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Degradation of Pentachlorophenol by White Rot Fungus (*Phanerochaete chrysosporium*-TL 1) Grown in Ammonium Lignosulphonate Media

C. Udayasoorian, P.C. Prabu and G. Balasubramanian
Department of Environmental Sciences, Tamil Nadu Agricultural University,
Coimbatore-3, Tamil Nadu, India

Abstract: The white rot fungus isolated from continuous pulp and paper mill effluent irrigated soil and identified as *Phanerochaete chrysosporium* (TL 1) was capable of degrading pentachlorophenol. ^{14}C synthetic lignin mineralization assays showed that the fungus assimilated 33.6% of the total label. Removal and degradation of pentachlorophenol (PCP) by the organism in static flask cultures was studied using ammonium lignosulphonate (LS), a waste product of paper mill industry, as a carbon and nitrogen source. After eight days, *Phanerochaete chrysosporium* (TL 1) grown in 2% LS (nitrogen sufficient) medium removed 85% of PCP which was comparable that of degradation in 2% glucose medium (93%). The presence of laccase (EC.1.10.3.2) extracellular activity suggested that the fungus secrete the enzyme into the extracellular medium, which was responsible for the degradation on PCP. The extracellular laccase activity was assayed on agarose plates containing ABTS.

Key words: Lignosulphonate media, laccase, PCP

INTRODUCTION

Microorganisms are known to utilize phenolic substances, recalcitrant molecules and even xenobiotic compounds as carbon source for their growth. *A. pseudomonas* sp. strain isolated from a consortium could be used very effectively for *in situ* bioremediation in an environment which is highly contaminated with PCP, other chlorinated phenols and hexadecane (Murialdo *et al*., 2003). White rot fungi efficiently degrade lignin, a complex aromatic polymer in wood that is among the most abundant natural materials on earth (Martinez *et al*., 2004). The white rot fungi have been widely studied for their ability to degrade variety of environmental soil pollutants, including pentachlorophenol (Aiken and Logan, 1996). Joyce *et al*. (1987) reported that whiterot fungi were able to degrade pentachlorophenol and 2, 4, 6-trichlorophenol at concentrations up to 250 mg L^{-1} and it could be reduced to less than 5 mg L^{-1} in 96 h. If white rot fungi are to be used for large scale bioremediation of PCP contaminated soils and waste waters, however, glucose media will need to be replaced by other, less expensive carbon sources. The fungus can degrade various other xenobiotics such as polyaromatic hydrocarbons and chlorinated aromatic compounds and also pollutants, which are covalently bound to humic substances (Leung and Ponting, 2002). The white rot fungus *Phanerochaete chrysosporium* can be used for bioremediation of phenolic, xenobiotic compounds and decolorization of textile effluents. The siderophores detected from the culture of the organism have been found useful in the decolorization and remediation of the effluent (Asamudo *et al*., 2005).

In this study we report the results of our work on examining the use of lignosulphonate (LS) as a fungal growth medium. LS is a waste product of paper mill industry generated during the bleaching and pulping process. These solutions are highly colored (dark brown) due to their lignin content and therefore usually disposed of by incineration. However, LS contain large concentrations of wood sugars, nitrogen (17.6 g L^{-1} as NH_3-N) and other trace minerals needed (Table 1) for growth of fungi making LS a logical choice as an alternative growth substrate. The persistence of the dark color of LS could pose a problem for its use in soil bioremediation or wastewater treatment processes. However, *Phanerochaete chrysosporium* can decolor sulphonized azo dye compounds which are structurally similar to the substructures of LS and chlorolignins in paper mill waste streams. We therefore speculated that LS might also sufficiently decolored during the growth of white rot fungi.

