Eco. Env. & Cons. 30 (Nov. Suppl. Issue) : 2024; pp. (S77-S83)
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ISSN 0971–765X

DOI No. http://doi.org/10.53550/EEC.2024.v30i07s.015

Evaluating the Efficacy of Biopulping on Poplar Wood Chips Using the White-rot Fungus *Trametes* SP.

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(Received 7 April, 2024; Accepted 13 June, 2024)

ABSTRACT

This study evaluated the novel application of the white-rot fungus *Trametes* sp. for biopulping of poplar wood chips as an environmentally-friendly alternative to chemical treatments. The isolated fungal strain *Trametes sp.* exhibited selective lignin degradation capabilities with high laccase production along with moderate xylanase and cellulase activities. The present experiment was conducted in three different mediums *i.e.* Solid (PDA), liquid (PDB), and liquid (PDB) + aeration. Among the mediums, solid-state conditions proved optimal and achieving 35.8% weight loss of poplar wood chips after 60 days treatment, significantly higher than PDB and PDB + aeration conditions. However, the lignin content decreased by 9.3 %, the cellulose remained largely intact at 38.9 % under solid culture, indicating the fungus's selectivity towards delignification over cellulose degradation. The scanning electron microscopy (SEM) visually confirmed the colonization and disruption of wood vessels and parenchyma cells by the fungal hyphae. The fourier-transform infrared spectroscopy (FTIR) analysis provided molecular evidence of substantial lignin degradation through diminished peaks corresponding to aromatic lignin components like guaiacyl and syringyl after fungal treatment. This pioneering use of *Trametes* sp. demonstrates promising biopulping potential by selectively removing lignin from wood chips while preserving cellulose. These results valorize this fungal strain for future industrial applications.

Key words: *Biopulping, White-rot fungus, Poplar wood chips, SEM, FTIR*

Introduction

Pulp and paper mills are significant contributors to water pollution, utilizing vast quantities of lignocellulosic materials in paper production (Bisht *et al.*, 2024). These materials, derived from forests, wood, and agro-industrial residues, are an affordable energy source with numerous applications (Junxian, *et al.*, 2023). The lignocellulose complex, which is a carbon-neutral resource, comprises three biopolymers: cellulose, hemicellulose, and lignin, along with some extractives and minerals (Su *et al.*, 2023). Lignin, a complex and hydrophobic component of the plant cell wall, is primarily found in the secondary cell wall (Ding *et al.*, 2006; Su *et al.*, 2023). It provides structural support, mechanical strength, and resistance against microbial attack (Junxian *et al.*, 2023). During conventional pulping processes, the resulting wastewater is heavily polluted, with high levels of biochemical oxygen demand (BOD), chemical oxygen demand (COD), and dissolved solids, mainly due to alkali-lignin and polysaccharide degradation residues. The untreated effluents, particularly lignin and phenolic compounds, significantly harm water quality (Bisht *et al.*, 2023).

Biopulping, which employs white rot fungi, offers environmental benefits by reducing the need for large quantities of potentially hazardous chemicals

in paper production. This process involves using white-rot fungi to biodegrade wood chips and other lignocellulosic materials before mechanical or chemical pulping (Singh *et al*., 2010; Junxian *et al*., 2023). *Trametes* sp. is versatile white-rot basidiomycetes showing a simultaneous degradation of lignin and holocellulose, that leaves the cells either pierced with bore holes or with thinned secondary walls (Shao *et al*., 2022). It is easily available and fast-growing, with the ability to use various lignocellulose materials as substrates. The ligninolytic enzymes of *Trametes sp.* have a broad range of commercial applications in the pulp and paper industry, such as bio-bleaching and biopulping (Junxian *et al.,* 2023). These fungi possess unique oxidative ligninolytic enzyme systems, as well as cellulases, lignin peroxidase (LiP), manganese peroxidase (MnP), laccase and xylanase, which enable them to degrade wood polymers (Giap *et al*., 2022). Various species of white rot fungi have been used for biopulping *Pycnoporus cinnabarinus*, *Pleurotus ostreatus*, *Phlebia radiata* (Junxian *et al.,* 2023). Therefore, the purpose of this preliminary research was to study effects of fungal treatment, *i.e. Trametes sp.* on structural and chemical properties of poplar wood chips. Also, this work highlights the production pattern of laccase, manganese peroxidase, lignin peroxidase, and cellulase enzymes during the lignin degradation activity under the three different conditions (solid, liquid and liquid + aeration) for 60 days. The chemical characterization of degraded wood chips were analysed by scanning electron microscopy (SEM) and fourier-transform infrared spectroscopy (FTIR).

