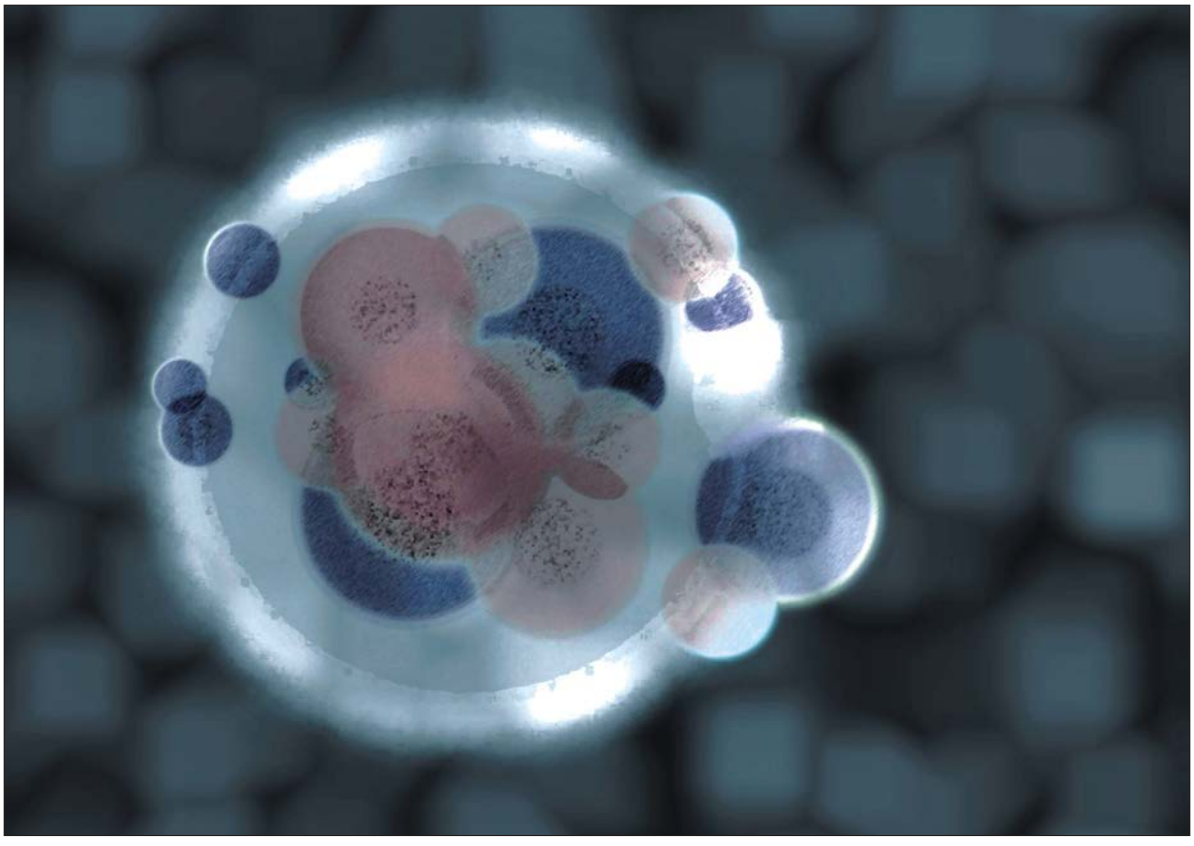

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Biodecolorization of Phenolic Paper Mill Effluent by Ligninolytic Fungus *Trametes versicolor*

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Abstract: A white rot fungus isolated from soil samples enriched by continuous pulp and paper mill effluent irrigation and identified as *Trametes versicolor* was capable of decolorization and degradation of phenol from paper mill effluent. ^{14}C synthetic lignin mineralization assays showed that *Trametes versicolor* assimilated 24.3% of the total label. There was 76% effluent decolourization along with 78% COD reduction. The effluent chlorinated phenol degradation was 85% by *Trametes versicolor*, when added with 1% glucose as co-substrate.

