

(An ISO 3297: 2007 Certified Organization) Website: <u>www.ijirset.com</u> Vol. 6, Issue 7, July 2017

Importance of Lignin Modifying Enzymes from Isolated White Rot Fungus in Lignin Degradation

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ABSTRACT: Lignin modifying enzymes is commonly found in white rot fungus. It decides the lignin degradation capability of white rot fungus. In this study lignin degrading fungus was isolated from decaying wood logs. After the screening the white rot fungus was identified as a *Trametes sp*. Their lignin degrading ability was tested with laccase assay, xylanase assay, and CMC assay. It was found that isolated fungus was efficient to use in biological pretreatment of wood chips. The whole experiment was conducted under two condition , one is the potato dextrose broth i.e. liquid media (PDB) and other is liquid + shaker (PDB+120 RPM) 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 lignin content was 9.8% and 15.8% while and cellulose content was 39.4% and 40.2% at 28°C. The results were confirmed by Fourier transmission infrared spectroscopic (FT-IR) showed that wood pretreatment in the liquid condition, removed large amount of lignin than shaken condition. Because of deep penetration of fungus mycelium inside the wood cell was indicated that complex structure of lignin was changed into their monomers and simple derivatives. So finally we concluded that, *Trametes sp*. had a great potential to degrade the recalcitrant lignin material inside the wood chips.

KEYWORDS: Lignin, White-rot fungus, Lignin peroxidase, Manganese peroxidase, Laccase, FT-IR.

I. INTRODUCTUON

The potential applications of lignin modifying enzymes have become more attractive and provide environmentally friendly technologies for the pulp and paper industries. The pulp and paper mills have been using large amount of lignocellulosic materials for the paper production subsequently discharging enormous amount of waste water in the water bodies. Effluent which comes from paper mills, contain high amount of lignin and other aromatic chemicals. Lignin biodegradation because of its complex structure and macromolecular features is complicated. Lignin is regarded as the most persistent and recalcitrant compound because of its complex structure (Li J, et al., 2009) which contain both non phenoloic and phenolic compounds. It has been cleared that there is no unique mechanism to achieve the process of lignin degradation except biological pretreatment. Many fungi and microorganisms have ability to decrease lignin by producing enzymes. Out of these, white rot fungi play major role in lignin degradation by producing extra cellular lignin degrading enzymes (Akhtar, M., 2000) viz. lignin peroxidase, which catalyses the oxidation of both phenolic and non-phenolic units, manganese-dependent peroxidase, laccase, which oxidizes phenolic compounds to give phenoxy radicals and quinones; glucose oxidase and glyoxal oxidase for H_2O_2 production (Hatakka, A., 1994).

The various white rot fungi have been reported such as *Pleurotus ostreatus, manganese peroxidase, Pycnoporus cinnabarinus, Phanerochates chrysosporium, Phlebiopsis gigantean* that produce laccase (Duque, et al., 2016 & Akhtar, et al., 1998, 2000). This paper describes that lignin degradation is influenced by different environmental factor such as growth rate of the fungus, temperature, aerobic condition, nutrient sources play major role in lignin degradation. In this



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study, we have focused on lignin degradation under nearly natural conditions, i.e., on wood chips using isolated white rot fungus, and essay of lignin modifying enzymes.

