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Lignin and colour removal of pulp and papermill wastewater by enzymatic action of different fungal strains

ABSTRACT

Wastewater from pulp and paper mill industries poses a major risk to the water and soil components of the ecosystem due to high concentration of organic as well as inorganic contaminants. In the present study, the performance of enzymatic activity of various fungal strains was investigated for the treatment of Pulp and paper mill effluent. Five effective white rot fungal strains were isolated which included Trametes versicolor recognized for the selective breakdown of lignin. Among all the strains, Trametes pubescens strain showed highest activity of lignin peroxidase, Fpase, xylanase, CMcase, protein, and sugar content at 120 h incubation period. Maximum removal efficiency of pollution load parameters such as colour (82%), COD (78%), lignin (83%) and adsorbable organic halides (AOX; 77%) was observed in Trametes pubescens on day 4 at 3.4 international unit per ml concentration of crude enzymes. The degradation and breakdown of high molecular weight contaminants such as lignin into small molecular weights compounds was established by FTIR and ^{13}C NMR analyses.

***Keywords**: Lignin peroxidase, pulp and paper mill, effluent, FTIR, ^{13}C NMR*

1. INTRODUCTION

Pulp and paper mill (P&P) industry is one of the major industrial sectors in India and worldwide which uses an enormous quantity of lignocellulosic resources as well as water throughout the manufacturing procedure [1]. It comes sixth among the world's most polluting industries. After agriculture, this industry uses the second-largest amount of water and is very high consumer of energy. In India there are approximately 900 pulp & paper mills and most of them practice the pulping and bleaching processes [2]. These industries generate varieties of wastewater depending upon the type of pulping method adopted (kraft process, sulphite process). There is also a massive upsurge in the release highly contaminated untreated effluent in water bodies as the number and capacity of these mills have doubled in the last 20 years, which has severe harmful impact on aquatic flora and fauna. Adverse effects include huge dip in dissolved oxygen, undesirable changes to color, turbidity, temperature and solids content of the water bodies which receives the effluents. It produces high values of Total suspended solids (TSS), alkalinity, biochemical oxygen demand (BOD), chemical oxygen demand (COD) in the effluent [3]. The toxic and xenobiotic matter present in the effluent includes degradation products of carbohydrates, lignin, and extractives along with chlorinated ligno-sulphonic acids, chlorinated resin acids, chlorinated phenols and chlorinated hydrocarbons (dioxins, furans, chlorophenols, chloroguicols, etc.), adsorbable organic halides (AOX), heavy metals etc [4]. Due to its complexity, the degradation of lignocellulosic waste is a great challenge for sustainable development. The absorption, accumulation and enrichment of these constituents leads to biomagnification which imbalances the aquatic ecosystem [5] and produces an alarming situation that needs utmost attention.

Physical, chemical, electrochemical, and biological procedures such as coagulation, flocculation, adsorption, ozonation, aerobic, anaerobic treatment, microbial fuel cell technology, etc. are among

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the different pulp and papermill effluent treatment practices currently in use [6-12]. Many of the effluent treatment systems now used in the pulp and paper sector have constrained intrinsic capabilities. They rely on a variety of factors, including the chemical properties of the effluent that needs to be removed, the purity standards for the effluent, the reactivity and selectivity of the chosen procedures, etc. For the majority of P&P mills, the economic and technological viability of advanced treatment methods may be limited.

Enzymatic degradation of has garnered intensive research interest as numerous microbes including bacteria, actinomycetes, fungi or yeast and algae have been reported for production of lignolytic enzymes [13-15]. These enzymes function as biological agents for the degradation of lignocellulosic waste present in several types of industrial wastes [16-17]. Fungi are regarded as nature's most active agents for decomposition of waste as they secrete a variety of extracellular lignolytic enzymes [18]. For instance, the most common fungal strains Trametes versicolor, T. reesei, Ganoderma lucidum, Aspergillus niger, Phanerochaete chrysosporium, Penicillium, Trichoderma are more efficient towards cellulose, hemicellulose, and lignin decomposition in a selective manner for wood decay [19].

