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The Emerging Role of White Rot Fungus *Pycnoporus Species* in Degradation of Lignocellulosic Materials

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Abstract—*During pulp and paper production, enormous amount of lignocelluloses materials are utilized and generate high amount of effluent into the water bodies. This effluent mainly contains lignin and phenolic compounds. Lignin is highly resistant towards chemical and biological degradation. In this research paper, we examined the biological pretreatment of poplar wood chips with isolated white rot fungus. In this study, eight isolates of white rot fungi were screened for biological pretreatment of poplar wood chips. After enzymatic essay, amongst these white rot fungi, two strains of white rot fungi that can produce lignin modifying enzyme (LME), cellulase, laccase (Lac) and xylanase were screened and then one strain of them i.e., Pycnoporus cinnabarinus showed most excellent enzymatic activity, was selected to grow on poplar wood chips. The experiment was conducted in the solid media condition to achieve the higher rate of degradation at different time period. Initially the lignin and cellulose content was 21.6% and 40.6 %. After 45 days the total weight loss was 20.7% in solid medium, while lignin and cellulose content was 14.52% and 38.6% at the optimal temperature. The results were confirmed by Fourier transmission infrared spectroscopic and scanning electron microscope. The FTIR results showed that wood pretreatment in the solid condition, removed large amount of lignin. On the other hand, scanning electron micrograph revealed that the pores and cracks were developed during pretreatment due to deep penetration of fungus mycelium. Finally, we concluded that, isolated white rot fungus had a great potential to degrade the recalcitrant lignin material inside the wood chips.*

Keywords*: Poplar wood chips, biological pretreatment, lignincellulose materials, lignin degrading enzyme, FT-IR, SEM*

1. INTRODUCTION

Global consumptions of lignocellulosic materials have been continuously increasing through pulp and paper mills. It creates negative impacts on water bodies. During the pulp and paper production, high amount of waste water is generated. This effluent mainly contains organic and inorganic compounds, lignin and phenolic compounds **[15]** which are responsible for decreasing the water quality. Lignin is a persistent chemical, shows highly resistant towards degradation **[14].** In wood biomass, it is found in the middle lamella, where it works as cement between wood fibers. Thus, the present study is based on an alternate technique, called biological pretreatment of wood. White rot fungi are vigorous in nature and having extracellular lignin mineralizing enzymes (LME). During lignin degradation, the white rot fungi produce ligninolytic enzymes including Lignin Peroxidase (LiP), Manganese dependent Peroxidase (MnP), laccases (Lac) and H_2O_2 producing oxidases **[9].** Therefore, pre-treatment of wood chips with white rot fungi before pulping process is more significant as well as environmental concern**.** Some reported white-rot fungi are *Trametes versicolor, hanerochates chrysosporium, Phlebiopsis gigantu etc* which have great potential to degrade lignin as well as other persistent chemicals through biological treatment **[1, 10]**.

2. MATERIALS AND METHOD

2.1 Materials

Poplar wood chips were collected from Lalkuan pulp and paper mill (Uttarakhand, India) and air dried wood chips were cut in to pieces from 0.5cm to 2.5cm, stored in polythene bags at 25^0 C.

2.2 Inoculums preparation

For fungus isolation, eight fungal fruiting bodies were collected from decayed wood from A.F.R.C. (Agro-Forestry Research Center, Pantnagar, Uttarakhand). Fruiting bodies were sterilized with 0.1% Mercuric chloride ($HgCl_2$) solution, and then frequently washed with distilled water. The fruiting bodies were inoculated in sterilized petri plates containing PDA (potato dextrose agar) media and incubated for 3-7 days at 28^0 C temperature.

2.3 Qualitative enzyme essay

Screening of enzymatic activity was done by substituting the malt extract agar medium (3%) with enzyme substrate tannic acid **[3].** Prepared Lignin basal media (LBM) was supplemented with 1.6% w/v agar and autoclaved. Add 1 ml

of sterilized 20% aqueous glucose solution and 1 ml of 1% w/v aqueous tannic acid solution. Lignin modifying enzyme (LME) production was recorded as the appearance of brown color zones around the fungal colony.