Key words: Color, COD, chlorinated phenol, paper mill effluent

INTRODUCTION

Contamination of the environment by chlorinated aromatic compounds has been the subject of increased concern in the last few years. Chlorinated phenols are common environmental contaminants and also formed as by products when chlorine is used for bleaching of pulp. The phenolic effluents produced by pulp and paper, coal conversion, petrochemical, dyeing and textile industries are toxic to mammals and fish[1]. Among these, effluent from pulp and paper plant is one of the major environmental bugbears in India, contributing to soil and water pollution. In India, pulp and paper industry ranks third in terms of fresh water withdrawal and is brought under the list of 17 most polluting sectors as identified by the Central Pollution Control Board[2]. The phenol rich waste waters from paper mill effluent are harmful to both flora and fauna, even at relatively low concentrations of 10-12 mg L^{-1}. Phenols in low concentrations impart carbolic odour and bitter taste to natural waters and high concentrations results in pain, renal irritation, severe shock, dermatitis, skin irritation, cancer, mutation, vomiting, capillary damage etc.[3]. At the same time continuous land application of effluent made the soil unfit for cultivation due to physical and biochemical constraints including sodicity problems and ground water contamination. Conventional treatment methods, such as aerated lagoons and activated sludge plants are ineffective in removing the colour. Microorganisms are known to utilize phenolic substances, recalcitrant molecules and even xenobiotic compounds as carbon source for their growth. Hence in the present study, the white rot fungus isolated from soil samples enriched with continuous paper mill effluent irrigation over 20 years for their color removal, COD reduction and phenol degradation of paper mill effluent were investigated.

MATERIALS AND METHODS

Chemicals: ^{14}C DHP (dehydro polymer) synthetic lignin, agar, dioxane, Czapek-Dox mineral medium, ethanol, DOPA (3,4- dihydroxy phenyl alanine, syringaldazine (Sigma Chemical Co., USA) guaiacol, ABTS (2,2'-azinobis-3 ethyl benzthiazoline-6 sulfonate) and sodium azide were used.

Microorganisms, inoculum development, culture medium and conditions: The white rot fungus was isolated from enriched soil samples with continuous pulp and paper mill effluent irrigation over 20 years by employing standard serial dilution plating technique[4]. The isolated fungus was screened based on the growth on media containing phenol red for its ligninolytic activity. Plates were observed for growth and colour change from yellow to red around the culture growth, which indicate the ligninolytic nature of the culture.

Degradation of lignin: Lignin degradation by fungal culture was confirmed by quantifying the $^{14}CO_2$ produced during the metabolism of ^{14}C labeled synthetic lignin obtained by polymerization of labeled coniferyl alcohol[5].

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Decolourisation of effluent using isolated fungi: Pulp and paper mill effluent was scanned in a spectrophotometer to ascertain the wavelength and the maximum absorbance was observed at 465 nm and the rate of decolourisation was monitored at this wavelength. For colour determination, the effluent sample was centrifuged at 10000 rpm for 30 min to remove all the suspended matter. The pH of the supernatant was adjusted to 7.6 with 2 N NaOH and then used for the measurement of absorbance at 465 nm against distilled water as blank. Absorbance values were transformed into Colour Units (CU) using the following equation:

$$CU = 500A_2/A_1$$

Where, A_1 is the absorbance of 500 CU platinum-cobalt standard solution (A_{465} = 0.132) and A_2 is the absorbance of effluent sample.