Materials and Methods

Chemicals required

Potato dextrose agar (PDA), potato dextrose broth (PDB), D-glucose, ammonium nitrate, (NH_4NO_3), magnesium sulfate ($MgSO_4$), calcium chloride ($CaCl_2.H_2O$), mineral solution, vitamin solution, trace elements solution, EDTA, zinc sulfate ($ZnSO_4$), iron sulfate ($FeSO_4$), manganese chloride ($MnCl_2$), trace elements, acetic acid, ethanol, poplar wood chips and distilled water.

Collection and isolation of *Trametes* sp.

Fungal fruiting bodies of white rot fungus were gathered from decayed wood trees at the Agroforestry Centre. These samples were appropriately labeled and transported to the laboratory in paper bags. The fruiting bodies underwent sterilization, washing, and were then inoculated onto Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) media under sterile conditions at 28 °C for 3-7 days. The collection of poplar wood chips was done from a local pulp and paper mill industry located in Lalkuan, Uttarakhand, India.

Enzyme assay

The Bavendamm test, involving tannic acid agar, was used to detect lignin-modifying enzymes (LMEs) by observing brown oxidation zones around the fungal colony, indicating polyphenol oxidase activity (Bains *et al.,* 2006). Similarly, carboxyl methyl cellulose (CMC) agar was used to detect cellulase activity, with the formation of dye-stained zones indicating cellulolytic ability. However, xylanase activity was analyzed by iodine staining and the laccase plate assay was employed to detect laccase enzyme by showing an intense bluish-violet color around the colony. Lignin peroxidase and manganese peroxidase activities were detected by observing the clearance of the blue color in an Azure-B agar medium (Asgher *et al.,* 2010).

Preparation of raw materials

For the experiments, poplar wood chips were used as the raw lignocellulosic material. The chips were chopped into pieces ranging from 0.5 to 1 cm in size. A 5 g sample of the chopped poplar wood was soaked in 20 mL of distilled water for 48 hours to rehydrate the material. After soaking, the wood chips were dried in an oven at 60 °C and then autoclaved at 121°C for 15 minutes for sterilization. To determine the most favorable conditions for lignocellulose degradation, three different culture media compositions were evaluated as given below:

1. Solid culture media (PDA)+ supplemented with nutrient media
2. Liquid culture media (PDB)+supplemented with nutrient media
3. Liquid culture media (PDB) + Aeration (120 RPM)

Wood loss (%)

The raw material was incubated for 20, 40, and 60 days with isolated fungal stain *Trametes sp.* After incubation period raw material were taken out, oven dried for 48 hrs at 80^{o}C and the dry weight was mea-

sured. These samples were then used for lignocelluloses degradation analysis.

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

Cellulose content (%)

The cellulose content in degraded wood was determined by treating 1 g of oven-dried sample with 80% acetic acid and concentrated nitric acid, followed by refluxing, filtering, and washing with ethanol. The residue (Sample A) was weighed, incinerated at 540 °C (Sample B), and the cellulose content calculated from the weight difference between Samples A and B (Gopinathan *et al*., 2011).

$$\textit{Cellulose content \%} = \frac{\textit{Sample A} - \textit{Sample B}}{\textit{Initial dry weight sample}} \times 100$$

Lignin content (%)

