Selectively enzymatic conversion of wood constituents with white and brown rot fungi

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ABSTRACT:

Lignocellulosic biomass is the most abundant renewable bioresource and can provide great potential in bioconversion and bioenergy production. This work studies the bioconversion processes of *Pinus yunnanensis* with white and brown rot fungi treatment, aiming to clarify the mechanism of enzymatic process and fungi's intrinsic motivation in fungal pretreatment during various conversion periods. Enzyme activity monitoring, chemical characterization and microstructure analysis were conducted to establish the mechanism. The results showed that fungi achieved the decomposition and conversion of

biomass through the generation of enzymes as well as their action within wood, in addition, fungi can secrete and employ various lignocellulosic enzymes to convert the wood. White rot fungi *T. versicolor* caused simultaneous rot could uniformly convert cellulose, hemicellulose and lignin. Brown rot fungi preferentially depolymerized carbohydrates and the biodegradation of polysaccharides disrupted lignin-carbohydrate complex (LCC) linkages, leading to an easier conversion of lignin. Furthermore, brown rot fungi biodegraded carbohydrates in their own selective path, namely *G. trabeum* could degrade hemicellulose selectively, while *R. placenta* had selectivity for degradation of cellulose. Morphology observation proved that both white and brown rot fungal pretreatments increased the porosity and improved the accessibility of wood cells. The research outcomes could provide insight into the mechanism of enzymatic process and role of fungal selectivity on *Pinus yunnanensis* biodegradation as well as the potential application of fungal pretreatment in future biorefineries and biochemical productions.

Keywords: Fungal selectivity; Pretreatment; *Pinus yunnanensis;* Bioconversion; Lignocellulolytic enzyme activities

1. Introduction

The bioconversion of lignocellulosic biomass (rich in carbohydrates and lignin) into various value-added biochemical has been considered as a most environmentally benign alternative to other traditional processes for biomass resources. The polysaccharides (cellulose and hemicellulose), which account for approx. 55-75% of the dry weight in plant cell wall, can be decomposed into monosaccharides and further fermented into alcohols, such as ethanol and butanol (Swana, Yang, Behnam, & Thompson, 2011), and lignin can be explored as a feedstock alternative to petrol-based chemicals (Aracri, Blanco, & Tzanov,

2014; Zeng, Yang, Yu, Zhang, & Ma, 2012). However, a bottleneck remaining in the biomass conversion is the recalcitrant nature of lignocellulosic materials, due to that lignocellulosic substrate is composed of cellulose, hemicellulose and lignin, which occur mostly in complex association and can be resistant to attack by various treatments. Some chemical and physicochemical pretreatment processes, such as acid pretreatment, alkaline pretreatment, steam explosion and ammonia fiber explosion, have been used to enhance the conversion rate of lignocellulose (Yu, Guo, Zhang, Yan, & Xu, 2009), but most of these pretreatments are energy-intensive, require expensive equipment and often create toxic compounds, which make the process environment unfriendly and economic uncompetitive (Alvira, Tomás-Pejó, Ballesteros, & Negro, 2010). In recent years, biological degradation process has received increasing attentions as the pretreatment path of lignocellulosic materials due to its environmentally friendly process compared with traditional pretreatment methods and its potential to reduce the natural recalcitrance and enhance the enzymatic digestibility of the biomass (Barbosa & de Carvalho Junior, 2020; Wu et al., 2021; Zhu, Li, Meng, Li, & Goodell, 2022). A wide range of microorganisms has been employed to process woody and/or cellulosic biomass, among which rot fungi has attracted growing attentions as a cost-competitive alternative to traditional pretreatments.

