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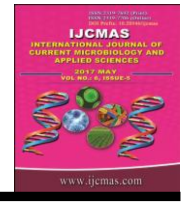


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# Ligninolytic Enzyme Production by White Rot Fungi *Podoscypha elegans* Strain FTG4

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**A B S T R A C T**

The aim of this study to investigate the laccase, lignin peroxidase and manganese peroxidase enzyme production by white rot fungi *Podoscypha elegans*, A white rot fungi *P. elegans* was collected from the Bilaspur district of Chhattisgarh, India, then fungi was cultivated in the ligninolytic enzyme screening medium containing indicator compound, after that quantify the production of enzymes. The maximum 1013 U/L laccase, 48.24 U/mg Specific laccase activity, 1509 U/L lignin peroxidase, 71.86 U/mg Specific lignin peroxidase activity, 14230 U/L manganese peroxidase, 677.62 U/mg Specific manganese peroxidase activity and 358 mg biomass were found in the mineral salt broth. Ligninolytic enzymes are responsible for the lignin and organic pollutant degradation therefore *P. elegans* can be used for the degradation of pollutants from the environment.

## Introduction

The white rot fungi are considered for their unique ability to degrade a heterogenous polyphenolic polymer lignin (Baldrian, 2003; Pointing, 2001). Besides of these, white rot fungi are also able to degrade several other organicpolluttants which are structurally similar to lignin (Tuomela and Hatakka, 2011). White rot fungi produced non-specific extracellular ligninolytic enzymes such as laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) (Kitamura *et al*., 2005). These ligninolytic enzymes play an important role in the transformation and mineralization of various organic pollutants (Lee *et al*., 2004; Casas *et al*., 2009; Wang *et al*., 2009), delignify different substrates like paper, animal feed (Lu *et al*., 2010; Salvachua *et al*., 2011) for the production of biofuels (Placido and Capareda, 2015).

Laccase is a multi copper containing enzyme (EC 1.10.3.2: benzenediol, oxygenoxido reductase or p-diphenol oxidase), catalyze the oxidation of aromatic and non-aromatic substrates by a radical catalyzed mechanism (Claus, 2004). Laccase is involved in the dye decolourization (Dominguez *et al*., 2005; Hou *et al*., 2004), food processing (Couto and Herrera, 2006), plant pathogenesis in nature (Geiger *et al*., 1986) paper and pulp industries, bioremediation and biodegradation (Pointing, 2001), and sporulation in fungi (Leatham and Stahmann, 1981). LiP (EC 1.11.1.14, 1, 2-bis (3,4-dimethoxyphenyl) propane-1,3-diol:hydrogen-peroxide oxido reductase) catalyze the hydrogen peroxidase dependent depolymerisation of lignin and other phenol, non-phenolic lignin model compounds (Tien and Kirt, 1983; Hammel *et al*., 1993). MnP (EC 1.11.1.13 Mn (II): hydrogen-peroxide oxidoreductase) catalyze the Mn dependent reaction (Orth and Tien, 1995). MnP oxidize Mn (II) to Mn (III), which catalyze the oxidation of phenolic compounds, including dyes (Wong, 2009).

The study of ligninolytic enzyme production has been carried out in different white rot fungi such as *Ganoderma lucidum*, *Trametes versicolor*, *Dichomitussqualens*, *Phanerochaete chrysosporium*, *Phlebia fascicularia*, *Pleurotus ostreatus*, *Armillaria* sp. F022 (Sasidhara *et al*., 2014; Arora and Gill, 2000; Hadibarata and Kristanti, 2013). But the production of ligninolytic enzyme is less studied in *P. elegans*. Due to the potential application of ligninolytic enzyme, our aim to study the ligninolytic enzyme production in *P. elegans*, isolated from Bilaspur district of Chhattisgarh, India, for the further use in the process of remediation of organic pollutants.

## Materials and Methods

### Chemicals

Guaiacol, Azure B, 2, 6 - dimethoxy phenol (2, 6 - DMP) was purchased from Himedia, India. All other chemicals and solvents used were of analytical grade purchased from Himedia, India.

### Microorganism and culture condition

A white rot fungi strain FTG4 (Accession no. KY464924.1), was collected from the wood surface from the Bilaspur district of Latitude 22º 7′ 50.1204″, Longitude 82º 8′ 31.2252″ and Elevation 280.0 meters) Chhattisgarh, India, fungi were isolated from the fruiting body by the spore drop method according to Choi *et al*., 1999. A piece of fruiting body was cut and transferred in the top of a petri dish containing Sabouraud Dextrose Agar (SDA) media composition (g/L): Dextrose (40), Peptone (10), Agar (15) and streptomycin (500 mg/L) antibiotic to inhibit the bacterial growth) then incubate at 27ºC, once the pure culture was obtained, culture was maintained in SDA media prior to use and stored at 4ºC. On the basis of morphological identification and molecular characterization through 18S rRNA cultured fungi are the member of family *Podoscyphaceae* and show 99% similarity to the genus *Podoscypha*.