The Klason method involved the hydrolysis and solubilization of cellulose and hemicellulose components from extracted wood or pulp samples using 72% sulfuric acid. The acid-insoluble fraction, known as Klason lignin, was then washed with distilled water, dried, and quantified gravimetrically. The acid-insoluble (Klason) lignin content in the samples was calculated using the following equation (Tappi, T222. 2011):

$$\textit{Lignin content (\%)} = \frac{\textit{Weight of lignin(g)}}{\textit{Oven dry weight of wood (g)}} \times 100$$

Chemical analysis of degraded wood

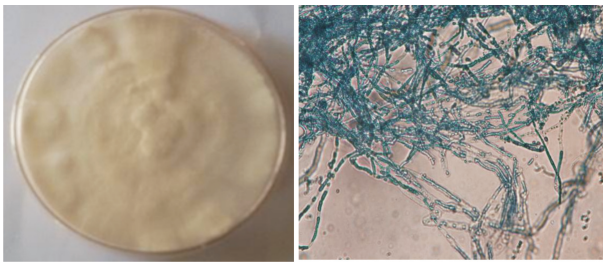
After the incubation period, the surface morphology and chemical composition of both the control (untreated) and treated poplar wood chips were analyzed using SEM and FTIR.

Result and discussion

The morphological characterization of the fungal strain of *Trametes* sp. was evaluated using a 7-day-old culture. Lacto-phenol blue staining revealed septate, filamentous, and branched hyphae under the microscope (100X) (Fig. 1a & 1b).

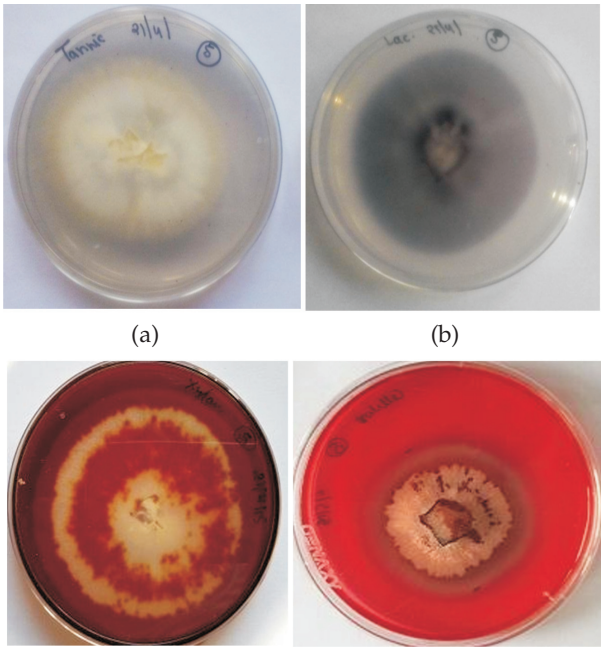
Screening of fungal strains for ligninolytic enzyme assay

The isolated fungal culture was screened for their ability to produce ligninolytic enzymes by incorporating the relevant substrates into the culture media plates. The initial screening for LME activity was conducted using the Bavendamm test, where the appearance of a brown color zone around the fungal colony indicates the presence of LMEs (Gopinathan *et al*., 2011; Su *et al*., 2021). However, LME activity alone is not sufficient to confirm the production of lignin-degrading enzymes (Bains *et al*., 2006). Therefore, these strains were further evaluated for the production of specific enzymes such as laccase, peroxidase, xylanase, and cellulase. The isolated *Trametes* sp. exhibited maximum laccase production, followed by xylanase and cellulase (Fig. 2). Goud (2011) found that significant lignin degradation was



(a) (b)

Fig. 1. The isolated fungal culture of (a) *Trametes* sp.; (b) Mycelia of isolated fungus examined under microscope at 100X



(a) (b)

(c) (d)

Fig. 2. Enzyme assay for degrading the lignocellulosic materials; (a) LME assay; (b) laccase assay; (c) cellulase assay; (d) xylanase assay

associated with high laccase activity rather than manganese peroxidase (MnP) activity. The white-rot fungus *Pycnoporus cinnabarinus* produced excellent laccase activity but not LiP or MnP. Similarly, *Trametestrogii*, another white-rot fungus involved in wood decay, produces several ligninolytic enzymes, with laccase being the dominant one. These findings suggest that the production of all ligninolytic enzymes is not essential for lignin degradation by white-rot fungi (Chuku *et al.*, 2021).