In fungal processes, white and brown rot fungi are considered to be the most effective microorganisms for the wood bioconversion (Rudakiya & Gupte, 2019). White rot fungi which can efficiently convert lignin are usually used in biological pretreatment of lignocellulose (Hakala et al., 2005). Members of white rot fungi are able to decompose all structural components in wood cell walls due to their complete range of enzyme systems, such as Li-peroxidase, Mn-peroxidase, and laccase, as well as hydrolytic enzymes, such as

cellulase, hemicellulase, and pectinase (Bari et al., 2015). Different white rot fungi vary greatly in the relative rates at which they convert lignin and carbohydrates in lignocellulose. White rot fungi can also be classified “selective” and “simultaneous” rot. Selective rot preferentially degrades hemicellulose and lignin, consequently cellulose is retained selectively. In contrast, simultaneous rot results in a rather uniform depletion of cellulose, hemicellulose and lignin (Bari et al., 2018). Thus, white rot treatment can enhance the overall chemical or enzymatic hydrolysis by partially or completely removing lignin to destroy its cross-linking with hemicellulose, so as to increase the accessibility of enzymes to the substrates, which can be used for biopulping, bioethanol or other industrial applications. Previous studies showed brown rot fungi could normally depolymerize carbohydrates and modify the lignin (Gao et al., 2016). If the aim is to maximize the proportion of valuable aromatic hydrocarbons of lignin for its valuable aromatic structures in the conversion for final biofuels or chemicals, brown rot fungi could be useful as an efficient microorganism. However, not all of these enzymes can be detected during fungal pretreatment. Due to the heterogeneous nature of lignocellulosic biomass and the complicated conversion mechanism of fungi action, the role of ligninolytic enzymes during the whole pretreatment stage of biomass still remains unclear, which limits its potential for bioconversion production.

This work aimed to unveil the underlying mechanism of enzymatic process and fungi’s selectivity by comprehensively studying the types and activities of lignocellulolytic enzymes, as well as the chemical structure, composition variations of wood during different bioconversion stages through multiple analyses. Crystalline structures and morphological transformations were also characterized to elucidate the structure-performance relationship.

In addition to achieving a thorough understanding of mechanism of the enzymatic process and fungi's intrinsic motivation and selectivity of *Pinus yunnanensis*, this current study could provide great potential and more feasibility for the production of bioenergy and bioengineering for value added products through fungal pretreatment of bioresources.

2. Materials and methods

2.1 Materials

The *Pinus yunnanensis* wood samples were cut into 20 mm (R) × 20 mm (T) × 10 mm (L) sections. The samples were free of knots and visible evidence of infection by mold, stain fungi. White rot fungus *Trametes versicolor* (*T. versicolor*), brown rot fungi *Gloeophyllum trabeum* (*G. trabeum*) and *Rhodonia placenta* (*R. placenta*) were obtained from China Forestry Microbial Species Preservation and Management Center.

2.2 Biodegradation decay assessment

The laboratory biodegradation was conducted according to the Chinese standard GB/T13942.1-2009. Mycelium of *T. versicolor*, *G. trabeum* and *R. placenta* was cultivated on Potato dextrose agar (PDA) at 28℃ and 85% relative humidity for 7-10 days. Two feeder strips (20 mm × 20 mm × 3 mm) of *Pinus massoniana* were placed on top of the mixture. Subsequently, the sterilized wood samples were transferred to the cultures of fungi, followed by incubating at 28±2℃, 85% relative humidity for 30, 60 and 90 days. The mass losses were calculated based on the oven-dried weight of the samples before and after the decay test.

2.3 Fungal pretreatment process

To assay the enzyme activities, erlenmeyer flask containing wood powder and sterile water in the ratio of 1:20 were prepared. After sterilization at 121℃ for 20 minutes, 10

pieces fungi (*T. versicolor*, *G. trabeum* and *R. placenta*) that cut from culture medium using 1 cm diameter punch were transferred into flask to mix with *Pinus yunnanensis* powder. Subsequently, incubation of fungi and powder were conducted using a rotary shaker (THZ-98AB, China) at 180 rpm and 30℃ for 90 days. Supernatants were taken out to determine enzyme activities every 10 days, then powder was used for chemical characteristics analysis at 30, 60 and 90 days.

