Enzyme Production and Lignin Degradation by Four Basidiomycetous Fungi in Submerged Fermentation of Peat Containing Medium

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Abstract

Biomass decomposing abilities of four basidiomycetous fungi (*Pleurotus ostreatus* sensu Cooke, *Coriolus versicolor* (L.) Quel., *Tyromyces albidus* (Schaeff.) Donk, and *Trametes gallica* Fr.) were studied using statically submerged media containing peat. The laccase, manganese-dependent peroxidase (MnP), lignin peroxidase (LiP), and total cellulase enzymatic profiles of the four fungi were assessed. The results revealed that laccase is the predominant ligninolytic enzyme secreted during the statically submerged fermentation process using peat as the substrate. *T. gallica* was the most active laccase producer with about 103 U/L at day 25. *C. versicolor* produced relatively higher levels of MnP than the other three basidiomycetous fungi (10 U/L). No significant LiP activity was detected in any of the four white rot fungi (WRF). Furthermore, the production of total cellulase by the four white rot fungi is low, which is beneficial to the selectivity of the bio-delignification. The Klason lignin content

of the peat sample decreased the most (from 57.5% to 51%) after the treatment by *C. versicolor* for 25 days. Scanning electron microscopy (SEM) experiments also proved the lignocelluloses in peat were degraded after white rot fungi (WRF) treatment for 80 days. This study provides evidence that WRF can convert peat into usable intermediates in biofuel production.

Keywords: Delignification, White-rot-fungi, Peat, Basidiomycetous fungi

1. Introduction

Peat is an early stage of coal formation. It forms by partial decomposition of accumulated vegetation dead bodies (i.e. trees and grasses) and histosols. Globally, the total area of peatlands approaches 2.7 million km^2, about 2% of the total land surface ("Survey of Energy Resources 2007" by World Energy Council 2007). Peat is of industrial value as a fuel in some countries, such as in Ireland and Finland. In Ireland and Scotland, peat is also used for cooking and domestic heating. The chemical composition of different types of peat varies, but it is usually rich in lignocellulose.

Lignocellulose is mainly composed of cellulose, hemicelluloses and lignin. Cellulose is a linear biopolymer consisting of anhydroglucopyranose molecules (glucose) connected by β-1,4-glycosidic bonds. Hemicelluloses are heterogeneous polymers of pentoses (including xylose and arabinose), hexoses (mainly mannose, less glucose and galactose) and sugar acids. Lignin is the second abundant biopolymer of lignocelluloses (Dashtban et al., 2010). Lignin generally contains three precursor aromatic alcohols including coniferyl, sinapyl and *p*-coumaryl alcohol (Wei et al., 2009). These precursors form the guaiacyl-(G), syringyl-(S) and *p*-hydroxyphenyl (H) subunits in the lignin molecule, respectively (Martinez et al., 2005). The subunits ratio, and consequently the lignin composition, varies between different plant groups (Dashtban et al. 2010). Lignin acts as a barrier to any solutions or enzymes by linking to both hemicellulose and cellulose. This prevents penetration of lignocellulolytic enzymes to the interior lignocellulosic structure (Dashtban et al., 2010).

Due to the structural complexity of lignocellulose, the removal of lignin by physical and chemical method is an energy intensive process. In nature, white rot fungi (WRF) are the most efficient microorganisms for degrading lignin. The ligninolytic enzymes produced by these fungi are mainly lignin peroxidase (LiP), manganese dependent peroxidase (MnP), and laccase. WRF variously secrete one or more of the lignin-modifying enzymes (Wesenberg et al., 2003). Du et al. (2006) reported the lignin degradation abilities of nine basidiomycetous fungi by using paddy straw as a substrate. They found *P. ostreatus* sensu Cooke, *C. versicolor* (L.) Quel., *T. albidus* (Schaeff.) Donk, and *T. gallica* Fr. had higher lignin degradation activities. The four WRF preferred to use lignin rather than cellulose when paddy straw was used as the substrate.