Effect of temperature and nutrient media on growth

Environmental conditions such as temperature, culture media composition, moisture content, and aerationsignificantly influence fungal growth and the ability to degrade wood. The effect of temperature and nutrient media on the growth of the fungal strain *Trametes* sp. was investigated in the present study using malt extract agar (MEA) andPDA media. *Trametes sp.* was grown on these media plates and incubated for 7 days at temperatures of 5 °C, 20 °C, 30 °C, and 40 °C. The results revealed that *Trametes* sp. exhibited a higher growth rate i.e. 37.7 mm at 30 °C on MEA compared to PDA (35.6 mm). Interestingly, at 40 °C, the fungal strain did not die but displayed a significantly slower growth rate (Table 1). These findings suggest that the isolated fungal strain *Trametes sp.* is a mesophilic organism, with an optimal growth temperature range between 20 °C to 30 °C. Corroborating these observations, Castoldi *et al*. (2014) reported that the specific growth rate of *Coriolus hirsutus*, a white-rot fungus, gradually increased with increasing temperature up to 35 °C, which is consistent with the mesophilic nature of these fungi. Consequently, temperature and media composition significantly influenced the growth pattern of the mesophilic *Trametes* sp. strain, highlighting the importance of optimizing environmental conditions for efficient fungal growth and wood degradation processes.

Table 1. Growth rate of isolated *Trametes* sp. on the two nutrient media at different temperatures

Fungus strain	Temperature	Media type	Growth diameter (mm)
Trametes SP.	5 ^{0}C	PDA	7.3±1.4
	5^{0}C	MEA	10.7± 1.3
	20 ^{0}C	PDA	23.3±1.8
	20 ^{0}C	MEA	25.7± 1.1
	30 ^{0}C	PDA	34.3±0.8
	30 ^{0}C	MEA	37.7± 1.4
	40 ^{0}C	PDA	37.3±1.2
	40 ^{0}C	MEA	41.3 ± 1.3

Values are given as mean ± S.E.

Wood degradation (%)

The weight loss experiments demonstrated that the degradation pattern depended on the environmental conditions. Among the three different culture conditions tested, the maximum weight loss of 35.8% was observed for the poplar wood chips treated under solid-state conditions after 60 days of incubation. This indicates that the fungal strain *Trametes* sp. exhibited the highest degree of degradation under solid-state conditions compared to the other two conditions. Wood degradation by *Trametes* sp. was less vigorous in liquid potato dextrose broth (PDB), with only 33.5% mass loss, and 32% mass loss in PDB with aeration (Fig. 3a). The solid-state condition was more conducive for *Trametes* sp. to degrade the poplar wood chips due to the uniform and extensive dispersion of the inoculum throughout the solid medium, which resembles the natural habitats to which fungi are adapted (Kim *et al*., 2005). In the liquid conditions, the excess PDB medium did not provide a natural environment for *Trametes sp.*to grow and penetrate the wood effectively.

