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Lignin in Paper Mill Sludge is degraded by White-Rot Fungi in Submerged Fermentation

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Abstract

This study investigated biological treatment of paper mill sludge and lignin degradation by fungi. Four different Basidiomycetes white-rot fungi (WRF) were used: *Coriolus versicolor*, *Tyromyces albidus, Trametes gallica* and *Pleurotus ostreatus*. The fungi were cultured in submerged fermentation cultures with paper mill sludge. The pH values of the cultures and ligninolytic enzymes production profiles of the four fungi were monitored and reported here. The highest laccase activity of 202 U/L was obtained in *T. albidus* at day 25 while the maximum manganese-dependent peroxidase (MnP) activity of 50 U/L was obtained in *C. versicolor* from day 15 to 25. The *P. ostreatus* showed highest total cellulase activity with the peak of 0.26 μmol at day 15 while the other three WRF showed negligible total cellulase activity. The lignin contents were significantly decreased in the four WRF-treated sludge samples and were confirmed by acetyl bromide and FTIR analyses. The results suggested that lignin was preferentially used and significantly degraded or converted in all the four WRF-treated sludge samples.

Keywords: Lignin; Biological treatments; Microbial processes; Lignocellulosic biomass; White-rot fungi (Wrf)

Introduction

The effluents from the pulp and paper industry cause considerable damage to the receiving waters if discharged untreated. They have high biochemical oxygen demand (BOD), chemical oxygen demand (COD) and contain chlorinated compounds, suspended solids (mainly fibers), fatty acids, lignin and sulphur compounds. Most of the solids are removed after the mechanical treatment resulting in a sludge that contains large quantities of fibers [1]. Because of more and more stringent environmental requirements, landfills are reaching full capacity and the development of alternative solutions or methods for treating paper mill sludge (PMS) are under pressure.

Paper mill sludge is a solid waste material composed of pulp residues and ash generated from the pulping and paper-making process [2]. It is a solid residue recovered from the wastewater. Paper mill sludges are distinctly different in composition, even between mills using the same pulp and paper manufacturing process. PMS consists primarily of polysaccharide material-cellulose, lignin and hemicelluloses [3]. The majority of the created sludge wastes (69%) are disposed in landfill while 21% are incinerated. Currently, most sludge produced by pulp and paper mills is dewatered and landfilled. It is both economically and environmentally beneficial to find treatment methods that cut down on landfill deposits.

Lignin is an amorphous highly-branched polymer present in vascular plants and accounts for up to 30% of the dry weight of the plant and biomass [4]. Lignin generally contains three precursor aromatic alcohols including coniferyl alcohol, sinapyl and *p*-coumaryl [5]. These precursors form the guaiacyl- (G), syringyl- (S) and *p*-hydroxyphenyl (H) subunits in the lignin molecule, respectively [6]. The subunits ratio, and consequently, the lignin composition, varies between different plant groups [7]. 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 [8]. The complex linking of lignin to cellulose and hemicellulose makes physical and chemical removal of lignin in biomass inefficient and energy intensive. Thermal, mechanical and chemical conversions are extensively reviewed by Bridgwater, Sun and Cheng and Hendriks and Zeeman [9]. Recently, biological methods for lignin removal or modification have received more and more attention by many research groups.

Biological treatments use microorganisms for the degradation and conversion of organic materials [9]. Dionisi et al. [10] reviewed the literature on three microbial processes and compared the rates of microbial processes with those of the alternative physio-chemical pretreatment processes. The best pretreatment method depends on many factors such as type of lignocellulosic biomass, process parameters, environmental impact, economical feasibility, etc [11].