The aims of this study are to (i) study the production of enzyme complexes by the four WRF at different incubation times under submerged fermentation, using peat as a substrate; (ii) to study the effect of the fungi on lignin degradation; and (iii) to study the effect of the fungi on modification of the lignocellulosic polymers.

2. Materials and Methods

2.1 Chemicals

All the chemicals and reagents were of analytical grade. Peat (harvested from Williams Bog, Thunder Bay, Canada) was dried at 37 °C then ground in a spice and nut grinder (Cuisinart Company, Canada). The powders were sieved through 0.5 mm mesh. The fine powders were used for making the media.

2.2 Fungal strains and culture conditions

The four WRF strains including P. ostreatus sensu Cooke, C. versicolor (L.) Quel., T. albidus (Schaeff.) Donk, and T. gallica Fr. were isolated by our collaborator Dr. Ruiqing Song's lab at Northeast Forestry University, China. The strains were grown and maintained on potato dextrose agar (PDA) containing 15.0 g/L potato dextrose, 20.0 g/L sucrose, 3.0 g/L KH_2PO_4, 2.0g/L $MgSO_4$ and 15.0 g/L agar. Strains were grown in 250 mL flasks containing 50 mL liquid peat containing medium: 15.0 g/L potato dextrose, 3.0 g/L KH_2PO_4, 3.1 g/L $MgSO_4·7H_2O$, 5.0 g/L ammonium tartrate, 20 g/L peat powder, and 50 ml/L trace element solution. Trace element solution contains 3.0 g/L $MgSO_4·7H_2O$, 0.5 g/L $MnSO_4$, 1.0 g/L NaCl, 0.1 g/L $FeSO_4·7H_2O$, 0.1 g/L $CoCl_2$, 0.1 g/L $ZnSO_4·7H_2O$, 0.1 g/L $CuSO_4$, 10 mg/L $AlK(SO_4)_2·12H_2O$, 10 mg/L H_3BO_3, 10 mg/L $Na_2MoO_4·2H_2O$, and 1.5 g/L nitrilotriacetic acid (NTA). The pH of the medium was adjusted to pH 5.0 by adding H_2SO_4. The recipe for the medium is from Du et al. (2006), except that paddy straw is replaced by 2% peat powder in our experiments.

2.3 Inoculum preparation

The four fungi were pre-grown in PDA liquid media for 5-7 days, then the mycelia clumps were cut to very

small pieces (the length of the pieces is shorter than 1 mm) and grown in a new PDA liquid medium to form pellets of mycelia (diameters of pellets were approximately 1 mm). Then a new liquid peat containing medium was inoculated using equal numbers of pellets. The fungi were grown statically in 250 mL flasks containing 50 mL liquid peat medium at room temperature for 25 days. Each fungus was grown in 15 flasks; three flasks of each fungus were used for collecting samples every 5 days for the enzyme activity measurements. The cultured fungi were filtered, and the filtrates were collected by centrifugation at 12,000 g for 2 minutes. The supernatants were used as the source of enzyme.

2.4 Enzyme activities

Laccase activity was determined by monitoring the oxidation of 2, 2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic Acid Ammonium Salt) (ABTS) at 436 nm. The reaction mixture contained 1 mM ABTS and 100 mM sodium acetate buffer (pH 4.5), ε436=29300 M-1cm-1 (Jauregui et al. 2003). MnP activity was determined by monitoring the oxidation of 2,6-dimethoxyphenol (DMP) at 469 nm. The reaction mixture contained 1 mM DMP, 1 mM $MnSO_4$, 100 mM sodium tartrate (pH 4.5) and 0.1 mM H_2O_2. MnP activity was corrected for manganese-independent peroxidase (MIP) activity by subtracting the activity obtained at pH 4.5 in the absence of $MnSO_4$, ε469=27500 M-1cm-1 (Heinfling et al. 1998). LiP activity was determined by monitoring the oxidation of azure B at 651 nm. The reaction mixture contained 0.032 mM azure B, 50 mM sodium tartrate (pH 3.0) and 0.4 mM H_2O_2, ε651=48800 M-1cm-1 (Park et al., 2007). One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μmol of substrate per minute.