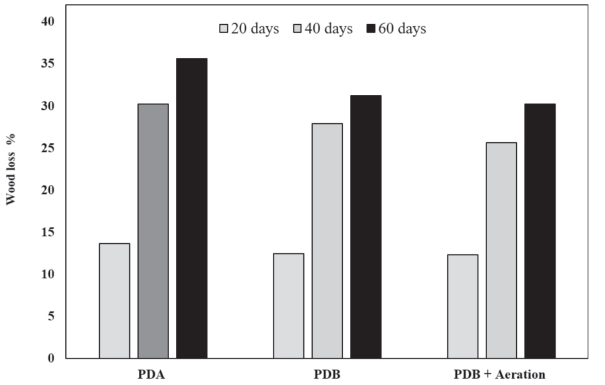
Cellulose and lignin content %

The untreated poplar wood chips contained 48.65% cellulose and 19.7% lignin, which falls within the typical range for hardwoods. During the investigation, the fungal strain *Trametes* sp. exhibited high cellulose content (38.5%) after 60 days incubation due to its low cellulolytic enzyme activity (Fig. 3b). Consequently, the cellulose content remained relatively unchanged across all treatment conditions (Liew *et al*., 2010) due to improper attachment and limited mycelial penetration into the wood cells. The lignin content showed a reduction of 7.4%, 9.8%, and 12% under different treatment conditions over the biodegradation period ranging from 0 to 60 days (Fig. 3c). The isolated *Trametes* sp. strain demonstrated maximum laccase enzyme activity, which contributed to the effective degradation of lignin in the poplar wood chips. Singh *et al*. (2010) observed that for biological pretreatment processes, the selected fungi should preferentially act on hemicellu-

lose and lignin while exhibiting low activity on cellulose. This selective degradation pattern observed with *Trametes* sp. where lignin was effectively degraded while retaining a high cellulose content, aligns with the desired characteristics for biological retreatment of lignocellulosic biomass.

SEM Analysis

The micrograph obtained from scanning electron microscope provided valuable information about wood degradation pattern and its relationship between ligninolytic enzymes activities produced by the *Trametes* sp. Both the untreated and treated wood sample were investigated and compared the microscopic view before and after the colonization fungus. It was reported that the most of the lumina of vessel and parenchyma cells of poplar wood chips were colonized with the *Trametes* sp. after the incubation periods (Fig. a). The hyphae present in the vessel cells are elongated and branched structure (Fig. 4b). The parenchymatous cells of the poplar wood chips was totally disrupted by the *Trametes* sp. because of vessels were provided large open spaces for fungal hyphae to grow and multiply.



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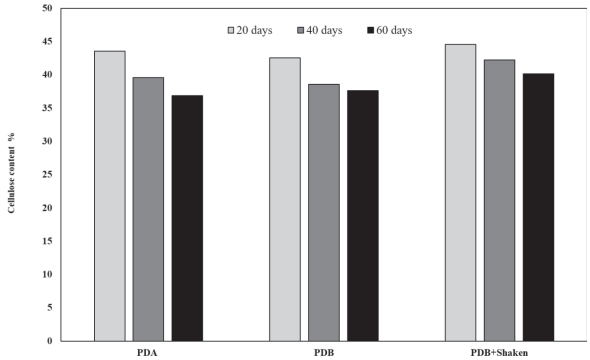

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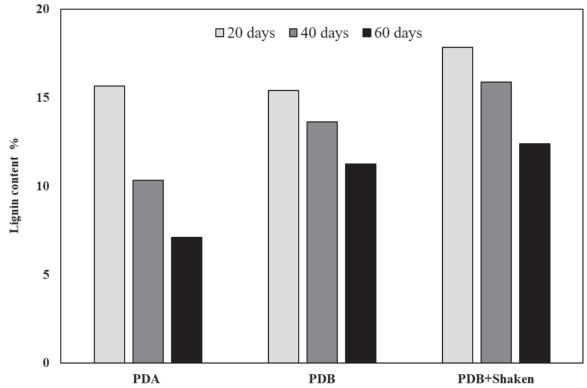
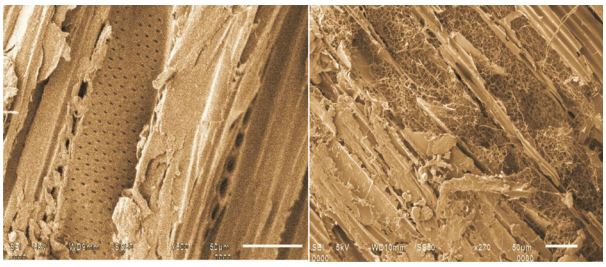


(c)

Fig. 3. Chemical properties of wood after degradation (a) Wood loss %; (b) Cellulose content %; (c) Lignin content %