### Qualitative screening for ligninolytic enzyme

The ability of the white rot fungi *P. elegans* to secrete extracellular ligninolytic enzyme was screened on the basis of oxidation of indicator compound in the screening medium. The method of enzyme production is as follows:

### Laccase enzyme activity

To access laccase assay 6 mm diameter one fungal disc (taken from the periphery of the 7 day old cultures grown in SDA media) of FTG4 strain was transferred on the petri dish (15 cm in diameter) containing 25 mL of Potato dextrose agar media (PDA g/L: Potato infusion - 200, Dextrose - 20, Agar - 20) with 0.01% Guaiacol (D'Souza *et al*., 2006). After that Plates were incubated at 27°C for 7 days in a static incubator and the change in the colour of the media around the mycellium was investigated.

### Lignin peroxidase enzyme activity

Lignin Peroxidase screening medium (g/L: Glucose - 4.0, Glycerol - 0.7, L histidine - 0.05, $CuSO_4$ - 0.01, $NaNO_3$ - 0.18, NaCl - 0.18, KCl - 0.05, $CaCl_2.H_2O$ - 0.05, $KH_2PO_4$ - 0.1, $FeSO_4.H_2O$ - 0.005, $MgSO_4.7H_2O$ - 0.05, Guaiacol – 10 mM (v/v), $H_2O_2$ – 10 mM, Agar - 2.0) was used for the lignin peroxidase enzyme assay. 6 mM diameter of fungal disc was transferred in the LiP screening medium, then incubated for 7 days and the colour change in the screening medium was analyzed (Atalla *et al*., 2010; Sivakami *et al*., 2012).

## Manganese peroxidase enzyme activity

To access MnP enzyme activity Czapek-Dox agar medium containing 0.0025% phenol red (w/v) was employed (Kuwahara *et al*., 1984; Ali *et al*., 2012). Fungal strain FTG4 (6 mm diameter one fungal disc) was inoculated in the Czapek-Dox agar medium at 27°C for 7 days and colour zone produced in the screening medium was analyzed.

## Quantitative estimation of ligninolytic enzyme production

To estimate the enzyme production in *P. elegans*, 8 mm fungal mycelium disc was transferred in the 20 mL mineral salt broth (composition g/L: Glucose – 10, $KH_2PO_4$ - 2, $MgSO_4.7H_2O$ – 0.5, $CaCl_2.2H_2O$ – 0.1, Ammonium tartrate – 0.2 and Trace element solution – 10 (mL). A trace element solution comprised of (in mg/L) $FeSO_4.7H_2O$ (12), $MnSO_4.7H_2O$ (3), $ZnSO_4.7H_2O$ (3), $CoSO_4.7H_2O$ (1), $(NH4)_6Mo_7O_{24}.4H_2O$ (1) (Hadibarata and Kristanti, 2012). Then incubate the culture at 27ºC in a rotatory shaker incubator for 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days, after that enzyme production was analyzed. All the experiments were performed in triplicates. The production of ligninolytic enzyme was investigated as the following procedure:

## Laccase assay

For the laccase enzyme production, take 3 mL of reaction mixture containing 0.5 mL of the enzyme extract, 1.5 mL sodium acetate buffer (10 mM, pH 5.0) and 1 mL guaiacol (2 mM), then incubated for 2 h and absorbance read at 450 nm. The laccase enzyme activity has been expressed in international units per liter of enzyme extract (U/L) (Sandhu and Arora, 1985).

## Lignin peroxidase assay

To investigate the lignin peroxidase enzyme production, take 0.5 mL of the culture filtrate, 1 mL of 125 mM sodium tartrate buffer (pH 3.0), 0.5 mL of 0.16 mM azure B, then add 0.5 mL of 2 mM hydrogen peroxide, after addition of hydrogen peroxide, the reaction was initiated. One unit of enzyme activity was expressed as an O.D. decrease at 651 nm of 0.1 units per minute per litre of the culture filtrate Archibald (1992).

## Manganese peroxidase assay

According to de Jong *et al.,* (1992) manganese peroxidase activity was accessed by the oxidation of 2, 6 - DMP at 468 nm. Take and 3 mL of reaction mixture contained 0.5 mL culture filtrate, 1 mL of sodium tartrate buffer (50 mM, pH 4.0) and 1 mL of 2 mM 2, 6-DMP. The reaction was started by the addition of 0.5 mL of 0.4 mM hydrogen peroxide.

