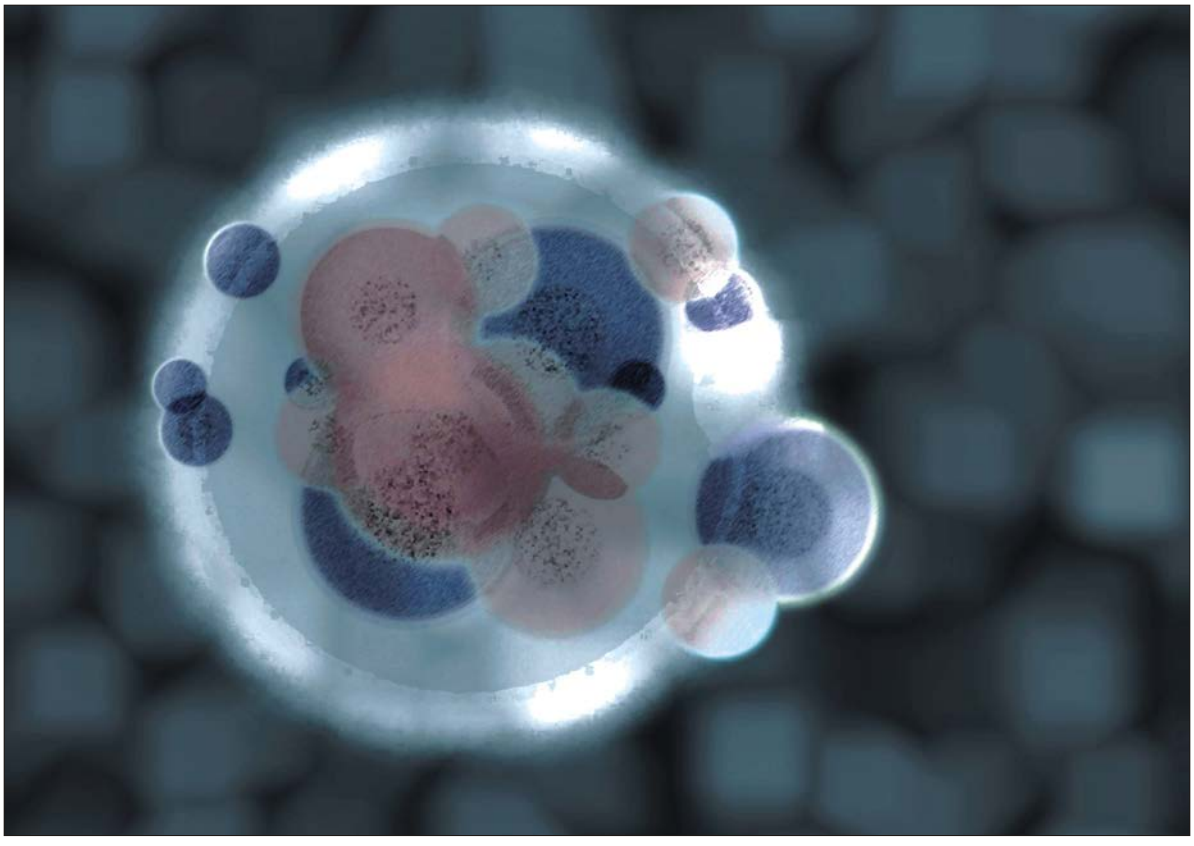

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Biodegradation of Phenols by Ligninolytic Fungus *Trametes versicolor*

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Abstract: The white rot fungus isolated from paper mill effluent enriched soil samples and identified as *Trametes versicolor* was capable of degrading phenol (Mono, di hydroxy and methoxy) compounds.^{14}C synthetic lignin mineralization assays showed that *Trametes versicolor* assimilated 24.3% of the total label. During five days of incubation period, 71% of para-hydroxy benzoic acid was utilized by *Trametes versicolor* when glucose used as a co-substrate and 56% degradation of protocatechuic acid was achieved using fructose. The presence of laccase (EC.1.10.3.2) and polyphenol oxidase (EC.1.10.3.0) extracellular activity suggested that the fungus secrete these enzymes into the extracellular medium and the extracellular laccase activity was assayed on agarose plates containing ABTS.