2.3.1 Cellulase assay

Prepared cellulose basal media (CBM) medium was supplemented with 2% w/v low viscosity Carboxyl Methyl Cellulose agar (CMC) and 1.6% w/v agar and then autoclaved. When the colony diameter reached 30 mm, the agar plates were stained by congo red and poured off after fifteen minutes and then flooded with 1M NaCl. It was observed that yellow opaque area against a red color CMC**[11, 13]**.

2.3.2 Xylanase assay

For essay of xylanase enzyme, xylan basal media (XBM) medium was prepared. Added 4 % w/v xylan and 1.6 % w/v agar and autoclaved. Inoculated with isolated fungus and incubated at 25^0C in darkness. When colony diameter reached 30 mm (2-5 days) then stained agar plates with iodine solution and left for 5 minutes and then it was washed with distilled water. Confirmation was observed a yellow opaque zone against a blue/ reddish purple color for undegraded xylan **[11, 13]**.

2.3.3 Laccase assay

Lignin basal media (LBM) was prepared and add 0.01 % w/v Azure B and 1.6 % w/v agar. Add 1 ml of a separately sterilized 20 % w/v aqueous glucose solution and inoculated with isolated fungus culture incubated for 10 days at 250C. The development of an intense bluish-violet color around the wells was considered as a positive test for laccase activity**[11, 13]**.

3. INOCULATION OF ISOLATED FUNGAL STRAIN

Morphological characterization was done in Department of Plant Pathology, G.B. Pant University of Agriculture & Technology, Pantnagar. The isolated fungal strain was *Pycnoporus species*. Experiment was carried in a 250 ml conical flask with 5 g of wood chips under 20 ml of distilled water for 24 hrs. These samples were sterilized in the autoclaved for 15 min at 121°C. The *Pycnoporu*s fungal culture was incubated in solid culture media (PDA)-supplemented with nutrient media(MSM) at 28^0C for 15, 30 and 45 days. The non-inoculated samples were served as the control. All experiments were performed in triplicate manner.

4. WEIGHT LOSS (%) EXPERIMENT

After completion of incubation period the samples were dried for 48 hrs at 80^oC. Dry weight of samples (control and treated) were measured.

$$\text{Wood loss (\%)} = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \times 100$$

5. CELLULOSE AND LIGNIN CONTENT (%)

For cellulose content, 1 g oven dried wood was taken and then add 15ml of 80% acetic acid and 1.5ml concentrated nitric acid and refluxed for 20 min, then filtered. Collected residue was washed with ethanol, dried in oven at 100-105^0C, finally weighed and labeled sample A. Then sample A was incinerated at 540^0 C and labeled sample B.

$$\text{Cellulose content (\%)} = \frac{sample\ A - sample\ B}{linital\ weight\ of\ sample} X100$$

Lignin content in poplar wood was determined by klason method, which is based on hydrolysis and solubilization of cellulose and hemicelluloses. 1g of grinded wood samples was taken and added 72% sulfuric acid. Then final hydrolysis was made with 3% sulfuric acid, and then acid-insoluble (i.e Klason) lignin washed with distilled water, dried, and measured. Acid-insoluble (Klason) lignin contents were calculated in the samples by using the following equation **[5].**

$$\text{Lignin content (\%)} = \frac{\text{Weight of lignin}}{\text{linital weight of wood chips}} X100$$

6. CHEMICAL ANALYSIS

It was done by Scanning Electron Microscope (SEM) [8, 9] and FT-IR Alpha Sample compartment (Bruker Optik GMbH) **[12]**

7. RESULTS AND DISCUSSIONS

Among the eight fungal strains only two fungal strains (WRF-3 and WRF-5) were given lignin modifying enzyme (LME) positive test (Table1). The LME activity could not be taken alone to confirm the production of lignin degrading enzyme **[2].** Further these strains were assessed for the production of laccase, peroxidase, xylanase and cellulase. Maximum production of laccase was given by WRF-5 followed by xylanase and cellulase but the production of these enzymes were absent in WRF-3 fungal strain while the production of peroxidase enzyme was negligible in both the strains. Results showed that lignin degradation was based on high laccase activity than MnP activity. Thus, it is not necessary, the production of all ligninolytic enymes by some white rot fungi **[3].**