# Results and Discussion

## Qualitative screening for the ligninolytic enzyme activity

### Laccase assay

On the basis of qualitative screening it was investigated that fungi *P. elegans* having the ability to produce ligninolytic enzyme. Due to oxidation of guaiacol by laccase enzyme, 70.00 mm diameter intense red colour zone (Fig. 1 a) appeared around the mycelium. Guaiacol is a very sensitive chromogenic compound, used for the screening of the production of enzymes on the basis of their oxidation reaction (Kiiskinen *et al*., 2004). Similarly Atalla *et al*., 2010 also screened the fungi on the basis of their oxidizing property, found that *Pleurotus ostreatus* and *Trematosphaeria mangrovei* showed 32.00 mm reddish brown colour zone due to oxidation of guaiacol.

### LiP assay

The production of LiP enzyme by *P. elegans* fungi was screened in the LiP screening medium.

After the oxidation of guaiacol by LiP enzyme in the presence of hydrogen peroxide in the LiP screening medium 70.00 mm diameter brick red colour zone (Fig. 1 b) appeared.

### MnP assay

In the MnP enzyme assay, due to the oxidation of phenol red by MnP enzyme the formation of yellow colour zone was investigated. Fig. 1 c showed that 50.00 mm diameter yellow colour zone (Fig. 1 c) appeared around the mycelium.

### Production of ligninolytic enzyme

The production of ligninolytic enzyme was investigated in the fungal strain FTG4 after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days of incubation in mineral salt broth. As the incubation period increases, the ligninolytic enzyme production also increases in the fungal strain FTG4.

**Fig.1** Qualitative screening for ligninolytic enzyme in FTG4 fungal strain, a - Laccase (red colour zone), b - Lignin peroxidase (reddish brown zone), and c - Manganese peroxidase (yellow colour zone) production by fungal strain FTG4