In the present paper, successful attempt has been made to remove lignin and colour and other pollution load parameters of P&P wastewater by enzymatic action of different fungal strains. The use of microbial or enzyme-based treatment offers some distinct advantages over physical and chemical AOX precipitation methods, in that only catalytic and not stoichiometric amounts of the reagent are needed, and the low organic concentrations and large volumes typical of bleaching effluents are, therefore, less of a problem.

2. EXPERIMENTAL

(i) Sampling sites and microorganisms

For the isolation of fungi, the soil sample along with decomposed wood parts of pulp & papermill (P&P) effluent was collected from the drainage passing through Daurala pulp and papermill, Meerut. But for evaluating the enzymatic action, the P&P effluent was collected in wide mouth glass bottles from the main canal receiving the effluent of kraft pulping, recovery plant located inside the industry and transported it to the laboratory by keeping the sample bottles in Styrofoam plastic cooler containing frozen gel packs. The effluent was immediately stored in a refrigerator at 4°C and the analysis was completed in a week time.

Soil sample along with decomposed wood parts was filtered through muslin cloth and filtrate was diluted ten times. Dilute sample (0.2 ml) was placed on the potato dextrose agar plates. The plates were then incubated for four days at 30°C. Fungal colonies developed on the plates were isolated, identified and purified. Five fungal discs with a diameter of approximately 1 cm were removed from the zone of active development and inoculated into conical flasks containing potato dextrose broth and 100 ppm streptopenicillin in order to treat the P&P effluent. After that, the flasks were shaken for four days at 30°C in an orbital shaker. After centrifuging the culture material for 15 minutes at 10,000 rpm, pellets measuring 1-2 mm were extracted using a sterile spatula. For treatment, the aforementioned pellets were suspended in the P&P effluent.

(ii) Culture conditions

The composition of the culture medium consisted of following (g/L) glucose, 10; yeast extract, 15; $(NH_4)_2SO_4$, 0.9; KH_2PO_4, 2; $MgSO_4{\cdot}7H_2O$, 0.5; $CaCl_2{\cdot}2H_2O$, 0.1; KCl, 0.5 and thiamine, 0.5 in citrate–phosphate buffer, pH 4.5[20].

(iii) Growth and Enzyme assay

Cellulose content of the pulp before and after degradation was determined according to Updegraff [21] using microgranular cellulose as a positive control. Protein content was estimated according to the method of Lowry *et al* [22]. Reducing sugar was estimated according to the method of Somagyi [23] using glucose as standard. Xylanase was determined by the dinitrosalicylic acid method [24]. CMCase and FPase activities determined according to IUPAC [25]. Lignin peroxidase activity was assayed according to the method Yee *et al* [26].

(iv) Effect of different enzymes on decolourisation

Effect of enzyme on decolourisation of P&P effluent was monitored. 100- unit/mg enzymes were mixed with effluent having pH and kept on rotatory shaker at 150 rpm. Colour, lignin, and AOX were determined at 2 h, 4 h, 6 h, 10 h, 12 h, 1-day and 4-day interval.

(v) Analytical methods

Samples (100 ml) were drawn from the batch study experiment conducted with fungus at an interval of 0-, 1- and 4-days interval. The sample was centrifuged at 10,000 rpm for 30 min to remove all the suspended matter. Reduction in colour was measured using a spectrophotometer using the method of Bajpai *et al* [27]. The lignin of

the effluent was estimated using the method of Pearl and Benson [28]. Adsorbable organic halogen was analyzed by IDC multi X-2000 AOX Analyzer. COD was determined by a dichromate reflux method [29]. FT-IR spectra were recorded in the range of 4000–500 cm^{-1} on Nicolet Magna FTIR-750 spectrometer. ^{13}C NMR spectra were obtained from a Bruker Ascend 500 MHz spectrophotometer operating at 125.8 MHz. The chemical shifts are reported on a ppm scale with respect to $CDCl_3$ as a solvent.

