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Phenol Metabolism by White Rot Fungus *Phanerochaete chrysosporium* Isolated from Indian Paper Mill Effluent Enriched Soil Samples

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Abstract: The white rot fungus *Phanerochaete chrysosporium* isolated from paper mill effluent enriched soil samples degrading various phenol (Mono, di hydroxy and methoxy) compounds. During five days of incubation period, 74% of para-hydroxy benzoic acid was utilized by the isolated *Phanerochaete chrysosporium* when glucose used as a co-substrate. There was 57% degradation of protocatechuic acid using fructose. The fungus degraded vanillin in the presence of co-substrates and the response was more in glucose and fructose than starch. The presence of laccase (EC.1.10.3.2) and polyphenol oxidase (EC.1.10.3.0) extracellular activity suggested that the microorganism secrete these enzymes into the extracellular medium.

Key words: Whiter rot fungus, phenol and extra cellular enzymes

INTRODUCTION

There is hardly any industry that does not add wastes into the environment. The introduction of contaminants through effluent and sludge to different environmental compartments, can often over helm the self-cleaning capacity of recipient ecosystems and thus result in the accumulation of pollutants to problematic or even harmful levels. Despite the toxicity of phenol and its derivatives, often present in effluents of many industrial processes[1], numerous microorganisms can degrade these compounds when they are present at low concentrations[2]. However, in media enriched with micronutrients, some *Pseudomonas* isolates can utilize phenol as the sole carbon source at concentrations up to 1000 ppm[3]. 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[4]. 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, CPDA medium, 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[5]. 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 its phenol degradation ability by using media containing 150 mg L^{-1} of phenol.

Identification of isolated fungi: The isolated fungal culture was identified based on colony and cell morphology, physiological and biochemical characteristics and verified at Mycology Department of Indian Agricultural Research Institute (IARI), New Delhi. 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

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(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 $FeSO_4$, 0.005g 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 fungi 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 mLErlenmeyer flask. Different carbon sources viz., glucose, fructose and starch were added individually to the Erlenmeyer flasks at 1% level and the whole content was sterilized by autoclaving and then inoculated individually 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 Inoculation (DAI). For analysis of phenol remaining in the medium, 1 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.

Assay of extracellular enzymes: The enzymes assayed were Laccase (p-diphenol oxygen oxido reductase) and Polyphenol oxidase (Tyrosine, Catechol oxidase or o-diphenol oxygen oxidoreductase). The culture was grown in MSM medium along with 150 ppm of phenol for enzyme production as stationary culture at 30°C for 3, 4 and 5 days. The cells were ruptured by freezing at -20°C and then ground in a pestle and mortar containing acid washed sand. Sand, cells, cell debris were removed by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant containing the enzyme with 0.1% sodium azide was stored at 4°C for further enzyme assays. Laccase assay was performed based on monitoring the rate of oxidation of syringaldazine in a spectrophotometer (ECIL, Hyderabad). The assay mixture contained 2.2 mL of 100 mM potassium phosphate buffer (pH 7.0), 0.3 mL of 0.216 mM syringaldazine (Sigma Chemical Co., USA) in methanol and 1.0 mL of the enzyme source at 24°C. 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. Polyphenol oxidase enzyme assay was performed[6]. The activity was estimated by measuring the formation of dopochrome 2-carboxy-2,3-dihydroindole-5,6 quinone) from DOPA (3,4-dihydroxy phenylalanine). The assay mixture consisted of 3.5 mL of 0.2 M phosphate buffer (pH 6.0) and 0.5 mL of L-DOPA. The mixture was equilibrated in water bath at 30°C and then 0.5 mL of the enzyme preparation was added after incubating the mixture at 30°C for 5 min. The absorbance was measured at 475 nm. Controls were maintained with heat killed enzyme. One unit of enzyme activity is defined as change in absorbance of 0.001 cm^{-1} min^{-1}, which was equivalent to $27x10^{-7}$ M of dopachrome formed using the molar extinction coefficient.

RESULTS

Identification of fungus: The isolated fungus was identified as *Phanerochaete chrysosporium* based on its morphology, physiological and biochemical characteristics and verified at Mycology Department of Indian Agricultural Research Institute, New Delhi.

Phenol degradation

Monohydroxy phenol: The effect of *Phanerochaete chrysosporium* on degradation of para-hydroxy benzoic acid indicated that the fungus could degrade the para-hydroxy benzoic acid from 150 to 42 ppm during the five days of experimental period. The mean para-hydroxy benzoic acid reduced from 109 ppm at 3 DAI to 42 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 39 ppm) of para-hydroxy benzoic acid (Table 1).