Table 1: Ligninolytic Enzymes essay

Enzymes	WRF-5	WRF-3
LME (Bavendamm test)	+ +	+ +
Laccase	+ + + +	-
Cellulase	+	+ +

Xylanase	++	-
Peroxidase	-	-

No of + sign indicates highly positive test, - sign indicates absence of enzyme

It was concluded that white rot fungus *Pycnoporus sp.* produced excellent laccase activity but did not show LiP and MnP activity **[4].**

7.1 Effect of pretreatment of wood

In solid state condition the most of the wood chips colonized by mycelia of fungal strain, Initially the lignin and cellulose content was 21.5% and 40.6 %. After 45 days the total weight loss was 20.7% in solid medium, while lignin and cellulose content was 14.52% and 38.6% at the optimal temperature.

Table 2. Weight loss (%), lignin and cellulose content (%) in different time period

Medium	Decay time (day)	Weight loss (%)	Lignin content %	Cellulose content %
Solid media (PDA)	0	0	21.5 ±0.08%	40.6±0.12
	15	16.4±0.12%	16.4±0.07%	44±0.17
	30	18.3±0.11%	15.3±0.05%	41.6±0.07
	45	20.7±0.13%	14.5±0.01%	38.6±0.10

*Values are given as mean ± S.E.

7.2 SEM analysis

The morphological characterization of control and treated wood samples were observed using SEM. SEM micrograph shows that inner layer of cell of the controlled wood chips is undisturbed by *Pycnoporous sp*. **(Figure 1).** The lumina of vessel and parenchyma cells is fully colonized with the *Pycnoporous sp* after 45 days due to presence of large open spaces [7]. Hence, the structure of parenchyma cells were changed after the treatment with *Pycnoporous sp* [6, 8].