White-rot fungi (WRF) are known as efficient lignin degrading microorganisms. Du studied the lignin degradation using wheat straw as a substrate by nine different basidiomycetous fungi [12]. Among the studied WRF, four of them including *P. ostreatus* sensu Cooke, *C. versicolor* (L.) Quel., *T. albidus* (Schaeff.) Donk, and *T. gallica* Fr. were reported to be more efficient in lignin degradation. The four aforementioned WRF preferentially work on lignin in a selective manner. Hong et al. examined four fungi including *Coriolus versicolor*, *Tyromyces albidus, Trametes gallica* and *Pleurotus ostreatus* in submerged media containing peat [8]. The enzymatic profiles of the fungi were assessed. They found the predominant ligninolytic enzyme during the fermentation process using a peat substrate was laccase. *T. gallica* was the most active one of the four. The production of total

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cellulase was for all four WRF [8]. These fungi produce ligninolytic enzymes such as lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and laccase. They also secrete other accessory and lignin-modifying enzymes.

The aim of this research was to (i) study the production of lignin-degrading enzyme complexes by the four WRF under different conditions in submerged fermentation using paper mill sludge as a substrate; (ii) to study the effect of these fungi in lignin modification and degradation.

Materials and Methods

Chemicals

The chemicals and reagents used in this research were analytical grade. Paper mill sludge (obtained from a paper mill in Canada) was dried at 37°C then ground in a coffee grinder. The powders were filtered through a mesh screen, and then were used for making the media to be used in the submerged fermentation process.

Fungal strains and culture conditions

Four WRF strains *P. **ostreatus*** sensu Cooke, *C. versicolor* (L.) Quel., *T. albidus* (Schaeff.) Donk, and *T. gallica* Fr. were used in the current study. They were isolated in the laboratory of Dr. Ruiqing Song at Northeast Forestry University, China**.** The fungi were grown and maintained on potato dextrose agar (PDA) supplemented with 20.0 g/L sucrose, 3.0 g/L KH_2PO_4, and 2.0g/L $MgSO_4$. Strains were grown in 250-mL flasks containing 50 mL liquid sludge medium modified from Du et al. [12]: 15.0 g/L potato dextrose, 3.0 g/L KH_2PO_4, 3.1 g/L $MgSO_4 \cdot 7H_2O$, 5.0 g/L ammonium tartrate, 20 g/L sludge and 50 ml/L trace element solution. The trace element solution contained 3.0 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $MnSO_4$, 1.0 g/L NaCl, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.1 g/L $CoCl_2$, 0.1 g/L $ZnSO_4 \cdot 7H_2O$, 0.1 g/L $CuSO_4$, 10 mg/L $AlK(SO_4)_2 \cdot 12H_2O$, 10 mg/L H_3BO_3, 10 mg/L $Na_2MoO_4 \cdot 2H_2O$ and 1.5 g/L nitrilotriacetic acid (NTA). The pH of the medium was adjusted to pH 5.0 by adding H_2SO_4.

Inoculum preparation

Fungi were pre-grown in potato dextrose agar (PDA) broth medium for several days. The mycelia were then cut to small pieces (shorter than 1 mm) and grown in a new PDA liquid medium until pellets were formed. These pellets were used as inocula for the liquid sludge medium. The fungi were grown in 250 mL flasks containing 50 mL liquid sludge medium and maintained at room temperature for 25 days statically. Each fungus was grown in 15 flasks. Three flasks of each fungus were used for collecting samples every 5 days to complete enzyme activity measurements. The broth cultures of fungi were first filtered and the filtrates were collected by centrifugation at 12,000 *g* for 2 minutes [8].The supernatant was used as the source of enzyme for all of the enzymatic assays**.**

Enzyme activities

One unit of enzyme activity was defined as the amount of enzyme that transformed 1 µmol of substrate per min. The activities of laccase, MnP, and Lip were measured by using a previously described method [8]. 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). The extinction co-efficient (ε) of ABTS at 436nm is 29300 $M^{-1}cm^{-1}$[13].

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. The MnP activity was corrected for MIP activity by subtracting the activity obtained at pH 4.5 in the absence of $MnSO_4$. The extinction co-efficient (ε) of DMP at 469nm is 27500 $M^{-1}cm^{-1}$ [14].