Dihydroxy phenol: The percent degradation of protocatechuic acid a dihydroxy phenol by *Phanerochaete chrysosporium* was lesser than monohydroxy phenol (Table 2). The protocatechuic acid reduced to 56 ppm 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 fungi *Phanerochaete chrysosporium* degrade vanillin, a methoxy phenol easily as that of monohydroxy phenol. The degradation of vanillin was 150 to 43 ppm within five days and the

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

	Phanerochaete chrysosporium			
Treatments	3 DAI	4 DAI	5 DAI	Mean
Control	148	147	145	146
Para-hydroxy benzoic acid+Fungus	98	40	25	54
Para-hydroxy benzoic acid+Fungus+lucose	92	15	10	39
Para-hydroxy benzoic acid+Fungus+ructose	105	34	18	52
Para-hydroxy benzoic acid+Fungus+Starch	102	37	12	50
Mean	109	54	42	

The degradation of monohydroxy phenol (para-hydroxy benzoic acid) was measured by the reduction in absorbance at the wavelength of maximal absorbance (248 nm) relative to the absorbance of the compound incubated with an identical heat killed culture (control)

	SEd	CD
Treatments (T)	1.50	3.00
Days (D)	1.16	2.33
Interaction (TxD)	2.61	5.23

Table 2: Degradation of dihydroxy phenol (protocatechuic acid) by *Phanerochaete chrysosporium*

	Phanerochaete chrysosporium			
Treatments	3 DAI	4 DAI	5 DAI	Mean
Control	150	149	147	148
Protocatechuic acid+Fungus	115	75	51	80
Protocatechuic acid+Fungus+Glucose	128	46	32	68
Protocatechuic acid+Fungus+Fructose	127	44	25	65
Protocatechuic acid+Fungu+Starch	132	41	27	66
Mean	130	71	56	

The degradation of dihydroxy phenol (protocatechuic acid) was measured by the reduction in absorbance at the wavelength of maximal absorbance (251 nm) relative to the absorbance of the compound incubated with an identical heat killed culture (control)

	SEd	CD (p=0.05)
Treatments (T)	1.73	3.50
Days (D)	1.34	2.71
Interaction (TxD)	3.01	6.06

Table 3: Degradation of methoxy phenol (vanillin) by *Phanerochaete chrysosporium*

	Phanerochaete chrysosporium			
Treatments	3 DAI	4 DAI	5 DAI	Mean
Control	148	147	146	147
Vanillin+Fungus	95	38	22	51
Vanillin+Fungus+Glucose	81	26	15	40
Vanillin+Fungus+Fructose	78	29	18	41
Vanillin+Fungus+Starch	108	22	16	48
Mean	102	52	43	

The degradation of methoxy phenol (vanillin) was measured by the reduction in absorbance at the wavelength of maximal absorbance (362 nm) relative to the absorbance of the compound incubated with an identical heat killed culture (control)

	SEd	CD (p=0.05)
Treatments(T)	1.46	2.94
Days (D)	1.13	2.27
Interaction (TxD)	2.53	5.09

degradation was enhanced by addition of co-substrates viz., glucose, and fructose than starch. The vanillin reduced from 150 to 40 ppm (Table 3) by addition of glucose and closely followed by fructose (41 ppm). The degradation was low in control (heat killed culture)

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

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

Table 4: Production of laccase by *Phanerochaete chrysosporium*

	Days of incubation (mean of three cultivations)		
Culture	3	4	5
Phanerochaete chrysosporium	0.144	0.392	0.725

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. 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

Table 5: Production of polyphenol oxidase by *Phanerochaete chrysosporium*

	Days of incubation (mean of three cultivations)		
Culture	3	4	5
Phanerochaete chrysosporium	0.078	0.380	0.205

One unit of enzyme activity is defined as the change in absorbance of 0.001 min^{-1} mg of $protein^{-1}$ at 495 nm. One unit of enzyme activity is defined as change in absorbance of 0.001 cm^{-1} min^{-1} which, was equivalent to $27x10^{-7}$ M of dopachrome formed using the molar extinction coefficient

biological material. The present study 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 confirmed as *Phanerochaete chrysosporium.*

Phenol degradation by the isolated fungus: During the five days of incubation, 74% of para-hydroxy benzoic acid was utilized by *Phanerochaete chrysosporium* when glucose was added as a co-substrate. Efficient degradation of para-hydroxy benzoic acid by the fungus in the presence of glucose might be due to high induction of peroxidase. This is supported by the findings of Peresteol *et al.*[7] and Elangovan *et al.*[8]who showed the necessity for the presence of glucose in the lignin degradation. The dihydroxy phenol (protocatechuic acid) degradation was more in fructose added treatment (57%) for *Phanerochaete chrysosporium* than other co-substrates. This showed the induction of lignin degradation enzymes by the fungus when added with fructose as reported by Elangovan *et al.*[8]. The fungus *Phanerochaete chrysosporium* 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, the fungus degraded vanillin easily. Vanillin was oxidatively decarboxylated by the fungus and subsequently demethylated to form hydroxyl quinol. The aromatic rings of the hydroxyl quinol was rapidly metabolized further by fungus as shown by Hata[9]. Present results revealed that there is a possibility of these 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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