2.5 Filter Paper Assay (FPA) and determination of reducing sugar content

A microplate based filter paper assay, similar to the one described by Dashtban et al (2011) and Cianchetta et al (2010), was carried out to measure the total cellulase activity. The cell free culture supernatant was diluted 50 times with 50 mM NaAc (pH 4.8). 60 μL of each sample was placed in a well containing a 6 mm diameter filter paper disk (Whatman No. 1, with average weight of 3.0 mg each, ThermoFisher Scientific, Canada). Filter paper disks were made by using a standard office hole punch. Reagent blank control containing only 60 μL of 50 mM NaAc buffer, and substrate control containing the filter paper and 60 μL of 50 mM NaAc buffer were also run. To exclude the background signal of reducing sugars in the enzyme supernatant from the results, a negative control without filter paper was run. A glucose standard curve with a range of 0 to 1.0 mg/mL was run. All of the samples and the standards were run in triplicate.

For incubation, the microplates were sealed with paraffin membrane, covered in a Ziploc bag and incubated at 50 °C in water bath for 60 min. To measure the released reducing sugar, 120 μL of 3,5-dinitrosalicylic acid (DNS reagent) was added to each well, and the plate was resealed with paraffin. The plate was placed in a boiling water bath for 5 min to develop the colour. A 100 μL aliquot was transferred to a new 96-well flat-bottom microplate, and the absorbance at 540 nm was measured using an xMark Microplate Spectrophotometer (Bio-Rad, Canada). Total reducing sugars generated during the assay was estimated as glucose equivalents. To calculate glucose equivalents, the absorbance of the samples was converted into a concentration of glucose using the standard curve.

2.6 Klason Lignin content measurement

The resins of peat were extracted with ethanol-benzene according to the Technical Association of the Pulp and Paper Industry (TAPPI) T204 protocol. Then proteins were eliminated by treatment with 1% pepsin solution (pH 2.0) for 24 h at 40℃ using liquid/solid ratio 40 (Dias et al., 2010). Cellulose and hemicelluloses in peat were removed according to TAPPI T222 protocol, with some modifications as described by Sharma (Sharma et al., 2010). Samples were briefly treated with 72% H_2SO_4 at 30 ℃ for 1 h at 200 rpm. Subsequently, they were diluted to 3% H_2SO_4 with distilled water and autoclaved at 121℃ for 45 min. Finally, the weight of ash was measured after burning the samples in a muffle furnace at 525℃, according to TAPPI T211 protocol "Ash in wood, pulp, paper and paperboard: combustion at 525 ℃".

2.7 Scanning Electron Microscopy (SEM) analyses

Anatomical characterization was carried out by using SEM. Woody chips of peat were fixed by exposing them to 0.1 M phosphate buffer (pH 7.0) with 2% gluteraldehyde solution for 2 h at 4 ℃. Then, the samples were washed three times with the same buffer, 10 min for each wash. Following this, the samples were dehydrated with a series of graded ethanol washings by increasing concentrations of alcohol (50%, 70%, 80%, 90% and three rounds of 100%), 10 min for each washing. Finally, the samples were dried using the critical point-drying process. Dried samples were then mounted on carbon tape and coated in gold for SEM experiments (Lin et al., 2009).

2.8 Data processing and statistical analysis

All results are the average values of three independent experiments. The data were collected in a Microsoft Excel spreadsheet where the average and standard error of the mean were determined. A one-way analysis of variance (one-way ANOVA) at a confidence level of 95% ($\alpha = 0.05$) was carried out with the software PRISM 5 to test the statistical significance of differences between lignin content of peat before and after fungal treatment.