3. RESULTS AND DISCUSSION

With the assistance of IMTECH, Chandigarh, five distinct species of fungal colonies were isolated from pulp mill effluent after 48 h incubation at 30°C and identified. They were further purified on PDA plates. Based on morphological traits and microscopic investigations, these pure fungal isolates were identified (Table 1). They are *Trametes versicolor* (F1), *Trametes pubescens* (F2), *Ganoderma adspersum* (F3), *Ganoderma lipsiense* (F4) and *Perenniporia tephropora* (F5). F1 was taken as control.

Table 1. Morphological traits and microscopic investigations of various fungal strains

Isolates	Characteristics	Growth
Trametes versicolor (F1)	off-white, abundant aerial hyphae, polyporous nature	Medium growth
Trametes pubescens (F2)	narrow hyphae, 2-3 layers of hyphae	Medium growth
Ganoderma adspersum (F3)	Dark brown at the center, reddish-brown at the second, and yellow nearly white at the border concentric zones, di-trimitric hyphal system	Slow growth
Ganoderma lipsiense (F4)	thin and acute margin of the pileus, and unbranched terminal endings of skeletal hyphae	Slow growth
Perenniporia tephropora (F5)	pale brown pore surface, dimitic hyphal system with branched skeletal hyphae	Medium growth

The five strains were cultivated in P&P effluent and produced lignolytic, hemicellulolytic, and cellulolytic enzymes. The efficiency of the degradation of pulp by the four isolates and *Trametes versicolor* was studied in this work to understand degradation of cellulose carbon and weight loss. The solid fractions were tested for weight loss, cellulose and organic carbon content, which are presented in Table 2.

The weight loss of pulp for all isolated strains increased during degradation. F2 (*Trametes pubescens* (F2) resulted weight loss of 20.2% at 24 h, reached up to 61.4 at 120 h followed by strains F1 (*Trametes versicolor*), F4 (*Ganoderma lipsiense*), F3 (*Ganoderma adspersum*), F5 (*Perenniporia tephropora*). The maximum cellulose loss was found to be 59.5% at 120 h for *Trametes pubescens* (F2). Carbon content loss increased progressively for all the fungal strains and reached a maximum of 49.3% for *Trametes versicolor* (F1) whereas it was slightly lower for *Trametes pubescens* (47.1%). Production of Cellulolytic (Fpase, CMCase), xylanalytic and lignolytic enzyme touched a maximum level at120 h in all the strains. Protein and reducing sugar content augmented towards 120 h after which the decline started (Table 2). The increase in protein and sugar was expected because the increase in microbial biomass would add to total protein availability and also production of hemicellulolytic and lignolytic enzymes [3, 30]. Among the four isolates, *Trametes pubescens* was quite effective in producing more protein and sugar. It was even higher than that of Trametes versicolor. Results indicated production of all four enzymes (xylanases, Fpase, CMCase and lignin peroxidase) by selected fungal strains is in conformity with the literature reports [31-34].

Table 2. Degradation of pulp and production of enzymes by various fungal isolates after 120 h

Parameters	Isolated fungal strains				
	F-1	F-2	F-3	F-4	F-5
weight loss (%)	52.1	61.4	36.2	40.3	31.1
cellulose loss (%)	58.4	59.5	31.6	34.3	30.6
organic carbon (%)	49.3	47.1	32.7	40.4	33.2
Xylanases activity (IU/ml)	0.67	0.83	0.43	0.28	0.35
CMCase (IU/ml) activity	0.41	0.46	0.13	0.21	0.28
FPase activity (IU/ml)	0.58	1.1	0.38	0.33	0.31
Lignin peroxidase activity (IU/ml)	2.9	3.2	2.1	2.4	2.9
Reducing sugar (mg/ml)	2.4	2.7	1.0	0.9	2.1
Total protein (mg/ml)	1.6	2.0	0.58	1.32	1.3

The analysis of the data indicated that *Trametes pubescens* (F2) produced significantly more lignin peroxidase than xylanases and cellulolytic enzymes than other isolated fungal strains. Fpase and carboxymethylcellulase activity was highest in *Trametes versicolor* (F1) followed by *Trametes pubescens* (F2) and other isolates. The dialyzed culture filtrate of all the fungal strains were tested for bioremediation of pulp and paper mill effluent. Degradation efficiency of all the isolates was also found to be in agreement with literature reports of other species of fungi [30, 35].