II. MATERIALS AND METHODOLOGY

- A. Sample Collection: The raw material i.e. poplar wood chips were collected from pulp and paper mill (Uttarakhand, India) and cut in to pieces from 0.5cm to 3 cm. The wood samples were stored in polythene bags at 25^o C. For fungus isolation, fungal fruiting bodies were collected from decaying wood logs and sterilized with 0.1% Mercuric chloride (HgCl₂). They were inoculated in sterilized petriplates containing PDA (potato dextrose agar) media and incubated for 3-7 days at 28^o C temperatures.
- B. Identification of fungus: Morphological characterization was done from the Department of Plant Pathology, G.B. Pant University of Agriculture & Technology, Pantnagar. A clean grease free slide was taken and a few drops of phenol cotton blue solution were put on it. Sterilized needle was taken to transfer fungus culture and pressed it gently and covered with cover slip. The slides were then observed under low (40X) to high power (100X) objectives of microscope.
- C. Screening of lignin modifying enzyme activity: it was done by Tannic acid agar (Bavendamm test). Prepared lignin basal medium (LBM) was supplemented with 1.6% w/v agar and autoclaved. Added 1 ml of separately sterilized 20% aqueous glucose solution and 1 ml of 1% w/v aqueous tannic acid solution. The medium was transferred aseptically into the petriplates and inoculated with isolated fungus after solidification of medium. The petriplates were incubated at 25 °C in darkness and examined plates daily for 10 days. Ligin modifying enzyme (LME) production was recorded as the appearance of brown oxidation zones around the colony (Bains, et al., 2006).
- D. Screening for laccase activity: The laccase plate assay was allowed rapid determination of the presence of laccase in the extracellular fluid. The media with the following ingredients: Glucose 1g, yeast Extract 0.1g, peptone 0.5 g, Agar 1.6 % w/v and α napthol 0.05 g; distilled water 1000ml. 100 ml of media was prepared and autoclaved at 121°C at 15 psi. The media was cooled at room temperature before pouring in sterile Petri plate. After pouring the media isolated fungus was inoculated and was incubated for 48 hours and the development of an intense bluish-violet color around the wells was considered as a positive test for laccase activity.
- E. Screening of cellulase activity: It was done by Congo red plate assay. Congo-Red agar media with the following composition: KH₂PO₄ 0.5 g, MgSO₄ 0.25 g, cellulose 2 g, agar 15 g, Congo-Red 0.2 g, and gelatin 2 g; distilled water 1000ml. 100ml of media was prepared and autoclaved at 121°C at 15 psi. The media was cooled at room temperature before pouring in sterile petriplate. After pouring the media with the help of sterile gel puncher small wells were made on the plates. The spore suspensions of the isolated microorganism were prepared and were inoculated on to the well and were incubated for 48 hours and the colour change of Congo red plate was observed (Levin, et al., 2013 & Buswell, et al., 1996)
- F. Inoculation of isolated fungal strain: This 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 at 121°C at 15 psi. The *Trametes* fungal culture was incubated in liquid media (PDB)-supplemented with nutrient media (MSM) and liquid plus shaker (120 RPM) at 28°C for 15, 30 and 45 days. The non-inoculated samples were served as the control. All experiments were performed in triplicate manner.
- G. Weight loss (%) experiment: After completion of incubation period the samples were dried for 48 hrs at 80°C. Dry weight of samples (control and treated) were measured.

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



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H. Lignin content (%): It was determined by klason method. This method is based on hydrolysis cellulose and hemicelluloses in the poplar wood chips. For calculating lignin content, 1g of grinded wood samples was taken and added 72% sulfuric acid. Then final hydrolysis was made with 3% sulfuric acid, and then acidinsoluble (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 [TAPPI, 2011].

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

I. Chemical Analysis: The chemical and structural changes were observed by FT-IR Alpha Sample compartment (Bruker Optik GMbH) (Panday and Pitman, 2003).

III. EXPERIMENTAL RESULTS AND DISCUSSION

A. Identification of lignin degrading fungus: The lignin degrading white rot fungus identified as *Trametes sp.* on the basis of lactophenol cotton blue staining.

Characteristics	Results			
Colony Characterization	Moist and cottony			
Colony Margin	Entire			
Growth	Fast			
Microscopic Characteristics	Septate, branched and filamentous structure,			
	nonsporulation fungus			
LME (Bavendamm test)	++			
Laccase	++++			
Cellulase	+			
Xylanase	+++			
MnP	-			

Table 1. Physiological and biochemical characteristic of white rot fungus

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

The isolated white rot fungal strains 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 (Bains, R., 2006). Further these strains were assessed for the production of laccase, cellulase and xylanase. Maximum production of laccase was given by Tramets sp. followed by xylanase. Results showed that lignin degradation was based on high laccase activity than MnP activity.



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A. Pure form of isolated white fungus



C. Laccase activity



B. LME activity



D. Cellulase activity

Fig 1. Lignin modifying enzyme assay in isolated white rot fungal

B. Effect of temperature and nutrient media: The temperature and nutrient media on growth of white rot fungus was examined in the MEA and PDA medium. Isolated fungus strain was grown on MEA and PDA plates and incubated for 7 days at different temperatures at 5°C, 28°C and 50°C (Figure 2) Results were indicated that higher growth rate was observed at temperature 28°C in MEA media than PDA (Figure 2) (Kim, et al., 2005). At 5° C temperatures the fungus strain did not dead but the growth rate was too slow. Thus, the isolated fungus strain was mesophiles.



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Table 4: Growth pattern of *Trametes sp.* on the two culture media at different temperatures

Fungus strain	Temperature	Media	Growth diameter (mm)
White rot fungus	$5^{\circ}C$	PDA	17.3±1.2
(Trametes sp.)		MEA	19.7 ± 1.4
	28 ⁰ C	PDA	78.0±16
		MEA	83.3±1.2
	50 ° C	PDA	26.3±1.5
		MEA	46.7±.1.1

Values are given as mean \pm S.E.