3. Results and Discussion

3.1 The pH value change in submerged fermentation process

The pH value changes during submerged fermentation process of the four WRF were monitored over the 25 days of incubation (Figure 1). In the fermentation of C. versicolor, T. albidus and T. gallica, their pH values decreased from pH 5.0 (starting value) to approximately pH 4.0 during the first ten days of incubation. The pH values then started to increase over the last 15 days of incubation and reached approximately pH 5.0 at day 25. Pellinen et al. (1989) suggested that the pH value of the unbuffered culture medium decreases during the incubation phase due to the releasing of acidic lignin degraded products. The subsequent pH increase may be due to microbial assimilation of organic acids (Botella et al., 2007). P. ostreatus showed a different pH pattern than the other three WRF: the pH slowly decreased over the 25-day experiment. The different pH trend in P. ostreatus could be due to its slow growth rate compared to the other three fungi. This may suggest that the pH of the medium would increase if the experiment were extended.

3.2 Laccase activities

Laccase activities of C. versicolor and T. gallica gradually increased over the experiment starting at day five with a peak at the last time point (25 days) (Figure 2). In the case of P. ostreatus, laccase activity was seen with a peak of approximately 67 U/L at day 15. The laccase activity of T. albidus showed a small peak (39 U/L) at day ten with a decrease at day 15; it then gradually increased over the last time points. Among the four fungi studied here, T. gallica was the most active laccase producer with about 103 U/L laccase activity at the last time point (day 25) (Figure 2). It is critical to determine the precise time point for maximum laccase production in industry. Except for P. ostreatus, laccase activities of the WRF continuously increased. The trend of laccase activity is highly variable among different WRF. For example, the maximum laccase activities were detected during the first ten days of wheat straw incubation by T. versicolor (C. versicolor) (Dinis et al., 2009). This is because lignin enzyme production by WRF is highly dependent on species, strains, lignocellulosic substrates, medium, and fermentation method (Elisashvili et al., 2008).

3.3 MnP activities

All four of the WRF studied secreted a notable quantity of manganese peroxidase (MP) (data is not shown here). However, they produced little manganese-dependent peroxidase except for C. versicolor. After the 25 days' submerged fermentation, C. versicolor produced relatively high yields of MnP and it had a maximum activity at 25th day of incubation with a value of 10 U/L. In a related study, C. versicolor also produced relatively higher levels of MnP, compared to the other fungi including P. ostreatus DSM 11191, Trametes hirsuta K 21a, and Cerrena unicolor T 71 (Winquist et al., 2008). However, the other three WRF studied here produced MnP activities less than 2 U/L. T. albidus also showed some MnP activity with a peak of approximately 1.3 U/L at day ten of the incubation (Figure 3). Lower MnP activity in T. albidus, T. gallica, and P. ostreatus could be due to growth conditions such as medium composition. In a previous study (Sun et al., 2004), HcLn (high amounts of carbon source and low amounts of nitrogen source) and LcHn (low amounts of carbon source and high amounts of nitrogen source) media were used to investigate the production of T. gallica ligninases under both agitated and stationary incubations. Although laccase activities were found in the four cultivation methods (HcLn and LcHN using agitated and stationary incubations) no LiP activities were detected using the four cultivation methods. MnP activity was presented, though only under stationary HcLn condition. Similar results with low MnP activity were reported using other WRF. For example, WRF secreted high laccase activity; but either no MnP or trace amounts of MnP activities were detected by the majority of the tested fungi (Elisashvili and Kachlishvili 2009; Songulashvili et al., 2007; Elisashvili et al., 2009)

3.4 LiP activities

No significant LiP activity was detected in any of the four WRF in submerged fermentation using peat as the substrate. Studies have shown that many WRF do not secrete LiP. For example, Pleurotus species have been recognized to produce no typical LiP (Kamitsuji et al., 2004); and no LiP activity was detected in the C. versicolor cultured medium using Olive mill wastewater as the substrate (Ergul et al., 2009). It has been suggested that LiP production is influenced by the C/N ratio (Tien and Kirk, 1988). For ligninase enzymes, some

WRF produce all of these enzymes while others produce only one or two of them. Our results demonstrated that none of our four WRF secretes detectable LiP.