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. The extinction co-efficient (ε) of azure B at 651nm is 48800 $M^{-1}cm^{-1}$ [15].

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

A microplate based filter paper assay, described by Sun et al. was carried out to measure the total cellulase activity as described by Hong et al. [8,16]. In brief, the cell free culture supernatant was diluted 50 times with 50 mM NaAc (pH 4.8). Sixty (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, Thermo Fisher Scientific, Canada). Disks were made using a standard office hole punch. Reagent blank controls 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. A negative control with no filter paper was run to exclude the background of reducing sugars found in the enzyme supernatant from the results. A glucose standard curve with a range of 0 to 1.0 mg/mL was run. Triplicates of samples and standards were used.

Following the procedures adopted by Hong et al. the microplates were sealed with paraffin membrane, then covered in a Ziploc bag and incubated in a 50°C water bath for 60 min [8]. Then 120 µL of 3,5-dinitrosalicylic acid (DNS reagent) was added to each well to measure the released reducing sugar, and the plate resealed with paraffin. The plate was placed in a boiling water bath for 5 min to develop the color. 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 sample was converted into a concentration using the standard curve.

Analysis of lignin in paper mill sludge by acetyl bromide digestion

Lignin contents of paper mill sludge were determined by acetyl bromide treatment (25% in acetic acid) and then the absorbance was determined at 280 nm [17].

FTIR analyses of untreated and treated paper mill sludge

To investigate the modification of lignin functional groups of WRF-treated paper mill sludge, Fourier transform infrared spectroscopic (FTIR) spectra (1,900 to 700 cm^{-1}) were obtained and compared to untreated paper mill sludge (Lakehead University Instrumentation Lab, Thunder Bay, Canada). A Tensor 37 FTIR (Bruker Optics, Germany) with a resolution of 4 cm^{-1} and 32 scans per sample was used. The experiment was done using approximately 2 mg of each sample and applying a MIRacle ATR accessory with high-pressure clamp (PIKE Technologies, Madison, WI, USA).

Data processing and statistical analysis

The average values of three independent experiments were used for

experimental results. Using a Microsoft Excel spreadsheet the average and standard error of the mean were calculated. To test the statistical significance of differences between lignin content of paper mill sludge (PMS) before and after fungal treatment PRISM 5 software was used to complete a one-way analysis of variance (one-way ANOVA) at a confidence level of 95% (α= 0.05).

Results and Discussion

The pH value change in submerged fermentation process

The pH values of the four WRF cultured using a submerged fermentation process using paper mill sludge as the substrate was studied. They were monitored every 5 days over a 25 day period (Figure 1). The pH value of cultured *C. versicolor* and *T. albidus* decreased from 6.0 to around 4.0 over the first 10 days and *T. gallica* over 15 days, then continuously increased over the remaining days. The pH value of cultured *P. ostreatus* slowly changed over the 25 days experiment with only a slight decrease by the end of the 25 day experiment. Pellinen et al. suggested that the pH value of the unbuffered culture medium decreases during the incubation due to the releasing of acidic lignin degraded products [18]. The different pH trend in *P. ostreatus* could be due to its slow growth rate comparing to the other three fungi used in the study. This would suggest that the pH of the medium would increase if the experiment were extended (Figure 1).