Key words: Extra cellular enzyme, phenol, white rot fungus

INTRODUCTION

Phenolic compounds are toxic to all organisms. It is listed as a priority pollutant by the US EPA (code U188) and the lethal dose for humans is reported as 5-10 mg kg^{-1} body weight. Phenols enter the surface water from variety of industries like pulp and paper mill, leather, polymer, oil refinery, coal cabonization etc.[1]. Phenolic wastes are treated by several physico-chemical methods like ozonisation, adsorption, reverse osmosis, electrolytic oxidation, photocatalysis[2]. But all the above methods have its own difficulties for e.g. electrochemical, reverse osmosis, photo chemical and ozonisation are costly process and physical adsorption results in sludge disposal problem etc. Despite the toxicity of phenol and its derivatives, often present in effluents of many industrial processes[3], numerous microorganisms can degrade these compounds when they are present at low concentrations[4,5]. The realization of the high cost of physico-chemical methods and ability of microorganisms on phenol degradation have opened the door of bioremediation methods.

Despite the fact that phenol degradation has been studied, there are no studies comparing extracellular enzyme levels responsible for phenol metabolism. However, some fungi show that extracellular phenol oxidases are utilized in degradation of lignin, a complex phenolic compound[6]. Hence in the present study, the fungus isolated from soil samples enriched with continuous paper mill effluent irrigation over 20 years and its phenol degrading ability along with the extracellular phenol degrading enzyme activities were investigated.

MATERIALS AND METHODS

Chemicals: Phenol (Mono hydroxy, Dihydroxy and methoxy phenol), 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 fungi was isolated from enriched soil samples with continuous pulp and paper mill effluent irrigation over 20 years by employing standard serial dilution plating technique[7]. 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. The culture was further tested for their rapid phenol degradation by using media containing 150 mg L^{-1} of phenol.

Degradation of lignin: Lignin degradation by fungal culture was confirmed by quantifying the $^{14}CO_2$ produced

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during the metabolism of ^{14}C labeled synthetic lignin obtained by polymerization of labeled coniferyl alcohol [8].

Identification of isolated fungi: The isolated fungal culture 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 g vitamin B1, 15.0 g agar powder and 1,000 mL potato extract liquid (20%)]. A glass slide was taken and a drop of lactophenol plus cotton blue fluid was mounted on the centre of a glass slide. A portion of mycelial mat from the colony was transferred into the drop of lactophenol plus cotton blue with the help of flamed and cooled needle. With the help of two needles the propagules were gently spread, so that the mycelia mixed with the stain. The slides were observed under low and high power objectives of a microscope (Nikon, Japan) and the types of conidia, hyphae and their arrangement were noted and based on colony and cell morphology, physiological and biochemical characteristics the fungus was identified. The pure strain was stored at 4°C in CPDA slants and inoculated once in every 3 months.

Phenol degradation: The phenol degradation efficiency of the isolated fungus was studied under lab conditions using different phenol compounds as sole source of carbon and nitrogen in Mineral Salt Medium (MSM) with (glucose, starch and fructose) and without co substrates. The MSM 1 L (pH 7.0) consisted of 30 g K_2HPO_4, 30 g KH_2PO_4, 20 g $(NH_4)_2Cl$, 0.5 g $MgSO_4.7H_2O$, 0.05 g $FeSO_4$, 0.005 g Na_2MoO_4 and 1000 mL distilled water. The initial experiment involves standardization of appropriate concentration of phenol for degradation. The different concentrations of phenol for optimization were 100, 150 and 200 ppm. From the growth of the fungus at different concentrations, the concentration of phenol was fixed at 150 ppm. The different phenol compounds used to confirm the degradation efficiency were monohydroxy phenol (p-hydroxy benzoic acid), dihydroxy phenol (protocatechuic acid) and methoxy phenol (vanillin).