2.4 Enzymatic activities assay

The reducing sugars were determined by using dinitrosalicylic acid (DNS) method (Elissetche, Ferraz, Freer, & Rodríguez, 2007). Glucose and xylose standard curves were used to calculate the content of glucose and xylose. Endoglucanase, exoglucanase, xylanase, amylase and pectinase were assayed using carboxymethylcellulose, Avicel, xylan, starch and pectinas as substrates, respectively. For oxidizing enzymes, $MnSO_4$, veratryl alcohol and ABTS (2,2-azino-bis-3-ethylthiazoline-6-sulfonate) were used to assay Mn-dependent peroxidase, Li-peroxidase, and laccase activities, respectively (Fen et al., 2014). All enzymes were assayed photometrically, in duplicate, using a spectrophotometer, and the enzymatic activities were expressed as the amount of enzyme that oxidized 1μmol substrate within 1min (U). (Lip: ε_{310}= 9.3 ×10^3 M^{-1} cm^{-1}, Mnp: ε_{240}= 6.5 ×10^3 M^{-1} cm^{-1}, Lac: ε_{420}= 3.6 ×10^4 M^{-1} cm^{-1}).

2.5 Crystallinity and chemical characterization changes

X-ray diffraction measurements were performed on the UItima IV diffraction analyzer with continuous scanning. The diffracted intensity of Cu Kα raditon was measured in a 2θ range between 5º and 40º, and the scanning speed was 8°/min.

The Fourier transform infrared spectra of samples were measured by Thermo Scientific

Nicolet. Wood samples were ground into 40-60 mesh powder and approximately 2 mg of sample in 200 mg of KBr at a weight ratio of 1:100 before the spectrum collection. For each sample, the spectrum was measured in absorption units from 4000^{-1} to 500 cm^{-1} at a spectral resolution of 4 cm^{-1} with 32 scans.

The X-ray photovoltaic spectra were collected using a K-Alpha XPS system (Thermo Fisher Scientific, USA) with a Kα alpha source. A base pressure of 5×10^{-9} mbar was held in the analytical chamber. X-ray beam intensity was varied by changing the electron current striking the anode (15 mA), while the acceleration voltage was held at 15 kV. High-resolution spectrum of the C1s region from 280 to 300 eV was collected.

Chemical compositions of the control and the biopretreated samples were determined according to the standard laboratory analytical procedures developed by the National Renewable Energy Laboratory. All the experiments were conducted in triplicate.

2D-HSQC NMR technique was used to track the structural changes of lignin during biodegradation stages. About 20 mg of lignin was dissolved in 0.5 mL of DMSO-d_6 (99.8% D). For quantitative 2D-HSQC spectra, the Bruker standard pulse program hsqcetgpsi2 was used for HSQC experiments. Data processing was performed using standard Bruker Topspin-NMR software.

2.6 Observation on microscopic structure

Using a scanning electron microscope (SEM, Hitachi TM 3000, Japan), wood blocks with 14-16 μm thickness were cut and applied to observe the changes of microscopic structural and cell voids in different decay stages. The acceleration voltage of SEM was 15-20 Kv.

2.7 Thermal stability analysis

163 The thermal stability of wood samples at different biodegradation stages were examined
164 by using a thermogravimetric analyzer type STA 2500 from Netzsch, Germany. Wood
165 samples were ground into approximately 200 mesh wood dust, weighed and placed in a
166 crucible with approximately 4 mg of sample and heated from room temperature to 600 °C
167 at a rate of 10 °C/min. The shielding gas was nitrogen, the equilibrium gas was 40 ml/min
168 and the sample gas was 60 ml/min.

169 **3. Results and discussion**

170 **3.1 Lignocellulolytic enzyme activities**

171 Lignocellulolytic enzyme activities during fungi pretreatment of *Pinus yunnanensis*
172 were shown in Fig. 1. It could be observed that the enzymatic activities varied among the
173 enzymes and fungi types during the biodegradation process.

