polysaccharides in nematode-infected pine sawdust can be used for the efficient cultivation of *F. velutipes*, which can improve the recycling value of nematode-infected pinewood.
4. In the future, optimization of the treatment method of the nematode-infected pinewood for cultivation of *F. velutipes* on the basis of this study and testing new *F. velutipes* varieties can also be tested to contribute in breaking the bottleneck of using pinewood for cultivation.

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REFERENCES CITED

Alexandropoulou, M., Antonopoulou, G., Fragkou, E., Ntaikou, I., and Lyberatos, G. (2016). "Fungal pretreatment of willow sawdust and its combination with alkaline treatment for enhancing biogas production," *Journal of Environmental Management* 203(2), 704-713. DOI: 10.1016/j.jenvman.2016.04.006

Auxenfans, T., Crônier, D., Chabbert, B., and Paës, G. (2017). "Understanding the structural and chemical changes of plant biomass following steam explosion pretreatment," *Biotechnology for Biofuels* 10(1), Article number 36. DOI: 10.1186/s13068-017-0718-z

Chen, L. J., Li, J. B., Lu, M. S., Guo, X. M., Zhang, H. Y., and Han, L. J. (2016). "Integrated chemical and multi-scale structural analyses for the processes of acid pretreatment and enzymatic hydrolysis of corn stover," *Carbohydrate Polymers* 141, 1-9. DOI: 10.1016/j.carbpol.2015.12.079

Chen, S. Y., Wu, Q. P., Xiao, Y., Gue, S. H., and Qiu, W. Y. (2002). "Review of studies of cultivation edible fungi with conifer chips," *Microbiology* 29(2), 49-52.

Chen, Y., Wang, L. F., Plao, C. G., Zhu, T. H., Shen, S. C., and Zheng, Y. Z. (2008). "Screening of wood-rotting fungi to treat stumps of dead pine trees caused by PWN (*Bursaphelenchus xylophilus*)," *Forest Research* 21(4), 548-554. DOI: 10.1016/S1872-2040(08)60061-4

Gan, Y. J., Zhang, Y. Z. H., and Hong, P. (2010). "Automatic control system and the process design of the heat treatment about pine wood nematode infected timber," *Biomass Chemical Engineering* 44(5), 41-44.

GB/T 742 (2008). "Fibrous raw material, pulp, paper, and board- Determination of ash," Standardization Administration of China, Beijing, China.

GB/T 2677.2 (2011). "Determination of moisture content in fibrous raw material," Standardization Administration of China, Beijing, China.

Jiang, P., Zhao, J. N., Chai, X. M., Wu, Z. L., and He, Z. H. (2001). "Integrated control techniques of *Bursaphelenchus xylophilus*," *Journal of Zhejiang Forestry Science & Technology* 21(4), 1-6.

Jiang, L. Y., Sheng, C. S., Ma, S. G., Wang, L., and Shi, J. (2006). "Study on processing timber infected with pine wood nematode using microwave," *Journal of Nanjing Forestry University,* 30(6), 87-90.

Jordan, S. N., Mullen, G. J., and Murphy, M. C. (2008). "Composition variability of spent mushroom compost in Ireland," *Bioresource Technology* 99(2), 411-418. DOI: 10.1016/j.biortech.2006.12.012

Kuroda, K. (1989). "Terpenoids causing tracheid-cavitation in *Pinus thunbergii* infected by the pine wood nematode (*Bursaphelenchus xylophilus*)" *Annals of the Phytopathological Society of Japan* 55(1), 170-178. DOI: 10.3186/jjphytopath.55.170

Laine, J., Stenius, P., Carlsson, G., and Ström, G. (1994). "Surface characterization of unbleached kraft pulps by means of ESCA," *Cellulose* 1(2), 145-160. DOI: 10.1007/BF00819664

Lara, V., Beata, K. T., Stefan, W., Jarl, H., Annele, H., Kari, S., and Marj, T. (2010). "Scots pine (*Pinus sylvestris*) bark composition and degradation by fungi: Potential

substrate for bioremediation," *Bioresource Technology* 101(7), 2203-2209. DOI: 10.1016/j.biortech.2009.11.052

Li, P., Cai, D., Luo, Z., Qin, P., Chen, C., Wang, Y., Zhang, C., Wang, Z., and Tan, T. (2016). "Effect of acid pretreatment on different parts of corn stalk for second generation ethanol production," *Bioresource Technology* 206(19), 86-92. DOI: 10.1016/j.biortech.2016.01.077

Lu, M., Yan, Q. L., Chi, Y., Zheng, H. Y., and Xiao, L. J. (2016). "Production of liquid spawn of an edible mushroom, *Sparassis latifolia* by submerged fermentation and mycelial growth on pine wood sawdust," *Scientia Horticulturae* 209, 22-30. DOI: 10.1016/j.scienta.2016.06.001