Corresponding Author: C. Udayasoorian, Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore-3, Tamil Nadu, India

Table 1: Analysis of ammonium lignosulphonates (Data by South India Viscose, Coimbatore)

Component	(%)
Total solids	48.56
Sodium	0.24
Potassium	0.11
Calcium	0.10
Magnesium	0.06
Sulphur (as S)	6.95
Sulphate (as S)	1.35
Ash	1.65
Galactose	1.25
Glucose	2.50
Mannose	9.50
Arabinose	3.45
Total free sugars	20.80
Hydrolysed galactose	4.30
Hydrolysed Glucose	5.10
Hydrolysed Mannose	12.80
Hydrolysed Arabinose	1.30
Hydrolysed xylose	2.00
Total hydrolysed sugars	25.50
Ammonium lignosuphonate	69.30
Manganese (2% LS solution)	2.9 mg L^{-1}

The presence of large concentrations of lignin in LS considered an asset for their use since the enzymes produced by fungi to degrade lignin have been implicated in the degradation of many toxic chemicals and lignin has been shown to enhance the enzyme activity (Leung and Ponting, 2002). Despite the fact that phenol degradation has been studied, there are no studies comparing extracellular enzyme levels responsible for phenol metabolism. Hence in the present study, the fungus isolated from soil samples enriched with continuous paper mill effluent irrigation over 20 years and its extracellular laccase enzyme activities during pentachlorophenol degradation was investigated using ammonium lignosulphonate.

MATERIALS AND METHODS

Chemicals: Pentachlorophenol (Sigma chemical Co, USA), agar, ^{14}C DHP (dehydro polymer) synthetic lignin, 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 (Jenson, 1968) at Bioremediation Laboratory of Tamil Nadu Agricultural University, Coimbatotre during 2003. 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 cultures. Lignin degradation by the fungus 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 (Ramasamy, 1993).

The isolated fungus was grown at 30°C for 5 days on CPDA medium [20.0 g glucose, 1.0 g KH_2PO_3, 1.5 g $MgSO_4.7H_2O$, 50 µg vitamin B1, 15.0 g agar powder and 1,000 mL potato extract liquid (20%)] for its growth. The pure strains were stored at 4°C in CPDA slants and inoculated once in every 3 months.

Media preparation and growth of fungus in LS: In order to determine the usefulness of LS for degradation of PCP, we measured the disappearance of PCP in static flask cultures using LS as either the sole carbon and nitrogen sources (2% LS) or as the sole nitrogen source (0.23% LS with 0.5% glucose). The other nitrogen deficient 2% glucose liquid medium used in the experiment was prepared according to Alleman *et al.* (1995). LS used in two other media were prepared by weight/volume basis (0.23 and 2%). A nitrogen deficient medium using LS as the sole nitrogen source was prepared by substituting 0.23% of LS for the 0.178 g L^{-1} of NH_4NO_3 in the nitrogen deficient 2% glucose liquid medium. This 0.23% LS medium therefore contained the same concentration of ammonia as a standard nitrogen deficient medium. No glucose or NH_4NO_3 were used for the 2% LS medium (a nitrogen sufficient medium defined as medium containing >2 g L^{-1} of NH_3 since ammonia and sugars already in the LS solution served as the nitrogen and carbon sources. In order to determine the effect of LS on the growth in static flask cultures, the fungus was incubated isothermally at 30°C with pH of 6. The flasks were incubated at 30°C for seven days under static conditions. After seven days of incubation, the biomass production was estimated. The mycelial growth was separated by filtration, dried at 80°C for 3 h and estimated as fungal biomass and expressed in g L^{-1}.

Pentachlorophenol degradation: The extent of PCP degradation in static flask cultures was evaluated based on the disappearance of the PCP. PCP was added by injecting 10 µL of a 15 mg PCP mL^{-1} ethanol solution into five Erlenmeyer flask 3 days after inoculation with fungi. On days 5, 6, 7 and 8, the contents of each flask including mycelium were poured into a stainless steel blender. To remove mycelium and PCP that was attached to the sides of the flask was rinsed with 3 mL ultra pure water, 1 mL 95% ethanol and 3 mL ultra pure water. In order to ensure

both the liquid and mycelia samples were homogenous, this mixture was blended on high speed for 2 min. This suspension was then centrifuged at 4° C for 30 min to separate mycelium from the liquid medium. The total PCP in liquid was measured directly by injecting 20 μL into a high performance liquid chromatograph (HPLC, Varian) equipped with a C-18 reverse phase column. The mobile phase was a 75:25:0.125 mixture of acetonitrile: water: acetic acid. PCP was monitored at 238 nm and peak counts compared to a standard calibration curve.