174 **3.1.1 Cellulase and xylanase enzymatic activities**

175 In white rot fungi *T. versicolor*, the highest endoglucanase (97.41 U/ml) and
176 exoglucanase activities (31.14 U/ml) reached their maximum activities on the 10th days,
177 and the highest xylanase activity (49.56 U/ml) was observed on the 40th day of incubation,
178 afterwards the activities of the three enzymes decreased after 50th days. In addition, the
179 activities of endoglucanase were higher than that exdoglucanase before the first 40 days,
180 suggesting that *T. versicolor* preferentially destroyed the amorphous area and then attacked
181 the crystalline area of cellulose. The results suggested that this white rot fungi had the
182 ability to attack and convert carbohydrate substrates of wood cell walls at the initial
183 pretreating process.

184 In brown rot fungi, after *G. trabeum* biodegradation, the highest endoglucanase (89.68
185 U/ml) and exoglucanase activities (75.36 U/ml) were occurred at 50th day, and the xylanase

reached the maximum activity (227.30 U/ml) at 40th days, suggesting that *G. trabeum* mainly disrupted carbohydrates at middle biopretreatment stage. Besides, different with white rot fungi *T. versicolor*, the activities of hemicellulase were always higher than that cellulase in the whole biological treatment process, indicating that *G. trabeum* could selectively depolymerize hemicellulose.

In *R. placenta* group, the highest endoglucanase (90.59 U/ml) was observed at 20th day, the exoglucanase and xylanase reached their maximum activities at 80th days, which enzymatic activities were 143.01 U/ml and 80.73 U/ml respectively. It could be inferred that *R. placenta* initially degraded cellulose and then the degradation of hemicellulose mainly occurred in the later stage. In addition, almost all exdoglucanase activities were higher than endoglucanase activities, and the activities of cellulase were always higher than hemicellulase activities during the biotreatment process, indicating that *R. placenta* had a strong destructive ability to cellulose crystal area, and could degrade cellulose selectively compared to *G. trabeum*.

3.1.2 Pectinase and amylase enzymatic activities

In white rot fungi, the highest pectinase and amylase activities were observed on the 10th day, which enzymatic activities were 319.84 U/ml and 44.90 U/ml, showing that pectin and starch were easily degraded into monosaccharides by this white rot fungi as nutrients at initial pretreatment stage (Daniel, 2016). In brown rot *G. trabeum* group, pectinase maintained high activities during the whole pretreating period just as in white rot fungi group. In brown rot *R. placenta* group, the activities of pectinase and amylase increased with the extension of decay time, and reached their maximum activities at 80th days, but the activities of pectinase were lower than that secreted by other two fungi.

In both white rot *T. versicolor* and brown rot *G. trabeum* groups, pectinase exhibited high activities during the whole biological treatment process, which proved that pectinase played a leading role to convert polysaccharides into monosaccharides. This was attributed to that the molecular weight of pectinase was about 20 kD, which could easily enter the cells interior and then decomposed pectin into monosaccharides although the wood cell void was small. In contrast, although the ability of *R. placenta* to degrade pectinase was weaker, the activities of amylase were stronger. Collectively, it could be concluded that the two enzymes played an important role in the whole biological treatment period, due to that these three fungi took pectin and starch as nutrients because pectin and starch were easily biodegraded into monosaccharides (Daniel, 2016).

3.1.3 Laccase, Mn-Peroxidase and Li-peroxidase activities

In white rot fungi *T. versicolor* group, laccase reached a maximum activity (50.92 U/ml) at 50th days, which native functions were to catalyze the demethylation and promote the depolymerization or polymerization in lignin (Mayer & Staples, 2002). The Li-peroxidase activities fluctuated and reached its maximum activity (79.11 U/ml) at the end of the incubation period, which Li-peroxidase could directly oxidize benzene ring substances and destroy the benzene ring skeleton of lignin macromolecules (Doria et al., 2014). These results suggested that *T. versicolor* initially disrupted the lignin side chains such as methoxy group, then attacked the aromatic ring structures. It was worth noting that the maximum activities of lignin enzymes occurred after middle stage. This fact could be attributed to the degradation of carbohydrates due to their high hydrolases activities in the early pretreatment stage, which disrupted LCC linkages, causing lignin more exposed and to be decomposed more easily (Janusz, Kucharzyk, Pawlik, Staszczak, & Paszczynski, 2013).