Lactase activities

Laccase activities of *C. versicolor* and *T. gallica* gradually increased over the experiment starting at day 5 (Figure 2). *P. ostreatus* showed a peak of laccase activity with 67 U/L at the day 15. The laccase activity of *T. albidus* has a peak (107 U/L) at 15th day, but decreased again for the 20th. The laccase activity gradually increased from that point onwards. Among the four fungi studied here, *T. albidus* was the most active laccase producer with about 202 U/L laccase activity at the last time point (day 25) (Figure 2). So it is critical to determine a right time point for maximum laccase production in industry. The trend of laccase activities of *P. ostreatus* is different with the other three strains. We assume this is because its production of laccase is more sensitive to medium acidification than the other three WRF. Except for *P. ostreatus*, laccase activities of the WRF were continuously increased. The trend of laccase activity is highly variable. Dinis et al. [19] found

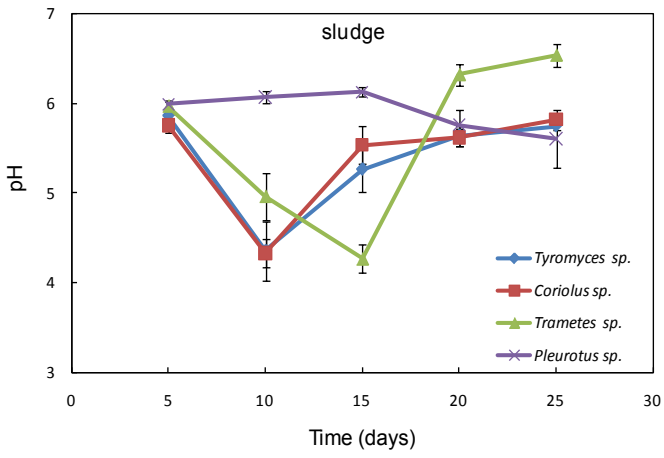

Figure 1: The pH value of the four white rot fungi in submerged fermentation process by using sludge as substrates. The pH of the medium was adjusted to pH 5.0 at the beginning.

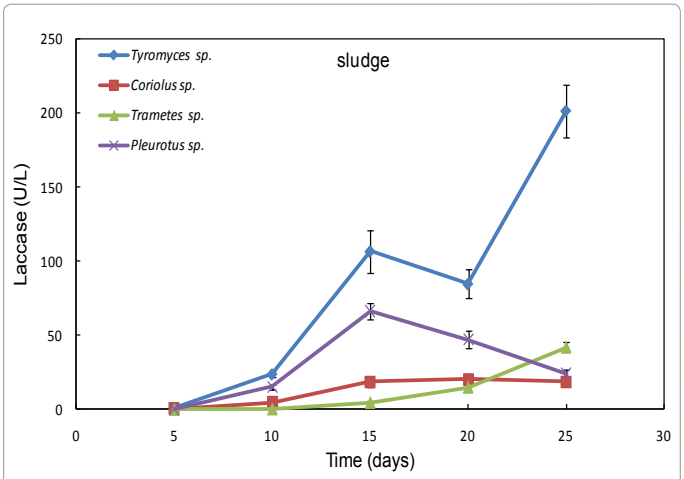

Figure 2: Laccase activities of the four white rot fungi in submerged fermentation using sludge containing media.

that maximum laccase activities were detected during the first 10 days of wheat straw incubation by *T. versicolor* (*C. versicolor*). This is because lignin enzyme production by WRF is highly dependent on species, strains, lignocellulosic substrates, medium and fermentation method [20] (Figure 2).

MnP activities

All four WRF secreted a notable quantity of manganese peroxidase (MP) (data not shown). 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. In Winquist's study, *C. versicolor* also produced relatively higher levels of MnP, compared with the other fungi including *Pleurotus ostreatus* DSM 11191, *T. hirsuta* K 21a and *Cerrena unicolor* T 71 [21]. The other three WRF studied here, produced MnP activities less than 2 U/L (Figure 3). Lower MnP activity in *T. albidus, T. gallica and P. ostreatus* could be due to the growth conditions such as medium composition. As in the study of Sun et al., 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 [16]. Laccase activities were found in the four cultivations. No LiP activities were detected in any of the four cultivations, consistent with Sun et al. [16]. MnP activity was detected only under HcLn. Similar results with low MnP activity were reported using other WRF. In these cases, WRF secreted high laccase activity but no MnP or trace amounts of MnP activities were detected by the majority of the fungi tested [22-24] (Figure 3).