3.5 Total cellulase activities (filter paper activities)

Total cellulase activity was only detected in two of our four studied WRF: T. gallica and P. ostreatus (Figure 4). Interestingly, no cellulase activity was detected for T. albidus and C. versicolor. For T. gallica the lowest activity was determined after ten days of incubation while for P. ostreatus the lowest values were observed within the first ten days of incubation. T. gallica showed a maximum total cellulase activity (0.04 μmol) at five days of incubation whereas P. ostreatus showed a peak (0.03 μmol) at 15 days of incubation. These relatively low values of cellulolytic activity by the four WRF are confirming the objectives of this work in their selective use of lignin as the substrate. This is important especially for maximum lignin breakdown during pre-treatment using fungi.

3.6 Klason lignin content measurement

The percentage of Klason lignin present in the peat samples subjected to the fungal pre-treatment decreased from 57.5% (controls) to 51.6% (T. gallica), 53.8% (P. ostreatus), 52.0% (T. albidus) and 51.0% (C. versicolor) after 25 days of incubation (Table. 1). These results are in accordance with previous work by Levin et al. (2007). Specifically, they observed a 3.1% decrease in the lignin content of pine chips after a 2 week pretreatment with Pycnoporus sanguineus, which was the most effective among the eight tested WRF (Levin et al. 2007).

In our study, the Klason lignin content of samples pre-treated with C. versicolor decreased the most. Similarly, Liew et al. (2010) found that lignin loss in Acacia mangium wood chips pre-treated with C. versicolor was the highest compared to three other studied fungi. Among the four WRF studied here, P. ostreatus was the only fungal strain that did not significantly reduce the lignin content of peat compared to the non-inoculated control ($P > 0.05$). A similar result was also obtained by Yang et al. (2010), who found that P. ostreatus did not significantly degrade the lignin of corn stover.

3.7 Scanning electronic microscopy analyses

T. gallica was chosen for SEM experiments due to its high laccase activity. The SEM pictures using both 800x and 2200x magnifications showed that the lignocellulosic structure of peat was degraded after 80 days treatment with T. gallica (Figure 5). As shown in Figure 5 b and 5 d, the wood surface became rough, with cracks forming after the treatment; whereas the non-treated wood fibers are smooth (Figure 5a and c). This supported the theory that the lignocellulases secreted by T. gallica could efficiently degrade lignocellulose of peat.

4. Conclusion

All of the four studied WRF produced good levels of laccase, whereas relatively higher levels of MnP was only produced by C. versicolor in submerged fermentation using a peat containing medium. The four fungi secreted low levels of cellulases in the submerged fermentation using a peat containing medium. The lignin content of peat significantly decreased after the treatment by the studied WRF for 25 days, except in the case of P. ostreatus. SEM pictures showed the lignocellulose structure of peat was degraded after the treatment using T. gallica for 80 days. High laccase activity, high MnP production (especially by C. versicolor), as well as low cellulase activity by the four studied WRF using a peat containing medium make them good candidates for pretreatment of lignocellulosic residues

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Table 1. Klason lignin content of the control (non-inoculated) and 25-days pre-treated peat (%, w/w of extractives)

Strains	Klason lignin content % (w/w)	Significance level (*P* value)
control	57.5±0.6	
Trametes gallica	51.6±2.7	0.025
Pleurotus ostreatus	53.8±1.3	0.214
Tyromyces albidus	52.0±2.0	0.036
Coriolus versicolor	51.0±2.3	0.014

*Total peat weight does not include resin as it was removed before measurement.