232 Therefore, the biodegradation of lignin increased gradually in the advanced stage.

233 In both brown rot groups, Li-peroxidase played a leading role in *G. trabeum*, but in a
234 low level. In contrast, *R. placenta* mainly secreted laccase and Mn-peroxidase during the
235 biodegradation process, which could disrupt the side chain of lignin. It was worth
236 mentioning that a similar situation was all the ligninase activities reached their maximum
237 levels after middle pretreatment stage whether in white rot or brown rot groups. This could
238 be due to the depolymerization of carbohydrates and the increase porosity of wood cell
239 walls at the initial biodegradation process for the high hydrolase activities, which was
240 conducive to the disruption of lignin in the advanced period. (Andre Aguiar, Gavioli, &
241 Ferraz, 2013) (Hunt, Kenealy, Horn, & Houtman, 2004) (Arantes & Goodell, 2014) (André
242 Aguiar & Ferraz, 2011).

243 The results of analysis on lignocellulolytic enzyme activities showed that enzymes
244 secreted by fungi, which could all generate carbohydrate hydrolase and ligninase, played a
245 leading role in the bioconversion of biomass. However, the enzyme activities were rather
246 different from various fungi as well as diverse stages, which could be attributed to the
247 fungi's instinctive motivation and selectivity.

248 **3.2 Mass conversion in relation to fungi pretreatment**

249 The mass losses of *Pinus yunnanensis* exposed to different fungi during different periods
250 were shown in Table 1. In *R. placenta*, the most remarkable change of mass loss occurred
251 at 30 days, reaching 7.17%, while the slight mass losses by *T. versicolor* and *G. trabeum*
252 were only about 1%, indicating that *R. placenta* was the most aggressive fungi. A sharp
253 increase could be detected at 90 days, with the mass losses of 10.59%, 13.03% and 35.21%
254 for white rot fungi *T. versicolor*, brown rot fungi *G. trabeum* and *R. placenta* respectively,

indicating that brown rot fungi were more vigorous than white rot fungi in depolymerizing *Pinus yunnanensis*. This fact could be due to the guaiacyl unit of lignin mainly in *Pinus yunnanensis,* which is more stable than syringyl structure, contributing to its less conversion by white rot fungi.

3.3 Crystalline structure and chemical characterization analysis

3.3.1 Crystalline structure analysis

The crystallinities of *Pinus yunnanensis* during different stages were determined by XRD analysis and the results were exhibited in Table 2 and Fig. 2. After exposed to white rot fungi *T. versicolor*, the crystallinities decreased through the pretreating stages, particularly at 60 days with the decrease by 5.30%, indicating that the crystal areas of cellulose were mainly attacked at middle and advanced biodegradation process. The main reason was that the exoglucanase activities were higher than endoglucanase activities after 50 days decay, which exdoglucanase could cut two glucose molecules from the end of the sugar chain and destroy the cellulose intensively (Irbe et al., 2006). In both brown rot fungi groups, the crystallinities continued to decline, with the decreased crystallinities by 4.96% in *G. trabeum* and 12.76% in *R. placenta*, suggesting brown rot fungi could cut cellulose macromolecular chain (Encinas, Henningsson, & Daniel, 1998) and decrease the crystallinities. It was worth mentioning that the greatest decline of crystallinity was caused by brown rot fungus *R. placenta*, suggesting that *R. placenta* could destruct the cellulose selectively, which was consistent with the results of enzyme activities.