LiP activities

No significant LiP activity was detected in any of the four WRF in submerged fermentation using paper mill sludge as the substrate. Studies have been shown that many WRF including *Pleurotus* species have been recognized to produce no typical LiP [25]. Additionally, LiP activity was not detected at all in medium cultured with *C. versicolor* using Olive mill wastewater as the substrate [26]. It has been suggested that LiP production is influenced by the C/N ratio [27]. It has been shown that some WRF are able to produce all different type of ligninase enzymes while other WRF only produces one or two types. Our results demonstrated that none of our four WRF secretes detectable LiP under the used experimental conditions.

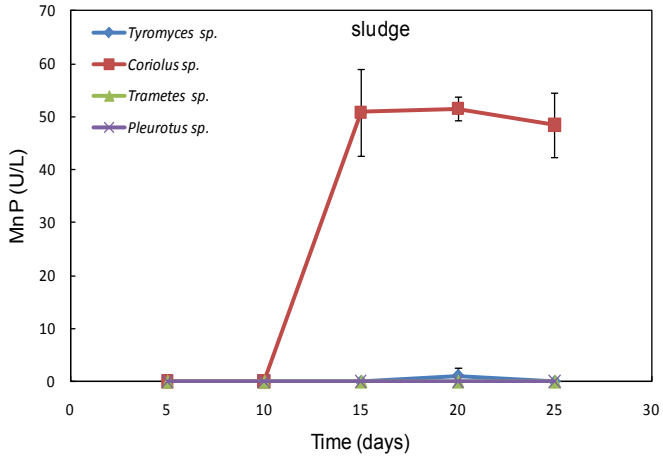

Figure 3: MnP activities of the four white rot fungi in submerged fermentation using paper mill sludge containing media.

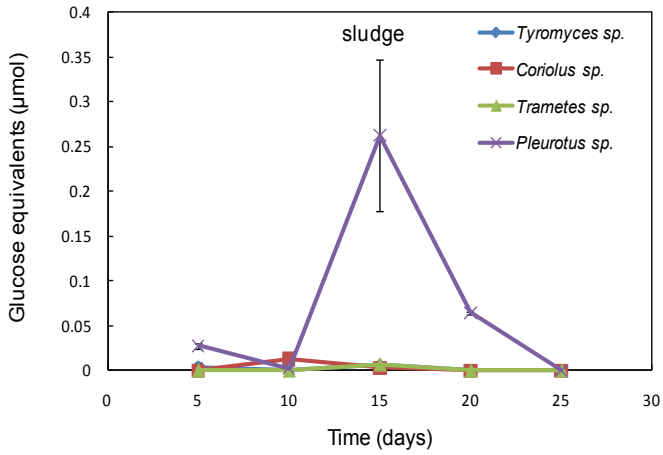

Figure 4: Total cellulase activities of the four white rot fungi in submerged fermentation using sludge containing media.

Total cellulase activities

Significant total cellulase activity was only detected in *P. ostreatus* (Figure 4). For the *P. ostreatus* the lowest values were observed in the first 10 days of incubation. *P. ostreatus* showed a maximum total cellulase activity of 0.26 μmol at 15 days of incubation. These relatively low values of cellulolytic activity were mainly due to the selectively use of lignin as the substrate by fungi. This is important, especially for the maximum lignin breakdown during pre-treatment using fungi (Figure 4).

Lignin content measurement

The acetyl bromide procedure for spectrophotometrically determining lignin in wood and wood pulp samples was used to measure the lignin content of paper mill sludge [17]. The lignin of woods and papermaking pulps contains the acid-insoluble (Klason) and acid-soluble lignin, the conventional method for lignin measurement uses sulphuric acid. This procedure requires large samples and complex steps. The acetyl bromide procedure only needs small samples and is very convenient. The lignin content can be dissolved in 25% acetyl bromide in acetic acid and then measured by absorbance at 280 nm. Our results showed that the lignin content of sludge samples treated by all the four WRF decreased after one month incubation (Figure 5).