C. Effect of biological pretreatment of wood: In liquid the media, mycelia of *Trametes sp.* was fully colonized on wood chips. It was found that the initial lignin and cellulose content was 21.5% and 40.6%. After 45 days the total weight loss was 33.5% in liquid medium, while lignin and cellulose content were 9.8% and 39.4% at the optimal temperature.

Thus, liquid state was more capable to degrade poplar wood chips because of uniform, highly extensive dispersion of inoculums in media which provided nearly natural habitats (Kim, et al., 2005). On the other hand, in shaken media, total weight loss was 24.6 %, while lignin and cellulose content was 15.8 % and 40.2 % at the optimal temperature. Thus, it was observed that liquid + aeration (120 RPM) did not provide uniform dispersion of fungal inoculums in the media throughout the experiment as well as improper attachment of fungal mycelium which showed that no deep was penetration was occurred inside the wood cell.

Medium	Decay time (day)	Weight loss %	Lignin %	Cellulose %
Liquid media	0	0	19.5±0.08	48.4±0.12
•	15	13.4±0.14	15.4±0.05	46.2±0.10
	30	27.6±0.14	13.5±0.04	42.5±0.12
	45	33.5±0.11	9.8±0.03	39.4±0.13
Shaken media (120 RPM)	15	12.2±0.10	17.5±0.03	46.2±0.15
	30	24.6±0.13	15.9±0.05	41.6±0.17
	45	32±0.15	12±0.02	40.2±0.16

Table 3. Weight loss (%), lignin and cellulose content (%) in different time period

D. FT-IR analysis: For qualitative and quantitative changes in lignin was assured by FTIR spectroscopy. Untreated and treated wood chips were recorded over a frequency range from 400 to 4000 cm⁻¹. The most degraded sample had smallest absorbance and least degraded sample showed highest absorbance (Pandey and Pitman, 2003). The sharp peaks were detected in untreated wood chips, which were disappeared after degradation of *Trametes sp*. The characteristic peaks of lignin and carbohydrates were found in the range of 1800 -700 cm⁻¹. Major signifying peaks of the lignin in untreated wood were present in the range at 1558, 1514.74, 1540, 1455cm⁻¹ due to raised aromatic skeletal vibration (C=C the lignin while at 1260–1270 and 1330–1375 cm⁻¹ showed the existence of guaiacyl and syringyl groups in the lignin. The gradually disappearing of peak band in the range 1330–1375 cm⁻¹ and 1260–1270 cm⁻¹ indicated the degradation of syringyl and guaiacyl groups. The most representative FTIR bands are summarized in Table 4.



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Results obtained from 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 changes of structure of poplar wood into the degraded wood. Finally, it observed that, treatment with *Trametes species* the peaks of lignin at 1800-500cm⁻¹ declined gradually in liquid static media, followed by shaken media. The peaks of lignin at the range of 1500- 1200 cm⁻¹ significantly declined in the liquid static media. Thus, *Trametes sp.* had shown great potential to reduce lignin and simultaneously reduced some amount of cellulose and hemicelluloses (Pandey and Pitman, 2003).

Table 4. Characteristic bands of FT-IR spectra observed in control and treated sample

Functional group (800- 1800 cm ⁻¹)	Control sample (wavelength cm ⁻¹)	Liquid culture media (wave length cm ⁻¹)	Liquid + shaken media (wave length cm ⁻¹)
Phenolic ring	1515, 1650	-	1513,1645-1670
Lignin (Aromatic ring)	1514, 1540, 1558	1595.94	1513,1546
Guaiacy	1226-1260	1240	1236.54
Syringyl	1320-1360	1320	1317.33
Cellulose	1027-1030, 900	899.39, 1030, 1155	1027.39, 1155, 837





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Fig. 3 FT-IR spectra: (a) Untreated; (b) Treated wood chips in liquid media; (c) liquid + shaker (120 RPM).



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IV. CONCLUSION

- 1. The current study was based on the comparative study of lignin degradation using isolated white rot fungus in different medium i.e., liquid (PDB) and shaken media. It was found that *Trametes sp.* showed the highest lignin degradation activity in liquid (PDB) + static condition when compared with shaken media.
- 2. In this paper we tried to suggest a alternative method, how fungal activity and growth pattern was made a successful biological pretreatment of wood.
- 3. Results obtained from FT-IR showed that lignin present in untreated poplar wood chips was either disappeared or transformed into the simpler compounds.
- 4. Therefore, it was concluded that *Trametes sp.* had excellent colonization and delignification capabilities on a different media culture. Therefore, it was considered as a most effective fungus in lignin degradation in pulping process.

V. AKNOWLEDGEMENT

The author is thankful to Dr. J.P.N. Rai Professor and H.O.D, Department of Environmental Sciences, G.B.P.U.A & T, Pantnagar for his salutary advice and kind co-operation.

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