3.3.2 FTIR analysis

To analyze the changes of chemical characterization after fungi biodegradation, the FTIR spectra of the samples were presented in Fig. 3. In samples after *T. versicolor*

biopretreatment, the bands at 1600 cm^{-1}, 1510 cm^{-1} and 1266 cm^{-1} assigned to lignin (Beck, Thybring, & Thygesen, 2018), and the absorption peaks 1735 cm^{-1}, 1050 cm^{-1} and 898 attributed to hemicellulose and cellulose (Xia et al., 2018), significantly shifted and declined, indicating that *T. versicolor* caused simultaneous rot which resulted in a rather uniform depletion of carbohydrates and lignin. In both brown rot fungi groups, the peaks at 1735 cm^{-1}, 1045 cm^{-1}, 898 cm^{-1} (attributed to hemicellulose and cellulose) decreased significantly and continuously during the biodegradation period, especially in *R. placenta* group. In contrast, the peak at 1510 cm^{-1} corresponding to lignin showed almost no variation, implying no remarkable damage in the benzene ring skeleton of lignin. This result indicated that both brown rot fungi mainly degraded cellulose and hemicellulose. Besides, *R. placenta* had more remarkable biodeterioration effect on the *Pinus yunnanensis* than the other two fungi, which was consistent with the result of mass conversion analysis.

3.3.3 XPS analysis

In order to obtain the surface chemical composition of complex organic materials, XPS analysis was carried out to investigate the changes of carbon atoms C1-C3, and the results were portrayed in Fig. 4 and Table 3. Among of carbon atoms, C1 mainly came from lignin and wood extracts (Xu, Wang, Liu, & Wu, 2013), C2 and C3 had been proved to be mainly derived from cellulose (Meng, Yu, Zhang, Yu, & Gao, 2016). In white rot fungi *T. versicolor* group, the ratio of C1 did not significantly differ, and the ratio of C2 increased slightly. It could be inferred that the white rot fungi not only converted lignin, but also biodegraded cellulose (Sun & Cheng, 2002), owning to that white rot fungi could secrete oxidative enzymes to convert lignin (Kellner et al., 2014), such as Li-peroxidase, Mn-peroxidase and laccase, as well as hydrolytic enzymes, such as cellulase, hemicellulase and

pectinase, being consistent with the results of enzyme activities analysis. After exposure to both brown rot fungi, the amounts of C2 and C3 decreased and C1 elevated significantly, and the changes were more obvious in *R. placenta* than in *G. trabeum*. This fact could be resulted from that carbohydrates biodegradation mainly occurred by the function of brown rot fungi, in which *R. placenta* had instinct selectivity on cellulose, which was consistent with the above analysis.

3.3.4 Reducing sugars analysis of supernatants

To analyze the reducing sugars and the relationships between reducing sugar contents and enzyme activities of supernatant during *Pinus yunnanensis* bioconversion process, the results were displayed in Table 4 and Fig. 5. It could be seen from Fig. 5 that the contents of reducing sugars positively correlated with enzyme activities, demonstrating that enzymes secreted by fungi played a leading role in the bioconversion of carbohydrates during the biodegradation of *Pinus yunnanensis*. However, in the initial biodegradation stage, especially before 10 days, the size of the enzymes was too large to enter the cell wall (Arantes & Goodell, 2014). At this time, fungi produced small molecular compounds such as free radicals and oxalic acid (Goodell et al., 1997), which could open the channels of cell wall, and as such promote macromolecular enzymes to penetrate through the cell wall due to their diffusivity (Larran et al., 2015).

In addition, in brown rot fungi groups, it should be noted that the contents of xylan were higher than glucan before 60 days in *G. trabeum*, while the glucans were higher than xylan in *R. placenta* during the whole biopretreatment process, suggesting brown rot fungi biodegraded carbohydrates through their own selective path. *G. trabeum* could decompose hemicellulose selectively, whereas *R. placenta* had selectivity for conversion in cellulose,

which was also proved by enzyme activities analysis.