Maximum removal efficiency of pollution load parameters such as colour (82%), COD (78%), lignin (83%) and adsorbable organic halides (AOX; 77%) was observed in *Trametes pubescens* on day 4 at 3.4 international unit per ml concentration of crude enzymes. (Figure 1-4). It has been reported that lignin peroxidases secreted by white rot fungi are involved in the degradation of a whole range of organic pollutants [19]. Arcand and Archibald [36] observed that a range of chlorophenolics could be partially dechlorinated by lignin peroxidase secreted by *T. versicolor*.

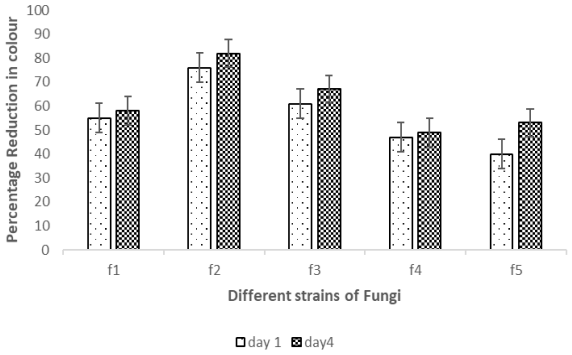

Figure 1. Reduction in colour (CU) of P&P effluent by enzymatic action of different strains of Fungi (F1-F5) after day 1 and day 4

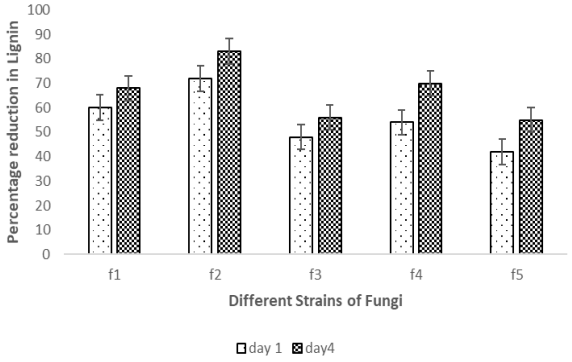

Figure 2. Reduction in lignin content (mg/L) of P&P effluent by enzymatic action of different strains of Fungi (F1-F5) after day 1 and day 4

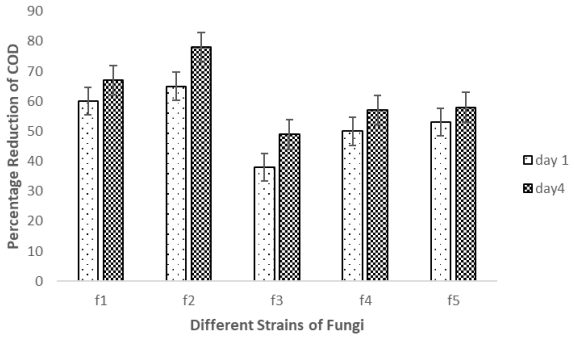

Figure 3. Reduction in COD of P&P effluent by enzymatic action of different strains of Fungi (F1-F5) after day 1 and day 4

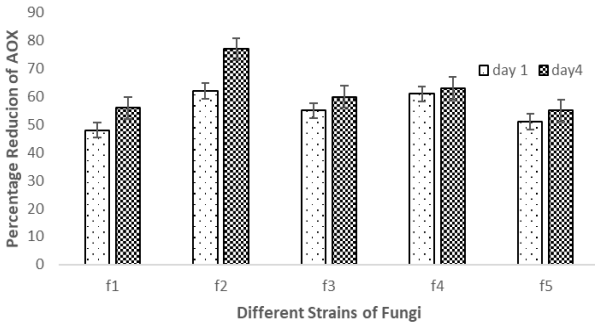

Figure 4. Reduction in AOX of P&P effluent by enzymatic action of different strains of Fungi (F1-F5) after day 1 and day 4