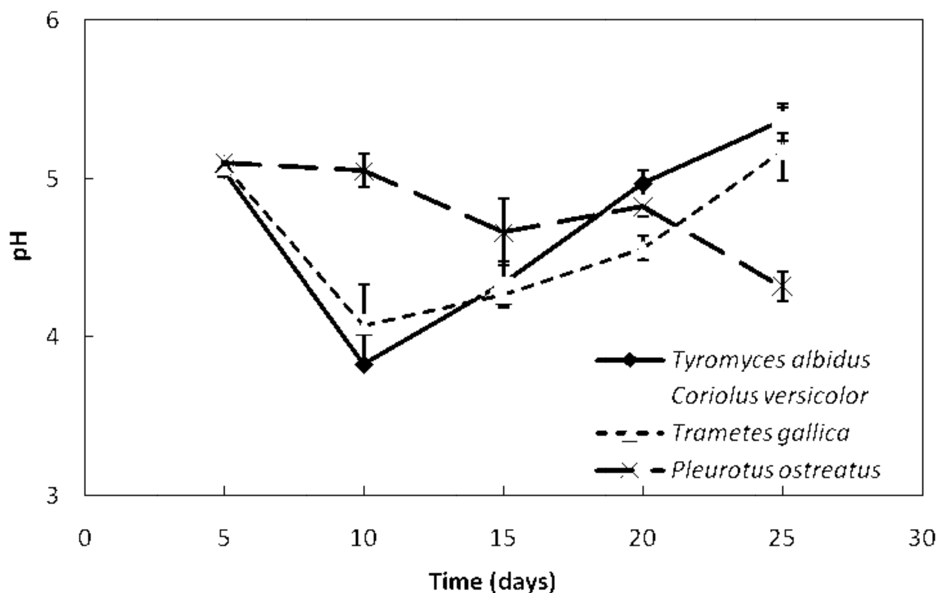

Figure 1. The change in pH value of submerged fermentation of the four WRF using peat as the substrate (three independent biological replicates represented by each data point and error bars denote standard error of the mean)

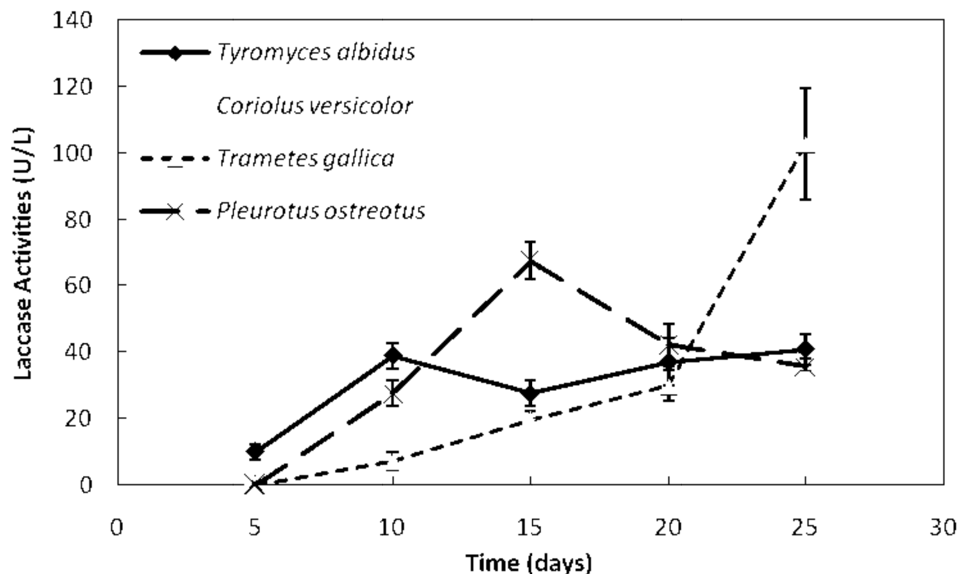

Figure 2. Laccase activities measured during peat submerged fermentation in the presence of the four WRF (three independent biological replicates represented by each data point and error bars denote standard error of the mean)