3.3.5 Chemical compositions analysis of wood powder

The chemical compositions of the control and pretreated wood were analyzed and the results were depicted in Table 5. The results showed the contents of glucan, xylan and lignin of control sample were 33.67%, 9.73% and 32.24% respectively. It could be seen as well that white rot fungi *T. versicolor* caused simultaneous rot and resulted in a rather uniform depletion of glucan, xylan and lignin. In contrast, both brown rot fungi preferentially degraded carbohydrates, but left lignin retained selectively. However, brown rot fungi attacked carbohydrates through their own selective path. It is interesting that *G. trabeum* degraded xylan from 9.73% to 2.97%, while *R. placenta* bioconverted glucan from 33.67% to 19.50%, revealing that *G. trabeum* could degrade hemicellulose selectively, while *R. placenta* could preferentially attack cellulose. It was worth noting that the lignin only reduced by 2%-4% after the enzymatic process and the fungi's instinct motivation and selectivity, and as such the residual lignin could be used to optimal final biofuels or biochemicals through these two brown rot fungi biopretreatment.

3.3.6 2D-HSQC spectral analysis

NMR technique was employed to acquire the structural information and fundamental chemistry (interunit linkages) of lignin. The DELs of control and fungal pretreated samples were characterized by the 2D-HSQC NMR (Fig. 6). The side-chain (δC/δH 2.5–5.7/48.0–92.0) and aromatic regions (δC/δH 5.7–8.0/100.0–135.0) in 2D-HSQC NMR spectra and the main substructures annotated with peak assignments referred to previous publications (Wen & Sun, 2013b). The protruding methoxyl groups (OMe), the noticeable aryl ethers (β-O-4, A) and visible phenylcoumarans (β-5, C) substructures could be observed in the

side-chain of control sample. For the chemical composition of lignin fractions, guaiacyl (G) and p-hydroxyphenyl (H) units were clearly presented in the aromatic region. After *T. versicolor* pretreatment, almost all the signal intensities were lower than those of control, especially phenylcoumarans (β-5, C), suggesting that *T. versicolor* could disrupt most of the lignin structural units during the fungi pretreatment process. In *G. trabeum* group, the Aα and Cα signals partly disappeared due to that the depolymerization and cleavage of lignin side-chain by the function of laccase and Mn-Peroxidase. In the aromatic region, the signals of guaiacyl (G) and *p*-hydroxyphenyl (H) were also weakened, indicating the main units of lignin could be partly biodegraded due to the presence of Li-peroxidase. In *R. placenta* group, in the side chain region, there was no significant difference of the signal intensities compared with control sample, whereas the signals were weakened in the aromatic region, indicating that *R. placenta* had ability to attack lignin aromatic region. In both brown rot fungi groups, the conversion of lignin was not serious, which could be attributed to insufficient oxidative enzymes secreted by two brown fungi to degrade lignin.

The relative abundances of different linkages were quantified according to the previous publications (Kim & Ralph, 2010), and the results were listed in Table 6. After *T. versicolor* bioconversion, the relative content of β-*O*-4 linkage decreased from 41.76 to 27.62/100 Ar, and the same tendency also occurred in phenylcoumarans (β-5, C) and guaiacyl (G) units (decreased from 6.90 to 2.75/100 Ar and from 98 to 87/100 Ar respectively), whereas the content of *p*-hydroxyphenyl (H) increased slightly, indicating *T. versicolor* had a tendency to convert native lignin. In consequence, the degradation of methoxy group of G-unit structure led to the relative increase of H-unit content. In both brown rot fungi groups, the destruction of lignin was mainly resulted from the cleavage of β-5 linkages, especially in

370 *R. placenta* group due to its more contents and higher activities of laccase and Mn-
371 peroxidase, which degraded the side chains in lignin structure. Besides, the G-unit
372 decreased from 98 to 93/100 Ar in *G. trabeum* group, which was mainly attributable to that
373 *G. trabeum* could secret higher Li-peroxcidase to cleave lignin aromatic region (Doria et
374 al., 2014).

375 In short, these results suggested that oxidative enzymes secreted by fungi determined the
376 lignin's conversion path, which was consistent with the results of hydrolases and
377 supernatants analysis. Collectively, it could be concluded that enzymes secreted by wood
378 rot fungi, which had different instinct motivation and selectivity, played a leading role
379 during the bioconversion of *Pinus yunnanensis*. Therefore, this finding can offer potential
380 for the production of biorefineries and biochemicals.