FTIR and ^{13}C NMR analyses of pulp and papermill effluent samples before and after fungal treatment were also conducted to spot the changes in the nature of the compounds and to consolidate the fact that enzymatic activity of various fungal strains has led to the degradation of complex high molecular weight compounds. The peaks obtained in FTIR spectra (Figure 5 and Table 3) were assigned to specific functional groups found in lignin as reported in the previous studies [37, 38].

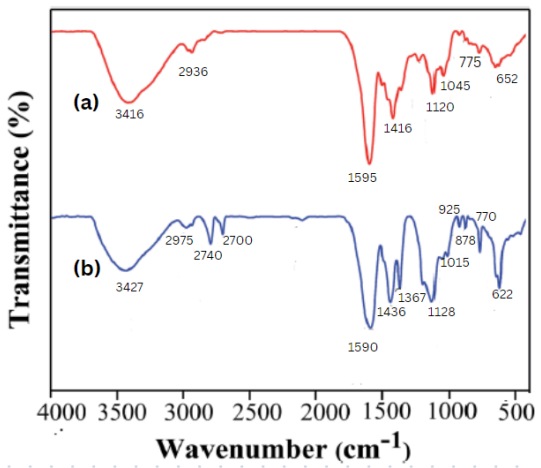

Figure 5. Comparison of IR spectra of P&P effluent (a) before treatment and (b) after treatment

Figure 5 shows the appearance of several additional peaks around 880-930 cm^{-1} and 2700-2750 cm^{-1}, which can be attributed to the C–H bending and C-H symmetric stretching vibrations respectively. It can happen only upon the breakdown of high molecular weight into smaller molecules which supports degradation. Besides, intensity of some peaks increased in IR spectrum of P&P effluent after treatment (1100-1130 cm^{-1} and 620 cm^{-1}).

Table 3. Assignment of bands in IR spectra of P&P effluent before treatment and after treatment (cm^{-1})

P&P effluent before treatment (cm^{-1})	P&P effluent after treatment (cm^{-1})	Functional group assignment	Intensity
3416	3427	O-H stretching (phenolic, alcoholic group)	Strong, broad
2936	2975	C-H asymmetric stretching	Weak
-	2740, 2700	C-H symmetric stretching	Weak
1595	1590	C=C stretching (aromatic)	strong
1416	1436	C-H bending in-plane vibration (aromatic)	medium
-	1367	C-O stretching	Weak
1120, 1045	1128, 1015	C-O bending (ether)	strong
-	925, 878	C-H bending out-of-plane vibration	medium
775, 652	770, 622	C=C bending	medium

The peaks obtained in ^{13}C spectrum (Figure 6 and Table 4) were assigned to appropriate chemical groups present in P&P effluent before and after the enzymatic treatment with the help of the literature data [39, 40].

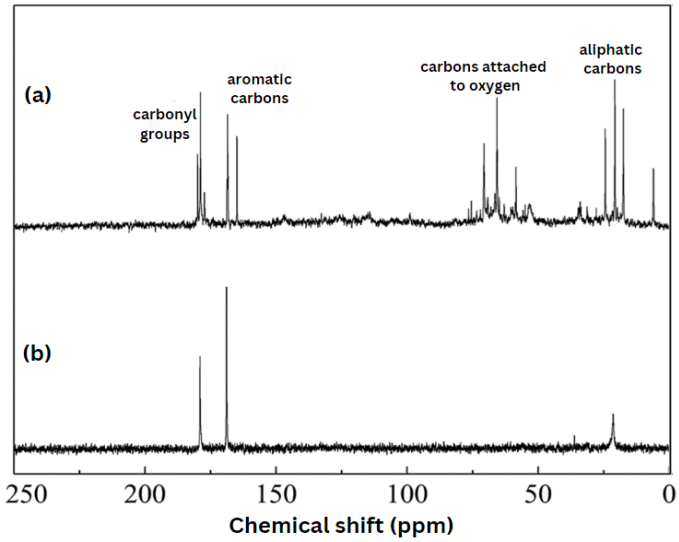

Figure 6. Comparison of ^{13}C NMR spectra of P&P effluent (a) before treatment and (b) after treatment