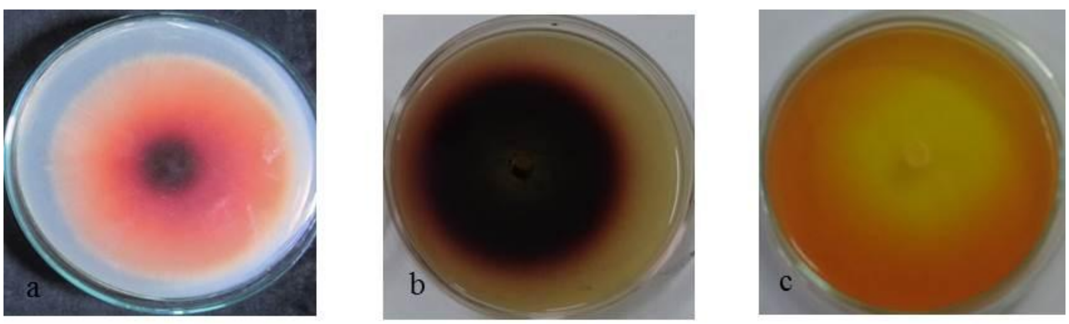



**Fig.2** Laccase enzyme production by *P. elegans*

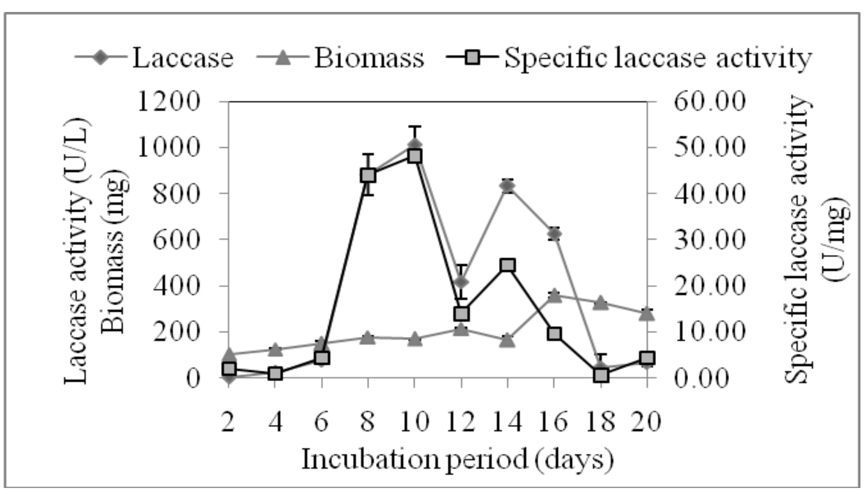



**Fig.3** LiP enzyme production by *P. elegans*

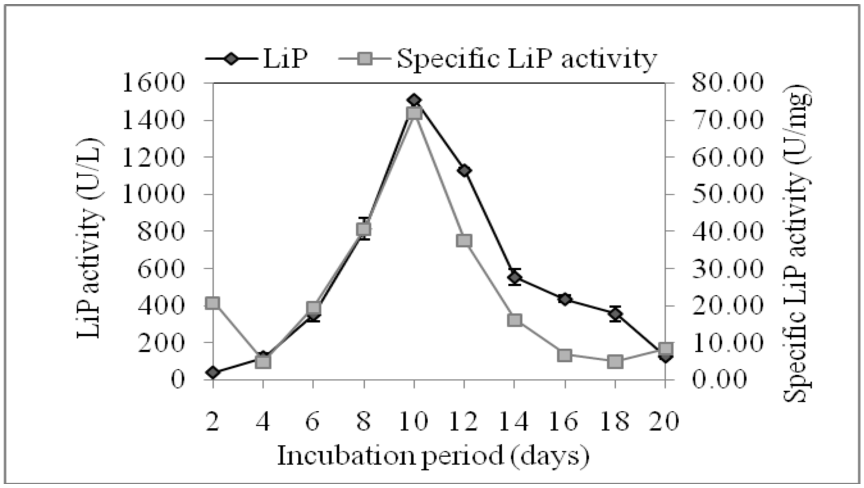
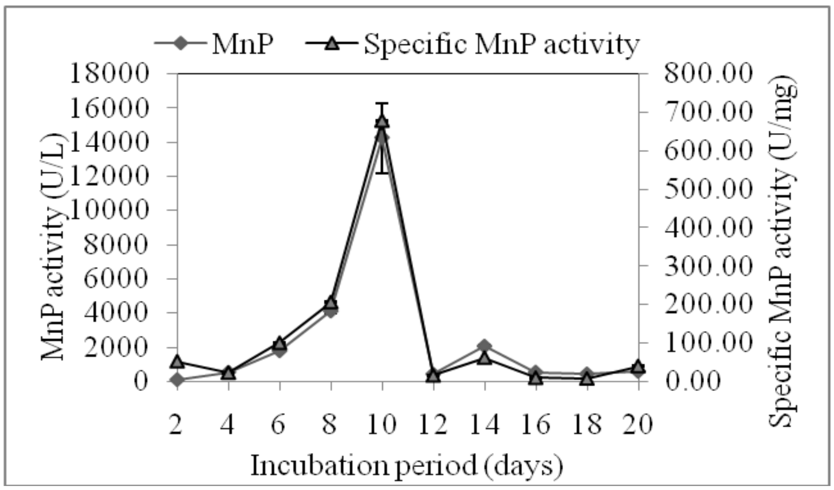



**Fig.4** MnP enzyme production by *P. elegans*




## Laccase enzyme production

The production of laccase enzyme rose to a maximum after 8-10 days of incubation, at 10$^{th}$day maximum 1013 U/L laccase enzyme production was investigated (Fig. 2), and then decreased, again increased, and after 20 days 66.73 U/L laccase enzyme activity was found. The maximum 48.24 U/mg Specific laccase activity was investigated after 10 days of incubation (Fig. 2).

## LiP enzyme production

LiP enzyme production was investigated by the oxidizing property of LiP enzyme, it oxidizes Azure B in the presence of hydrogen peroxidase, the change in the absorbance was read at 651 nm. After the 10$^{th}$ day of incubation maximum 1509 U/L LiP enzyme activity was found, and then the activity of LiP enzyme decreases and71.86 U/mg Specific LiP activity was investigated (Fig. 3).

## MnP enzyme production

The oxidation of 2, 6 DMP by MnP enzyme is a suitable method to quantify for the MnP enzyme. The maximum 14230 U/L MnP, 677.62 U/mg Specific MnP activity was found (Fig. 4).

In this study it was found that *P. elegans* produced efficient amount of ligninolytic enzyme. Therefore, we can use *P. elegans* for the remediation of organic pollutants, because many researchers (Bogan and Lamar, 1995; Hadibarata and Tachibana, 2010) also suggested that ligninolytic enzyme plays a significant role in the degradation of organic pollutants.

In conclusion, in this study ligninolytic enzyme producing white rot fungi *P. elegans* strain FTG4 was isolated. *P. elegans* produced efficient amount of enzyme in the mineral salt broth, therefore *P. elegans* can be used for the organic pollutant degradation study after successful investigation.

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