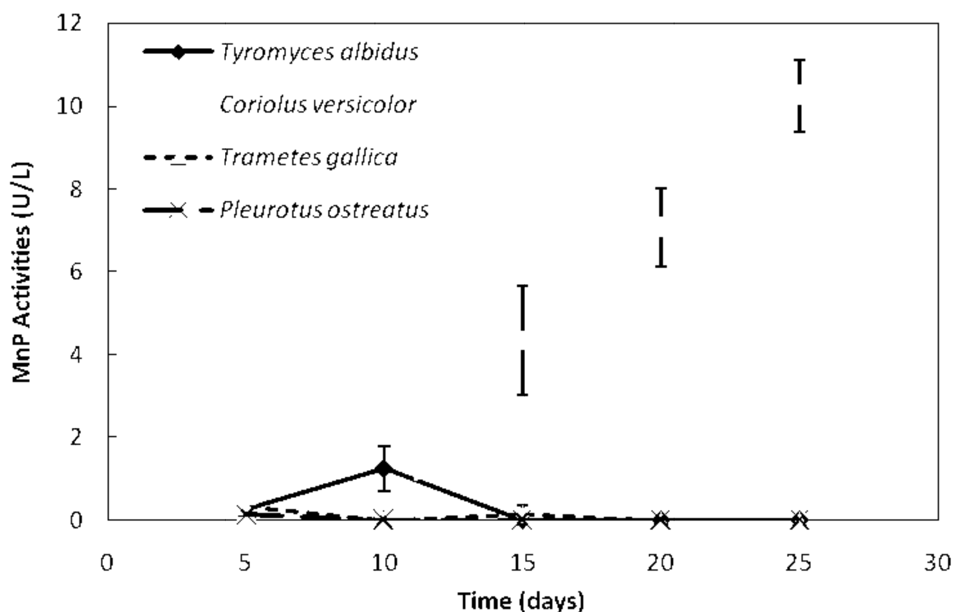

Figure 3. MnP activities measured during peat submerged fermentation in the presence of the four WRF (three independent biological replicates represented by each data point and error bars denote standard error of the mean)

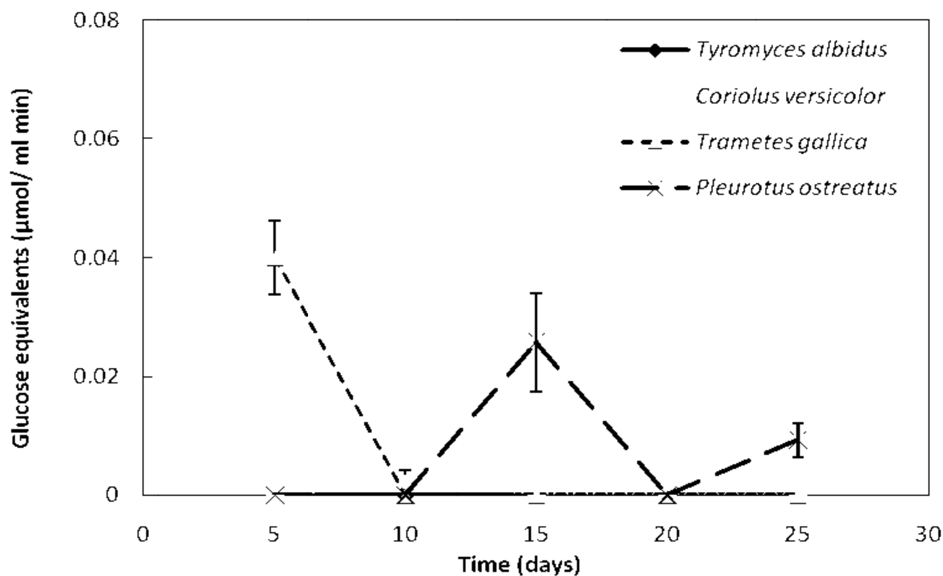

Figure 4. Total cellulase activities of the four WRF in submerged fermentations using peat containing media (three independent biological replicates represented by each data point and error bars denote standard error of the mean)