Table 4. Assignment of Chemical shifts (ppm) in ^{13}C NMR spectra of P&P effluent before treatment and after treatment

Chemical Shifts (ppm)	Chemical Group assignment
170-180	CO(O)–(H, R) carboxylic and ester
160-165	Aromatic carbons
70-77	C-α in G type b-O-4 units
64-66	β-O-4 with CO in G and S units
54	OCH_3
20-35	Aliphatic carbons (not linked with oxygen atom)

The major lignin linkages such as β-O-4, β -5 and β-β (carbon attached to oxygen atom) were present in the ^{13}C NMR spectrum of P&P effluent before treatment but were absent in the spectrum of effluent after the treatment signifying that degradation of lignin like giant molecules has taken place. Moreover, the intensity of peak corresponding to carbonyl carbon (170 ppm) increased after the treatment. This may be due to the formation of low molecular weight compounds having carboxylic groups which degradation of high molecular weight lignocellulosic waste present in effluent.

4. CONCLUSION

Bioremediation reduces the pollutant load present in pulp and papermill effluent by changing recalcitrant and xenobiotic and high molecular weight components into less toxic and acceptable form. Enzymatic colour removal has potential advantage over microbial treatment due to producing less sludge during treatment. Among all the enzymes (lignin peroxidase, xylanases, Fpase, carboxymethylcellulase) secreted by the selected fungal strains, lignin peroxidase has the highest activity. *Trametes pubescens* (F-2) was found to have the highest removal effectiveness of pollutant load metrics, including colour (82%), COD (78%), lignin (83%), and adsorbable organic halides (AOX; 77%) at 3.4 international units per millilitre concentration of crude enzymes. Therefore, it can be used in the large-scale bioreactor treatment of pulp and papermill effluent. Use of fungal consortium of most efficient fungal strains can result in higher degradation rates and increased stability than using each strain alone.

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IZVOD

UKLANJANJE LIGNINA I BOJE IZ OTPADNIH VODA PULPI I PAPIRA ENZIMSKIM DELOVANJEM RAZLIČITIH SOJEVA GLJIVICA

Otpadne vode iz industrije celuloze i papira predstavljaju veliki rizik za komponente vode i zemljišta ekosistema zbog visoke koncentracije organskih i neorganskih zagađivača. U ovoj studiji, ispitivana je aktivnost enzima različitih sojeva gljivica za tretman otpadnih voda fabrike celuloze i papira. Izolovano je pet efikasnih sojeva gljivica bele truleži, uključujući Trametes versicolor koji je prepoznat po selektivnoj razgradnji lignina. Od svih sojeva, soj Trametes pubescens pokazao je najveću aktivnost lignin peroksidaze, Fpaze, ksilanaze, CMcase, proteina i šećera u periodu inkubacije od 120 h. Maksimalna efikasnost uklanjanja parametara opterećenja zagađenjem kao što su boja (82%), COD (78%), lignin (83%) i adsorbirajući organski halogenidi (AOKS; 77%) primećena je u Trametes pubescens 4. dana pri 3,4 međunarodne jedinice po ml koncentracija sirovih enzima. Degradacija i razlaganje zagađivača visoke molekulske težine, kao što je lignin u jedinjenja male molekulske težine, ustanovljeno je FTIR i 13C NMR analizama.
Ključne reči: *lignin peroksidaza, fabrika celuloze i papira, efluent, FTIR, 13C NMR*

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