Mamiya, Y. (1983). "Pathology of the pine wilt disease caused by *Bursaphelenchus xylophilus*," *Annual Review of Phytopathology* 21(1), 201-220. DOI: 10.1146/annurev.py.21.090183.001221

Ohgren, K., Bura, R., Saddler, J., and Zacchi, G. (2007). "Effect of hemicellulose and lignin removal on enzymatic hydrolysis of steam pretreated corn stover," *Bioresource Technology* 98(13), 2503-2510. DOI: 10.1016/j.biortech.2006.09.003

Pérez, J. A., Ballesteros, I., Ballesteros, M., Sáez, F., Negro, M. J., and Manzanares, P. (2008). "Optimizing liquid hot water pretreatment conditions to enhance sugar recovery from wheat straw for fuel-ethanol production," *Fuel* 87(17), 3640-3647. DOI: 10.1016/j.fuel.2008.06.009

Samuel, R., Pu, Y., Raman, B., and Ragauskas, A. J. (2010). "Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment," *Applied Biochemistry and Biotechnology* 162(1), 62-74. DOI: 10.1007/s12010-009-8749-y

Sánchez, C. (2009). "Lignocellulosic residues: Biodegradation and bioconversion by fungi," *Biotechnology Advances* 27(2), 185-194. DOI: 10.1016/j.biotechadv.2008.11.001

Shi, J., Luo, Y. Q., Wu, H. W., Kari, H., and Liang, L. Z. (2008). "Impact of the invasion by *Bursaphelenchus xylophilus* on forest growth and related growth models of *Pinus massoniana* population," *Acta Ecologica Sinica* 28(7), 3193-3204. DOI: 10.1016/S1872-2032(08)60068-2

Sluiter, A. R. R., Scarlata, C., Sluiter, J., and Templeton, D. (2008). *Determination of Extractives in Biomass* (NREL/TP-510-42618), National Renewable Energy Laboratory, Golden, CO, USA.

Song, Y. S., Zang, X. Q., Liu, Y., Ge, M. G., and Zhao, J. L. (1994). "Study on the effect of fumigation on the wood infested by pine wood nematode with methyl bromide," *Forestry Scientific Research* 7(6), 671-676.

Song, Y. S. (1993). "Visual examination of diseased wood of pine wood nematode," *Plant Quarantine* 7(1), 23-25.

Syaichurrozi, I., Kurnia Villta, P., Nabilah, N., and Rusdi, R. (2018). "Effect of sulfuric acid pretreatment on biogas production from *Salvinia molesta*," *Journal of Environmental Chemical Engineering* 7(1), Article ID 102857. DOI: 10.1016/j.jece.2018.102857

Wu, S., Lan, Y., Wu, Z., Peng, Y., Chen, S., Huang, Z., Xu, L., Gelbič, I., Guan, X., and Zhang, L. (2013). "Pretreatment of spent mushroom substrate for enhancing the conversion of fermentable sugar," *Bioresource Technology* 148(8), 596-600. DOI: 10.1016/j.biortech.2013.08.122

Wu, Y. Z. (2013). "A preliminary study on cultivation of *Poria cocos* on nematode affected pine wood," *Journal of Fujian Forestry Science and Technology* 2013(4), 51-55.

Yu, R. L., Qin, X. S., and Song, F. G. (2004). "The survey of *Flammulina velutipes* research," *Acta Edulis Fungi* 11(4), 63-68.

Zhao, X., Luo, K., Zhang, Y., Zheng, Z., Cai, Y., Wen, B., Cui, Z., and Wang, X. (2017). "Improving the methane yield of maize straw: Focus on the effects of pretreatment with fungi and their secreted enzymes combined with sodium hydroxide," *Bioresource Technology* 250, 204-213. DOI: 10.1016/j.biortech.2017.09.160

Zheng, Q., Lin, B., Zhang, Q., Wu, S., Wang, S., He, X., Wang, Y., Guan, X., and Huang, X. (2015). "Treating distillers' grain from the Luzhou aroma/flavor industry to degrade fiber," *Energy & Fuels* 29(7), 4305-43107. DOI: 10.1021/acs.energyfuels.5b00250

Zheng, Q., Zhou, T., Wang, Y., Cao, X., Wu, S., Zhao, M., Wang, H., Xu, M., Zheng, B., and Zheng, J. (2018). "Pretreatment of wheat straw leads to structural changes and improved enzymatic hydrolysis," *Scientific Reports* 8(1), Article number 1321. DOI: 10.1038/s41598-018-19517-5

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