The isolated fungus (*Trametes versicolor*) was maintained in the potato dextrose agar slants. The fungal isolate was grown in 250 mL Erlenmeyer flask containing 100 mL of Czapek-Dox mineral solution (pH 6.5). After five days, the mycelial pellets were washed with sterile phosphate buffer (pH 7.0) and then the mycelial mats were homogenized by grinding with acid washed sand via pestle and mortar. Two milliliter of homogenized fungal mycelial mats of isolated culture was added to 250 mL Erlenmeyer flask containing 100 mL effluent with different carbon sources viz., glucose, fructose and starch and nitrogen sources viz., ammonium sulphate, sodium nitrate and diammonium phosphate alone and in combination along with control (effluent alone). The treatment schedule is indicated below: T_1-Control (Effluent alone); T_2-Effluent + Glucose (2.5 g L^{-1}); T_3-Effluent + Starch (2.5 g L^{-1}); T_4-Effluent + Fructose (2.5 g L^{-1}); T_5- Effluent + Ammonium sulphate (1.25 g L^{-1}); T_6-Effluent + Sodium nitrate (1.25 g L^{-1}); T_7-Effluent + Diammonium phosphate (1.25 g L^{-1}); T_8-Effluent + Glucose (2.5 g L^{-1}) + Ammonium sulphate (1.25 g L^{-1}); T_9-Effluent + Glucose (2.5 g L^{-1}) + Sodium nitrate (1.25 g L^{-1}); T_{10}-Effluent + Glucose (2.5 g L^{-1}) + Diammonium phosphate (1.25 g L^{-1}); T_{11}-Effluent + Starch (2.5 g L^{-1}) + Ammonium sulphate (1.25 g L^{-1}); T_{12}-Effluent + Starch (2.5 g L^{-1}) + Sodium nitrate (1.25 g L^{-1}); T_{13}-Effluent + Starch (2.5 g L^{-1}) + Diammonium phosphate (1.25 g L^{-1}); T_{14}-Effluent + Fructose (2.5 g L^{-1}) + Ammonium sulphate (1.25 g L^{-1}); T_{15}-Effluent + Fructose (2.5 g L^{-1}) + Sodium nitrate (1.25 g L^{-1}); T_{16}-Effluent + Fructose (2.5 g L^{-1}) + Diammonium phosphate (1.25 g L^{-1}). The treatments were replicated twice with control. Five milliliter samples were drawn from the inoculated and uninoculated treatments and centrifuged at 7000 rpm for 10 min. The supernatant was collected and the CU values were measured for seven days at 0, 2, 4 and 7 days after inoculation at 465 nm in the spectrophotometer.

Colour removal percentage: The percent colour reduction was measured as per the method[6].

$$\text{Colour removal (\%)} = \frac{\text{(A)-Absorbance of residual broth}}{\text{Absorbance of uninoculated broth (A)}} \times 100$$

The effluent treated with fungal isolate for decolourisation and pollutant degradation was analysed for the physico-chemical properties as per the methods detailed in standard methods for the estimation of water and waste water[7].

Degradation of chlorinated phenol in effluent: The assay for degradation of chlorinated phenol in the effluent was carried out by taking 100 mL of effluent in 250 mL Erlenmeyer flasks. Glucose was added at one per cent level and the whole content was sterilized by autoclaving and then inoculated with 2 mL of fungus spore suspension along with heat killed culture. The inoculated flasks were incubated at room temperature and samples were collected at 3, 5, 7 and 9 Days After Inoculation (DAI). For the analysis of remaining phenol, the effluent was extracted by acidifying 10 mL effluent sample with 5 N HCl. The chlorinated phenol was extracted three times with 10 mL of dichloromethane medium. The organic phase was re-extracted with 0.5 N NaOH. The aqueous phase was taken and analysed by 4-amino antipyrine method.

RESULTS

Identification of fungus: The isolated fungus was identified as based on their morphology, physiological and biochemical characteristics and verified at Mycology department of Indian Agricultural Research Institute, New Delhi.

Confirmation of ligninolytic activity: The lignin degrading ability of the fungus was also confirmed by measuring the quantity of $^{14}CO_2$ evolved from degradation of ^{14}C DHP synthetic lignin (Fig. 1). The fungus *Trametes versicolor* released 24.3% of $^{14}CO_2$ and the percent utilization of synthetic lignin was 32.7 and fixed as cell carbon.