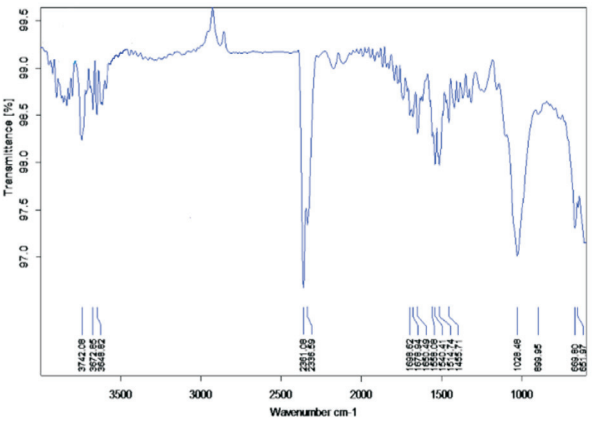
(a) (b)

Fig. 4. SEM micrograph poplar wood chips (a) control wood chips; (b) treated wood chips

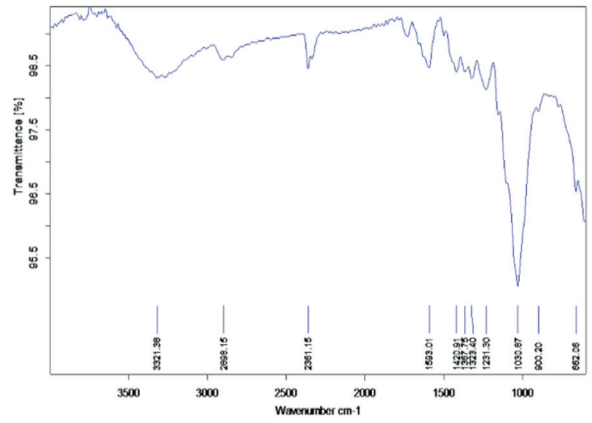
FTIR analysis

The FTIR analysis was conducted to investigate the changes in the chemical components of lignocellulose present in decayed and non-decayed wood samples over a frequency range of 400 to 4000 cm^{-1}. The most representative FTIR bands for untreated Poplar wood chips were observed in the spectral region of 1800–550 cm^{-1}. The results showed that as the degradation progressed, the absorbance of the major peaks decreased (Bouacem *et al*., 2021). (Fig. 5a). The peaks corresponding to lignin in the untreated wood chips, such as those at 1558, 1514.74, 1540, and 1455 cm^{-1}, which are attributed to aromatic skeletal vibrations (CP%C), gradually disappeared after treatment with the *Trametes* sp. Strain (Fig. 5b). This indicated the breakdown of aromatic rings in the lignin structure during the degradation period. Furthermore, the peaks associated with guaiacyl and syringyl groups in the lignin, typically observed in the ranges of 1,260–1,270 and 1,330–1,375 cm^{-1}, respectively, also diminished, indicating the degradation of these groups by *Trametes* sp.. The FTIR

analysis confirmed that the lignin present in the untreated poplar wood chips was either degraded or transformed into simpler compounds, resulting in structural changes in the degraded wood (Barnette *et al*., 2011).



(a)



(b)

Fig. 5. FTIR spectra of poplar wood chips (a) control; (b) treated wood chips of solid (PDA) condtion

Conclusion

In conclusion, this study successfully demonstrated the potential of utilizing the white-rot fungus *Trametes* sp. for an eco-friendly biopulping process on poplar wood chips. The solid-state culture conditions facilitated optimal colonization and selective degradation of lignin by the fungal strain's potent ligninolytic enzyme system, especially laccase. While effectively reducing the lignin content, the cellulose was largely preserved, making this an attractive pretreatment for valorizing the cellulosic fraction. Future research should focus on scaling up this biological pretreatment process to pilot and industrial scales. Optimizing operational parameters like moisture content, aeration, and nutrient supplementation could further enhance delignification efficiency. Overall, this green biopulping approach using *Trametes* sp. presents promising prospects for integration into sustainable biorefinery processes for paper, biofuel, and biochemical production from lignocellulosic biomass.

Conflict of Interest: None

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