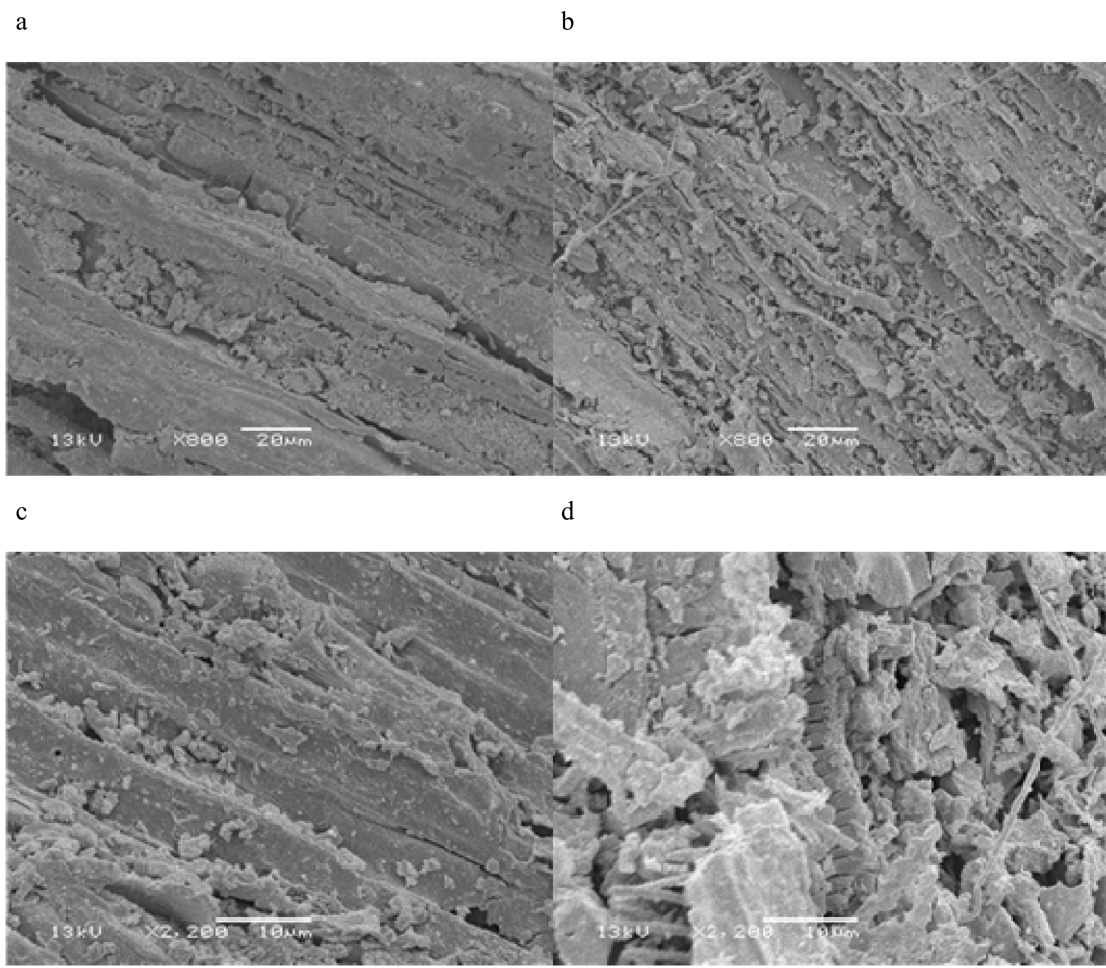

Figure 5. **S**EM pictures showing peat lignocellulose enzymatic degradation treated with *Trametes gallica* for 80 days [800x magnification, A = untreated but incubated for 80 days, B = treated with *Trametes gallica* for 80 days; 2200 x magnification, C = untreated but incubated for 80 days, D = treated with *Trametes gallica* for 80 days]