FTIR analyses of untreated and pretreated paper mill sludge

The FTIR was used to qualitatively determine the chemical changes in sludge with or without WRF treatment. The FTIR spectra for WRF-treated sludge samples were compared to untreated sludge and shown in Figure 6, while the main assignments of functional groups in FTIR bands are listed in Table 1. All of the four WRF-treated sludge samples showed decreased adsorption in bands at 1,373 cm^{-1} –1,529 cm^{-1} compared to the untreated sample. The band at 1,512 cm^{-1} are attributed to aromatic skeletal stretching in lignin, and the bands at 1,424 cm^{-1} and 1,462 cm^{-1} are attributed to C–H deformation within the methyl groups of lignin. Our results suggested that lignin was removed in all four of the WRF-treated sludge samples.

This is a prescreening test about the waste degradation abilities of the four white rot fungi. The four white rot fungi are good lignin degraders, for future development of fungal bioreactor, more studies about the fermentation parameters, include incubation temperature, media, pH and so on are needed (Figure 6) (Table 1).

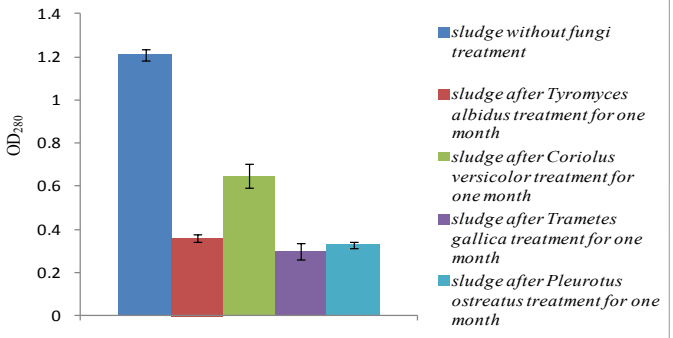

Figure 5: Lignin of paper mill sludge was degraded by white rot fungi (by acetyl bromide method).

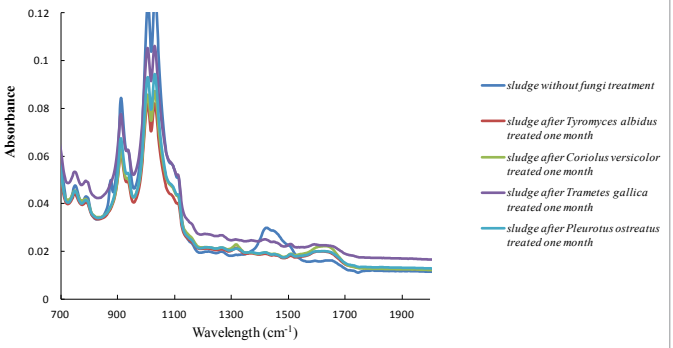

Figure 6: 1373-1529 cm-1 regions were all decreased after fungal treatment.

Band assignment	Wavenumbers (cm^{-1})
C=C of aromatic skeletal (lignin)	1512
C-H deformation in lignin and carbohydrate	1462
C-H deformation in lignin and carbohydrate	1424

Table 1: Main assignments of lignin FTIR bands of lime wood (Carmen et al, 2010).

Conclusions

The interest in utilizing ligninolytic enzyme producing fungi such as WRF for biological pretreatment of lignocellulosic residues has increased rapidly. In this study, PMS was used as the substrate for submerged fermentation cultures of four different WRF. All four WRF showed relatively high laccase activity whereas MnP activity was only remarkably produced by *C. versicolor*. Laccases are widely distributed in different WRF with high potential industrial applications. The cellulase production of the WRF was very low with the exception of *P. ostreatus*. The lignin contents of PMS-treated samples were significantly decreased after the treatment. This suggests the studied WRF are able to selectively degrade lignin.

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