The assay for degradation of phenolic compounds was carried out by the addition of 150 ppm of phenolic compounds in 100 mL MSM in a 250 mL Erlenmeyer flask. Different carbon sources viz., glucose, starch and fructose were added individually to the Erlenmeyer flasks at one percent level and the whole content was sterilized by autoclaving and then inoculated with 2 mL of fungal spore suspension along with heat killed culture (control). Phenol compounds without carbon sources were also taken as one treatment. The inoculated flasks were incubated at room temperature and samples were collected at three, four and five Days After Incubation (DAI). For analysis of phenol remaining in the medium, one mL of culture filtrate was removed and diluted with 5 mL of 95% ethanol. After 4 h, the UV visible absorption spectrum was recorded. The percent degradation for each compound was measured by the reduction in absorbance at the wavelength of maximal absorbance (248 to 362 nm) relative to the absorbance of the compound incubated with an identical heat killed culture (control). Heat killing was accomplished by autoclaving cultures at 116°C for 5 min. The treatments were replicated four times. The heat killed fungus was kept as control and incubated with phenol.

Assay of extracellular enzymes: The enzymes assayed were Laccase (p-diphenol oxygen oxidoreductase) and Polyphenol oxidase (Tyrosine, Catechol oxidase or o- diphenol oxygen oxidoreductase). Laccase assay was performed based on monitoring the rate of oxidation of syringaldazine [9]. Polyphenol oxidase enzyme assay was performed following the method of Mahadevan and Sridhar[10].

RESULTS

Identification of fungus: The isolated fungus was identified as *Trametes versicolor* based on their morphology, physiological and biochemical characteristics and verified at Mycology Department of Indian Agricultural Research Institute, New Delhi and the fungus has been referred many times in the literature as phenol degrader.

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.

Phenol degradation:

Monohydroxy phenol: The effect of *Trametes versicolor* on degradation of para-hydroxy benzoic acid indicated that the fungus could degrade the para-hydroxy benzoic

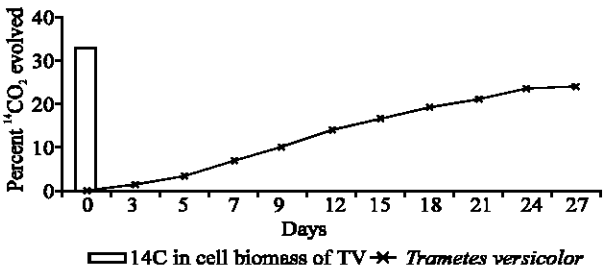

Fig. 1: Confirmation of ligninolytic activity by *Trametes versicolor*

acid from 150 to 46 ppm during the five days of experimental period. The mean para-hydroxy benzoic acid reduced from 114 ppm at 3 DAI to 46 ppm at 5 DAI. The addition of co-substrates viz., glucose, fructose and starch increased the degradation than addition of para-hydroxy benzoic acid alone and glucose addition along with the fungus caused significantly higher degradation (150 to 44 ppm) of para-hydroxy benzoic acid (Table 1).

Dihydroxy phenol: The percent degradation of protocatechuic acid a dihydroxy phenol by *Trametes versicolor* was lesser than monohydroxy phenol (Table 2). The protocatechuic acid reduced to 61 ppm by the fungus during the five days of incubation period and addition of co-substrates did not cause much degradation as that of parahydroxy benzoic acid.

Methoxy phenol: The fungus *Trametes versicolor* degrades vanillin, a methoxy phenol easily as that of monohydroxy phenol. The degradation of vanillin was 150 to 48 ppm within five days and the degradation was enhanced by addition of co-substrates viz., glucose and fructose than starch. The vanillin reduced from 150 to 44 ppm by addition of glucose than fructose (47 ppm). The degradation was low in control heat killed culture (Table 3).

Table 1: Degradation of monohydroxy phenol (para-hydroxy benzoic acid) by *Trametes versicolor*

	Trametes versicolor			
Treatments	3 DAI	4 DAI	5 DAI	Mean
Control	150	149	148	149
Phenol + Fungus	105	45	29	59
Phenol + Fungus + Glucose	95	22	15	44
Phenol + Fungus + Fructose	112	39	22	57
Phenol + Fungus + Starch	108	42	18	56
Mean	114	59	46	

	Sed	CD (p = 0.05)
Treatments (T)	1.56	3.15
Days (D)	1.21	2.44
Interaction (T x D)	2.71	5.46

(Phenol - Para-hydroxy benzoic acid; F-Fungi; G-Glucose; Fr- Fmctose; S- Starch; DAI- Days After Inoculation)