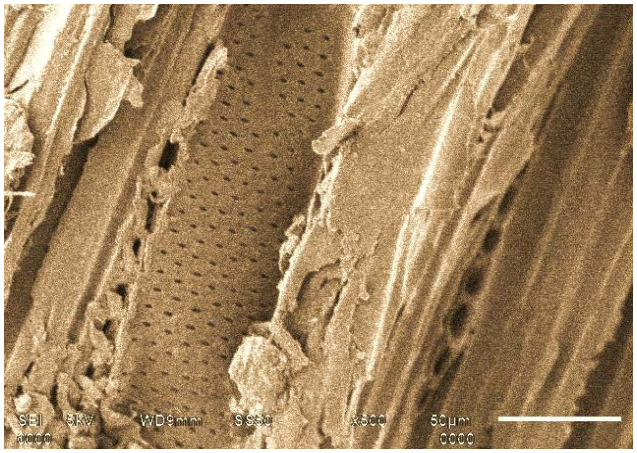


Fig 1. L.S of untreated poplar wood chips

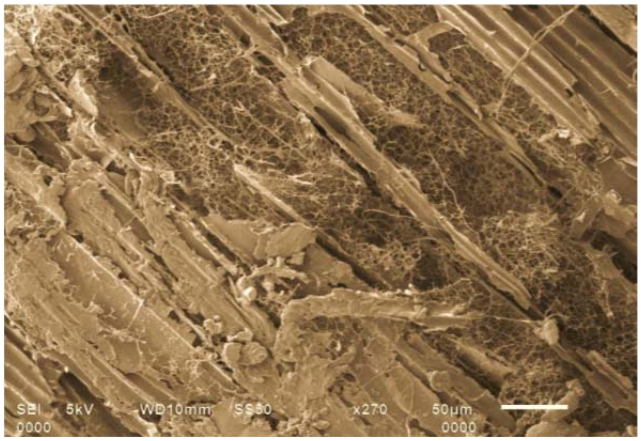


Fig 2. L.S of treated poplar wood chips

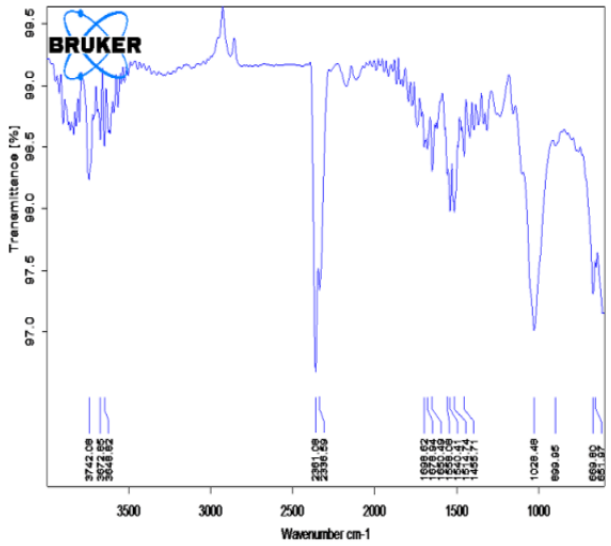
7.3 FT-IR analysis

For qualitative and quantitative changes in lignin and carbohydrate component is determined with FTIR. Control and treated wood chips were recorded with FTIR instrument over a frequency range from 400 to 4000 cm-1. In untreated wood chips, appearance of the bands in the range of 1,260–1,270 and 1,330–1,375 cm−1 shows the existence of Guaiacyl and Syringyl units in lignin (Table 3 & Figure3b). Pretreatment with *Pycnoporus sp*. the peaks gradually disappeared and converted this peak into flat peak of band in the range 1330– 1375 cm^{-1} and 1,260–1,270 cm^{-1} which indicate degradation of Syringyl and Guaiacyl units by *Pycnoporus sp*. It was concluded *Pycnoporus sp*. possesses great potential to degrade the lignin and its monomers. The most representative FTIR bands are summarized in Table

3.

Table 3. Characteristic bands of FT-IR spectra observed in control and treated sample

Functional group (800-1800 cm^{-1})	Control sample (wavelength cm^{-1})	Treated sample (wavelength cm^{-1})
Phenolic ring	1515, 1650	-
Lignin (aromatic ring)	1514, 1540, 1558	1595
Guaiacy	1226-1260	1232
Syringyl	1320-1360	1320- 1368
Cellulose	1027-1030, 900	899.39, 1030, 1155



a)

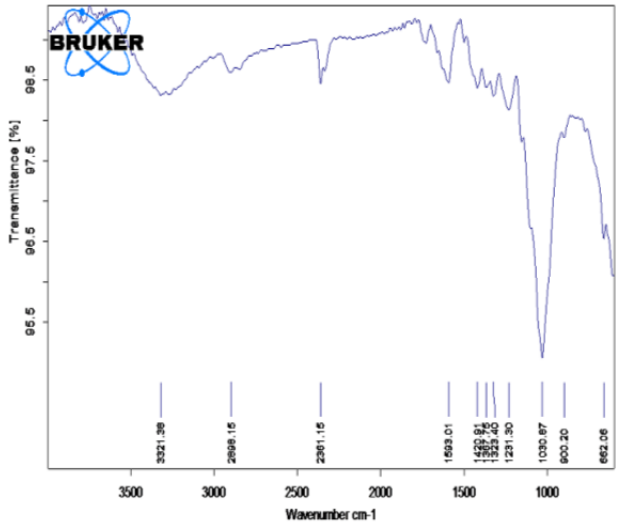


Fig. 3 FT-IR spectra: a) Untreated b) Treated wood chips in solid media

8. CONCLUSION

In this research work we tried to suggest a method how to evaluate the fungal activity and growth pattern to make a successful fungal pretreatment of wood.This study reports that how the composition and structure of lignocellulose in poplar wood changed during white rot fungus treatment. The results showed that *Pycnoporus sp*. Can be used in pre-treating of wood chips before pulping. In the results of the experiments, it has excellent colonization and delignification capabilities on solid media. By FT-IR analysis confirmed that lignin present in the untreated poplar wood chips was either disappeared or transformed into the simpler compounds resulted in the change of structure of poplar wood into the degraded wood. Therefore, it is considered that *Pycnoporus sp*. is the most effective fungus in lignin biodegradation.

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