Assays of extracellular enzyme: The laccase (p-diphenol oxygen oxidoreductase) enzyme was assayed during PCP degradation and the assay was performed based on monitoring the rate of oxidation of syringaldazine (Sealey and Ragauskas, 1998) in a spectrophotometer (ECIL, Hyderabad). The laccase plate assay was done as per the method of Srinivasan *et al.* (1995).

RESULTS AND DISCUSSION

Identification of fungus: The isolated fungus was identified as *Phanerochaete chrysosporium* (TL 1) based on morphology, physiological and biochemical characteristics and verified at Mycology department of Indian Agricultural Research Institute, New Delhi. 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 released 33.6% of $^{14}CO_2$ and the per cent utilization of synthetic lignin for *Phanerochaete chrysosporium* (TL 1) was 44.3 and fixed as cell carbon. 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 (D'Souza *et al.*, 1999). The non-specific oxidation caused by enzymatic combustion leads to the formation of CO_2.

Growth of *Phanerochaete chrysosporium* (TL 1) in lignosulphonate medium: Growth of *Phanerochaete chrysosporium* (TL 1) in lignosulphonate and glucose medium is presented in Fig. 2. In general, the growth of *Phanerochaete chrysosporium* is not affected much in lignosulphonate medium. The initial growth was more in glucose medium compared to lignosulphonate medium and later the growth was comparable that of glucose medium. Although a separate experiment showed a 2% LS solution could support fungal growth with no other additions to the medium (data not shown). We used LS to replace only carbon and/or nitrogen so the comparison of PCP removal with a standard nitrogen limited glucose

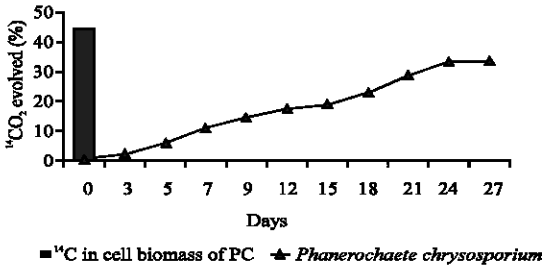

Fig.1: Degradation of ^{14}C DHP synthetic lignin by *Phanerochaet chrysosporium* (TL 1)

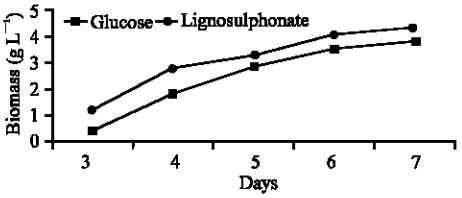

Fig. 2: Growth of *Phanerochaete chrysosporium* (TL 1) on glucose and LS medium

medium reflected only one variable. The growth of fungi mainly depends on the biomass production and the PCP removal by the fungi for growth was also a function of PCP dose. Similar results were reported by Alleman *et al.* (1992), who stated that Trametes versicolor was able to grow up to 100 ppm of PCP.

Pentachloro phenol removal and degradation: We compared the ability of fungus grown in a LS medium to degrade PCP with fungus grown in a nitrogen limited 2% glucose medium by measuring the disappearance of PCP from solution. Pentachlorophenol was successfully degraded by *Phanerochaete chrysosporium* (TL 1) grown in media containing LS but lesser extent than cultures grown in glucose medium. Cultures of *Phanerochaete chrysosporium* (TL 1) grown on nitrogen limited glucose medium removed 93% of initial PCP during five days of incubation. When 2% LS was used as the nitrogen source and carbon source, PCP removal was 85%. Similarly, when LS was used as a nitrogen source, PCP removal was 86% (Table 2) and the overall PCP degradation in the two media containing LS (2 and 0.23%) was more or less similar. Recovery of PCP from heat killed control cultures appeared to be unaffected. It is reasonable to assume that mineralization would follow dehalogenation since aromatic compounds such as phenol have been shown to serve as sole carbon sources for growth of *P. chrysosporium* (Krivobok, 1994). Other investigators have generally observed more rapid removals of PCP by *Phanerochaete chrysosporium* in liquid cultures (Lamar *et al.*, 1993) than

Table 2: Degradation of pentachloro phenol by *Phanerochaete chrysosporium* (TL 1)