381 **3.4 Microscopic structural changes during conversion**

382 In order to observe microscopic structure changes of wood cell walls during fungi'
383 bioconversion, SEM images were shown in Fig. 7. Images of control samples showed wood
384 cells were highly ordered and closely intact, and the cross-field pits were regular in the
385 longitudinal section with no breakage. In preliminary bioconversion stage, mycelium
386 mainly presented in the tracheid. Mycelium could secrete some small molecular
387 compounds such as oxalic acid and small molecular polypeptides (André Aguiar & Ferraz,
388 2012), which could penetrate into and break wood substrates. This could enlarge the pores
389 of cell walls, so that it became easier for macromolecular enzymes to penetrate through the
390 wood cell walls (Arantes & Milagres, 2009). The results herein proved that overcoming
391 the natural lignocellulosic recalcitrance was possible by the application of fungal
392 pretreatments, which could alter the microstructures and chemical constituents, therefore

393 enhance the accessibility to enzymatic digestibility.

394 **3.5 Thermal analysis of *Pinus yunnanensis***

395 Thermal analysis is important to evaluate materials thermal stability. The TG (in wt.%)
396 and DTG (in %/℃) curves of control and biopretreated wood were displayed in Fig. 8. It
397 could be observed that after pretreatment by fungi (white and brown rot fungi), lower
398 temperatures were required to reach the initial decomposition, especially the lowest
399 temperature corresponding to the maximum pyrolysis rate in *R. placenta* group. This result
400 suggested biopretreatment could convert thermal stability and decrease beginning
401 pyrolysis temperature of wood substrates, attributing to the reduced molecular weights
402 resulted from wood depolymerization during bioconversion process (Chu, Masyuko,
403 Sweedler, & Bohn, 2010; Wen, Sun, Xue, & Sun, 2013a), which means the thermal
404 decomposition could start at a relatively lower temperature and consume less energy as
405 well. In addition, it was observed that the contents of "char residues" of samples
406 biodegraded by *G. trabeum* and *R. placenta* increased to 20.59% and 26.26%, comparing
407 with that of control wood at 15.03%. The increased content of "char residues" was
408 attributed to high content of lignin in brown rot fungi pretreated wood. This result indicated
409 that cellulose and hemicellulose were preferentially removed by brown rot fungi, and as
410 such the residual lignin could be used to final biofuels or chemicals due to the maximized
411 proportion of valuable aromatic hydrocarbons, which was also proved by chemical
412 compositions analysis.

413 **4. Conclusions**

414 Enzymatic process and fungi's selectivity by white and brown rot fungi on *Pinus*
415 *yunnanensis* during various conversion periods were elucidated. The mechanism of

enzymatic process and transformation of chemical components and microstructural features of biopretreated wood were systematically investigated, especially by exploring their relationships with lignocellulolytic enzyme activities during various conversion stages. Enzymes played a leading role in the bioconversion of *Pinus yunnanensis*. These three fungi could all secrete carbohydrate hydrolase and ligninase, whereas the enzyme activities generated by different fungi varied considerably at different stages, mainly arisen from the fungi's motivation and selectivity. Different fungi bioconverted wood in their own selective path. White rot fungi *T. versicolor* caused simultaneous conversion which resulted in a rather uniform depletion of cellulose, hemicellulose and lignin. Both brown rot fungi *G. trabeum* and *R. placenta* preferentially degraded carbohydrates and left lignin retained selectively. However, they converted carbohydrates in their own selective path. *G. trabeum* could convert hemicellulose selectively, whereas *R. placenta* could preferentially attack cellulose. Furthermore, white and brown rot fungal pretreatment increased the porosity and improved the accessibility of the biopretreated wood biomass. The outcomes of current study indicated that fungal pretreatment could provide great potential for biorefineries and biochemical productions by effective bioconversion with the use of designed fungi.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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