Table 1: Effluent colour removal and COD reduction by *Trametes versicolor*

	Trametes versicolor							
	Colour removal (colour units)				COD reduction			
Treatments	0 DAI	2 DAI	4 DAI	7 DAI	0 DAI	2 DAI	4 DAI	7 DAI
T_1	1487	1301 (12.5)	1177 (20.8)	964 (35.5)	3935	3581 (9.00)	3055 (22.4)	2650 (32.7)
T_2	1591	1239 (22.1)	1028 (35.4)	708 (55.5)	4055	3125 (22.9)	2371 (41.5)	1618 (60.0)
T_3	1494	1173 (21.5)	1032 (30.9)	790 (47.6)	3852	2920 (24.2)	2495 (35.2)	1915 (50.3)
T_4	1467	1166 (20.5)	975 (33.5)	776 (47.1)	3887	2800 (27.9)	2300 (40.8)	2010 (48.3)
T_5	1495	1132 (24.3)	923 (39.5)	624 (59.5)	3781	2850 (24.6)	2100 (44.5)	1700 (55.0)
T_6	1514	1209 (20.1)	981 (35.2)	704 (53.5)	4121	3157 (23.3)	2508 (39.1)	1987 (51.8)
T_7	1590	1111 (30.1)	823 (48.2)	508 (68.0)	4058	3089 (23.8)	2417 (40.4)	1810 (55.3)
T_8	1622	1208 (25.5)	780 (51.9)	397 (75.5)	3815	2494 (34.6)	1418 (62.8)	850 (77.7)
T_9	1547	1177 (23.9)	658 (48.2)	499 (67.7)	4219	2980 (29.4)	1810 (57.0)	1150 (72.7)
T_{10}	1581	1161 (26.5)	673 (57.4)	434 (72.5)	3987	2625 (34.1)	1450 (63.6)	985 (75.2)
T_{11}	1589	1241 (21.9)	882 (44.5)	448 (71.8)	3721	2750 (26.1)	1917 (48.5)	1120 (69.9)
T_{12}	1617	1290 (20.2)	936 (42.1)	562 (65.2)	3815	2820 (26.1)	2011 (47.3)	1450 (61.9)
T_{13}	1537	1108 (27.9)	704 (54.2)	422 (71.5)	3727	2825 (24.2)	2150 (42.3)	1185 (68.2)
T_{14}	1459	1101 (24.5)	766 (47.5)	426 (70.8)	3851	2950 (23.3)	1950 (49.3)	1255 (67.4)
T_{15}	1465	1118 (23.7)	870 (40.6)	428 (63.8)	3955	2850 (27.9)	1924 (51.3)	1597 (60.6)
T_{16}	1517	1078 (28.9)	735 (48.5)	417 (72.5)	4218	3015 (28.5)	2017 (52.2)	1470 (65.1)

(Figures in parenthesis indicate percent colour reduction; DAI-Days after Inoculation)