Table 2: Degradation of dihydroxy phenol (protocatechuic acid) by *Trametes versicolor*

	Trametes versicolor			
Treatments	3 DAI	4 DAI	5 DAI	Mean
Control	150	148	148	148
Phenol + Fungus	119	84	58	87
Phenol + Fungus + Glucose	130	55	38	74
Phenol + Fungus +Fructose	125	46	28	66
Phenol + Fungus + Starch	130	54	35	73
Mean	130	77	61	

	Sed	CD (p=0.05)
Treatments (T)	1.77	3.57
Days (D)	1.37	2.76
Interaction (T x D)	3.07	6.19

(Phenol-Protocatechuic acid; F-Fungi; G-Glucose; Fr- Fructose; S-Starch; DAI- Days After Inoculation)

Table 3: Degradation of methoxy phenol (vanillin) by *Trametes versicolor*

	Trametes versicolor			
Treatments	3 DAI	4 DAI	5 DAI	Mean
Control	150	148	147	148
Phenol + Fungus	102	42	28	57
Phenol + Fungus + Glucose	85	30	19	44
Phenol + Fungus +Fructose	83	35	24	47
Phenol + Fungus + Starch	105	27	22	51
Mean	105	56	48	

	Sed	CD (p=0.05)
Treatments (T)	1.49	3.01
Days (D)	1.16	2.33
Interaction (T x D)	2.59	5.23

(Phenol-Vanillin; F-Fungi; G-Glucose; Fr-Fructose; S- Starch; DAI- Days After Inoculation)

Table 4: Production of laccase and polyphenol oxidase by *Trametes versicolor*

	Days of incubation (Mean of three cultivations)		
Enzyme	3	4	5
Laccase	0.091	0.234	0.611
Polyphenol oxidase	0.065	0.301	0.162

The assay of enzymes: The assay of enzymes laccase and polyphenol oxidase involved in ligninolytic system of *Trametes versicolor* were assessed in the crude cell extract of the culture. The maximum laccase activity was observed at 5 days of growth. Whereas the peak activity of polyphenol oxidase was observed at 4 days of growth and later it reduced (Table 4).

DISCUSSION

The multitude of inter unit 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 14 C 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[11]. The non-specific oxidation caused by enzymatic combustion leads to the formation of CO_2.

Phenol degradation by the isolated fungi: During the five days of incubation, 71% of para-hydroxy benzoic acid was utilized by *Trametes versicolor,* when glucose was added as a co-substrate. The degradation was similar in other co-substrates viz., fructose and starch and was more or less equal with or with out co-substrate addition. Thus fructose and starch have negative effect on their utilization by the fungus during the degradation process.

Efficient degradation of para-hydroxy benzoic acid by the fungus in the presence of glucose due to high induction of peroxidase. This is supported by the findings of Perestcol *et al.*[12] who showed the necessity for the presence of glucose in the lignin degradation. This corroborates with the findings of Elangovan *et al.*[13] who confirmed that there was 87% of monohydroxyphenol degradation by *Flavobacterium* sp. in the presence of glucose.

The dihydroxy phenol (protocatechuic acid) degradation was more in fructose added treatment (56%) for *Trametes versicolor* than other co-substrates. This is due to induction of lignin degradation enzymes by the fungus added with fructose as reported by Elangovan *et al.*[13]. The fungus *Trametes versicolor* degraded the methoxy phenol (vanillin) in the presence of co-substrates. Among them, the fungus responded more to glucose and fructose than starch. Since vanillin and related acids are prominent intermediate of lignin degradation enzymes, vanillin was degraded easily by the fungus. Vanillin was oxidatively decarboxylated by these fungi and subsequently demethylated to form hydroxyl quinol. The aromatic rings of the hydroxyl quinol were rapidly metabolized further by the fungus as shown by Hata[14]. Present results revealed the possibility of this phenol degradation by extra cellular enzymes and confirmed by laccase and polyphenol oxidase activities in the supernatant. Working with pH measurements on crude cell extracts, they showed that the enzymatic activity depends on the micro organism and the phenol quantity in the growth medium during sample collection. Since, phenol oxidation is an inductive process; enzyme production could vary under different growth conditions.

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