	Pentachloro phenol degradation				
Treatments	5 DAI	6 DAI	7 DAI	8 DAI	Mean
T1 - Control	150	150	150	150.0	150.0
T2 - 2% LS + *P. chrysosporium*	29.2	22.0	20.3	17.1	22.1
T3 - 0.23% LS + 0.5% Glucose + *P. chrysosporium*	25.5	21.0	18.0	15.0	19.9
T4 - 2% Glucose + *P. chrysosporium*	14.5	11.5	9.50	9.0	11.1
Mean	54.8	51.1	49.5	47.7	
		SEd	CD (p = 0.05)		
	Treatments (T)	1.08	2.16		
	Days (D)	1.06	2.13		
	Interaction (T×D)	2.15	4.31		

Table 3: Production of laccase by *Phanerochaete chrysosporium* (TL 1) during PCP degradation

	Laccase activity			
Treatments	5 DAI	6 DAI	7 DAI	8 DAI
T1 - Control	-	-	-	-
T2 - 2% LS + *P. chrysosporium*	0.135	0.285	0.581	0.540
T3 - 0.23% LS + 0.5% Glucose + *P. chrysosporium*	0.152	0.305	0.624	0.598
T4 - 2% Glucose + *P. chrysosporium*	0.184	0.401	0.751	0.720

One unit of enzyme activity is defined as that amount which at optimal pH caused a change in absorbance of 0.001 min^{-1} mL^{-1} at 530 nm. Laccase assay was performed based on monitoring the rate of oxidation of syringaldazine in a spectrophotometer. The assay mixture contained 2.2 mL of 100 mM potassium phosphate buffer (pH 7.0), 0.3 mL of 0.216 mM syringaldazine in methanol and 1.0 mL of the enzyme source at 24°C

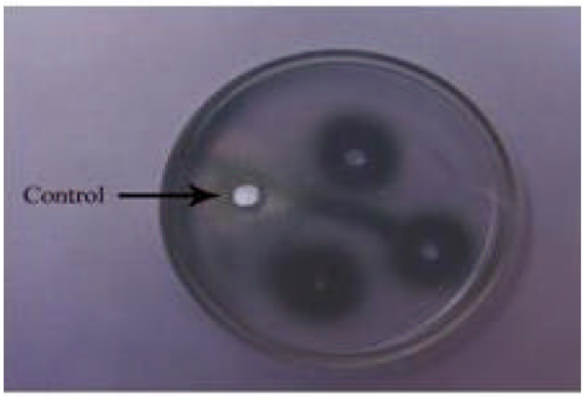

Fig. 3: Plate assay for laccase activity by *Phanerochaete chrysosporium* (TL 1)

observed here. Since the LS inhibit Laccase enzyme activity, the percent PCP degradation was reduced for fungi grown in LS media compared to nitrogen deficient glucose medium.

Effect of Ls on laccase activity: Since laccase enzyme has been shown to be important in the breakdown of some chemicals, we examined the effect of LS on laccase activity. In all the cases the addition of LS to culture media inhibited laccase activity. The activity was more in 2% glucose medium compared to 0.23% LS + 0.5% Glucose medium. Lesser activity was measured in 2% LS medium (Table 3). The possible reason for low laccase activity in LS medium was since, LS are extracted from wood, and there may be sufficient manganese, which suppress laccase production (Aiken and Logan, 1996). The higher concentration of manganese also causes the preferential production of MnP over laccase (Bonnarme and Jeffries, 1990).

Plate assay for laccase activity: The production of extracellular laccase by *Phanerochaete chrysosporium* (TL 1) was assayed on agarose plates containing ABTS. The results of laccase plate assay showed the presence of laccase activity in the extracellular fluid of *Phanerochaete chrysosporium* (TL 1) but not in the heat treated extracellular fluid (negative control). The development of an intense bluish green color showed the presence of laccase activity (Fig. 3).

CONCLUSIONS

In spite of the limitations resulting from the presence of lignosulphonates (lesser laccase activity and PCP degradation) the isolated *Phanerochaete chrysosporium* (TL 1) was able to degrade a significant fraction of PCP when grown in media containing LS. Since the fungus will grow on LS alone, LS could be useful in PCP removal and pretreatment of LS waste streams.

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