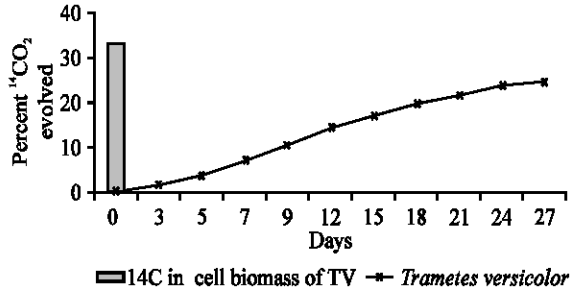

Fig. 1: Confirmation of ligninolytic activity by *Trametes versicolor*

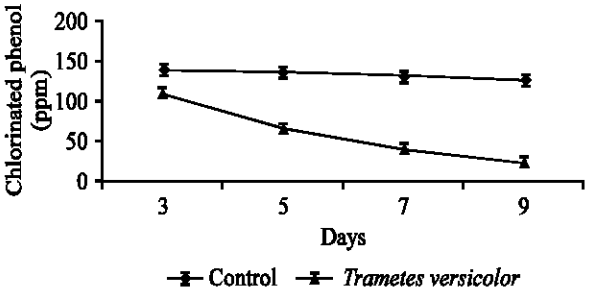

Fig. 2: Degradation of effluent chlorinated phenol by *Trametes versicolor*

Color removal of paper mill effluent using the isolated fungi with co-substrates: The fungus *Trametes versicolor* responded more to glucose among the carbon sources and the colour removal was 55.5%, whereas addition of fructose and starch the colour removal was 47.6 and 47.1%, respectively (Table 1). Among the N sources tested, *Trametes versicolor* responded more to DAP and the colour removal was 68%, whereas addition of ammonium sulphate and sodium nitrate it was 59.5 and 53.5%, respectively. Combination of carbon and nitrogen sources caused maximum decolourisation than individual addition of carbon or nitrogen sources. The maximum decolourisation was achieved in glucose + ammonium sulphate (75.5%) combination followed by glucose + DAP (72.5%), starch + DAP (72.5%) and the lowest (35.5%) was recorded in control (without co-substrate).

Effluent COD reduction: The COD of the effluent declined continuously up to 7 days of experiment (Table 1). In the absence of any co-substrate the maximum reduction in COD was 32.7% by *Trametes versicolor* and 77.7% of COD reduction achieved in combination of glucose and ammonium sulphate

Chlorinated phenol removal of pulp and paper mill effluent using *Trametes versicolor*: By using *Trametes versicolor* for chlorinated phenol degradation of effluent there was 85% degradation (Fig. 2).

DISCUSSION

Lignin is the single most important activity in the biological cycle of carbon. The multitude of interunit bonds and functional groups and the heterogeneity of the polymer is the main reason for the resistance of lignin to microbial attack and it is in fact one of the most recalcitrant naturally occurring biological material. In the present study, we describe the isolation of a lignin degrading fungus from the soil enriched by irrigation over 20 years by paper mill effluent. This fungus is a whiterot basidiomycete and able to oxidize phenol red and 14C synthetic lignin. It is unequivocally accepted that the ^{14}C DHP mineralization to $^{14}CO_2$ is considered as the

confirmation of ligninolytic activity of the organism and it convincingly demonstrate the rate and extent of lignin degradation[8]. The non-specific oxidation caused by enzymatic combustion leads to the formation of CO_2.

Color removal of pulp and paper mill effluent: The fungus *Trametes versicolor* showed better color reduction when glucose was used as a carbon source. There was 56% effluent color reduction when glucose was added as a co-substrate. It was reported that addition of glucose @ 1% (W/V) triggered the decolourisation potential of *Phanerochaete chrysosporium*[9,10]. Among the N sources, the fungus responded more to DAP. The nitrogen addition in small quantities increased ligninolytic activity through increased Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) activity and therefore enhanced decolourisation rates. Among the combination, glucose 2.5 g L^{-1} + ammonium sulphate 1.25 g L^{-1} recorded the highest colour removal (75%). Similar results were also reported by Jothimani and Prabakaran[11], who found that glucose at 0.15% and urea at 0.05% increased decolourisation of the dye effluent by 65 and 70.3%, respectively when *Bacillus* and *Phanerochaete chrysosporium* cultures were used.

The microbial system along with co-substrates were found to be efficient in reducing COD and pH of the pulp and paper mill effluent. In the present study the fungus reduced the COD of the effluent considerably when added with co-substrates. The addition of glucose induced the LiP and MnP enzyme activity of fungal system and enhanced the COD reduction and in line with the findings of Nagarathnamma and Pratima[12].

Chlorinated phenol removal of pulp and paper mill effluent: There was 85% chlorinated phenol degradation of pulp and paper mill effluent by *Trametes versicolor*, when added with 1% glucose. The glucose added in the effluent used as the energy source for the rapid growth of fungi and degraded the chlorinated phenol present in the effluent. Also the extracellular enzyme produced by the fungi is responsible for the chlorinated phenol degradation. Similar results were reported by Peralta-Zamora *et al.*[13], who observed the decolourization and pollutant degradation of pulp mill effluents with immobilized lignin and MnP from *Phanerochaete chrysosporium.*

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