Secretion of ligninolytic enzymes by the white rot fungus *Stereum ostrea* on saw dust under the influence of chlorpyrifos

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Abstract: The white rot fungus – *Stereum ostrea* is known to secrete the ligninolytic enzymes – laccase (LAC), manganese peroxidase (MnP) and lignin peroxidase (LiP). Exposure of *S. ostrea* to an organophosphorus pesticide - chlorpyrifos may affect secretion of ligninolytic enzymes by the white rot fungus. The present study compared secretion of ligninolytic enzymes by *Stereum ostrea* on Koroljova liquid medium with /without saw dust in the presence and absence of chlorpyrifos at 20ppm level under shaking conditions for 10 days. Under free state (devoid of saw dust) conditions, higher LAC (301.165 U/ml), MnP (133.1422 U/ml) productions were observed on chlorpyrifos-amended medium on 10^{th} day of incubation. Very low yields of these enzymes were attained from medium amended with both saw dust and chlorpyrifos and medium containing only saw dust. But, maximum production of LiP to the extent of 2.6654 U/ml was recorded on medium amended with only saw dust as against 2.084 U/ml culture grown on medium with both saw dust and chlorpyrifos on 10^{th} day of incubation. Under free state conditions, LiP production on chlorpyrifos-amended medium was 0.9538 U/ml as against 0.6486 U/ml on control under free state conditions on 10^{th} day of incubation.

Key words: Chlorpyrifos, LAC, Ligninolytic enzymes, LiP, MnP, Saw dust of *Tectona grandis*, *Stereum ostrea*, white rot fungi.

I. Introduction:

White rot fungi (WRF) are known efficient organisms to degrade lignin by secreting extracellular oxidative lignin modifying enzymes (LMEs) enzymes (Singh and Chen, 2008; Lomascolo*et al.*, 2011) - Lignin peroxidase (LiP) (EC.1.11.1.14), Manganese dependent peroxidase (MnP) (EC.1.11.1.13) and Laccase (Lac) (EC.1.10.3.2) (Hatakka, 2005; Martínez*et al.*, 2005; Abdel-Hamid *et al.*, 2013; Rouches *et al.*, 2016). *Stereum ostrea*is one of important white rot fungi isolated from wood logs and is shown to secrete three ligninolytic enzymes -Lac, MnP and LiP (Viswanath *et al.*, 2008; Praveen *et al.*, 2011). Laccase enzyme is a dominant enzyme in ligninolytic system of *S. Ostrea* (Usha *et al.*, 2014). These three enzymes are involved in degradation of complex lignin, the second most abundant renewable biopolymer after the cellulose in nature (Rahman *et al.*, 2013). Entry of a diversity of xenobiotics - into environment occurs due to anthropological and industrial activities causing environmental pollution (Guliy *et al.*, 2003). After reducing usage of longer persistent organochlorine insecticides, organophosphorus insecticides have become the most widely used

compounds with their share of about 38% in total sales of pesticides in the world (Singh and Walker, 2006). Chlorpyrifos [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate)], is one of the most widely used organophosphate (OP) pesticide in agriculture and forestry system to control a broad spectrum of insect pests of economically important crops (Kale *et al.*, 1999; Mallick *et al.*, 1999; Fang *et al.*, 2006; Singh and Walker, 2006; Maya *et al.*, 2011). The WRF - *Anthracophyllum discolor* produces ligninolytic enzymes - mainly MnP in the presence of pollutants such as chlorophenols, pentachlorophenol (PCP), polycyclic aromatic hydrocarbons (PAHs) and synthetic dyes (Tortella *et al.*,2008; Elgueta and Diez, 2010; Rubilar *et al.*,2011; Acevedo *et al.*, 2011; Elgueta *et al.*,2016).Impact of pesticides on WRF cultures in particular towards secretion of ligninolytic enzymes is least understood. The present study has mainly focussed on the influence of chlorpyrifos on secretion of ligninolytic enzymes by the white rot fungus *Stereum ostrea* on saw dust in liquid medium and in free state (devoid of amendment of saw dust) under shaking conditions.

II. Materials and Methods:

1. Chlorpyrifos

Chlorpyrifos of commercial formulation {20% E.C. (Emulsified concentrate), Cheminova India Limited, Mumbai, India} was purchased from a local fertilizer shop in Anantapuramu. Stock solution of chlorpyrifos (1000 ppm concentration) was prepared with commercial formulation using sterilised distilled water. This stock solution was used in the present study after filter-sterilisation through Millipore membrane filter with a pore size of 0.45 µm in experiments for studying effect of chlorpyrifos on secretion of ligninolytic enzymes by the white rot fungus – *S. ostrea*.

2. Saw dust:

Lignocellulosic materials such as, Powder (dust) form of *Tectona grandis* wood selected for the present study, were procured from a local market and dried.

3. Inoculum preparation

The white rot fungal culture *Stereum ostrea* was grown on slants of Koraljova solid medium. After 6 days of incubation, the fungal mycelial suspension was prepared by adding 5ml of sterile distilled water to the slant. The homogenised suspension of fungal culture was used as inoculum.

4. Comparison of secretion of ligninolytic enzymes by *S. ostrea* on saw dust and in free state under influence of chlorpyrifos

In order to compare the secretion of ligninolytic enzymes by *S. ostrea*, 250 ml of Erlenmeyer flasks with 50 ml of Koroljova broth were divided in to 2 sets. Powder (dust) form of *Tectona grandis* wood at 3% level was added to flasks of the first set. Flasks with broth devoid of any lignocellulosic material in the second set were meant for free state culture. The first half of the flasks in both sets received chlorpyrifos from stock solution of chlorpyrifos E.C grade at the final concentration – 20 ppm level. Another half of the flasks in each set with broth devoid of chlorpyrifos served as control. All flasks were inoculated with homogenized mycelial suspension of *S. ostrea.* All flasks were incubated at 30^{o}C in the incubator cum shaker (Scigenic Orbitek, Chennai, India) at 160rpm. All flasks with growing culture of *Stereum ostrea* from each set were withdrawn at different time intervals and processed for measurement of extracellular protein content and activities of ligninolytic enzymes in the culture filtrate.

5. Analytical methods

i. Processing of culture filtrate

Culture filtrate derived after separation of fungal biomass in different experiment was first checked for pH with a pH meter. For finding out the extent of secretion of proteins including ligninolytic enzymes such as laccase, Manganese peroxidase and Lignin peroxidase in extracellular medium, enzyme titers in culture filtrate were determined following standard protocols. The culture filtrate was centrifuged at 4°C in a refrigerated centrifuge (Remi C-24, Mumbai) at 1500 rpm for 15 min. Supernatant was used as a source of extracellular medium/ligninolytic enzymes.

ii. Extracellular (secretory) protein estimation

Appropriate dilution of culture filtrate derived from different experiment was used for the estimation of soluble protein content according to the Lowry *et al.* (1951). Bovine serum albumin was used as protein standard.

iii. Laccase enzyme assay

Laccase enzyme assay was determined according to method of Das *et al.* (1997). Assay solution contained 100 mM acetate buffer (1.2 ml), 10 mM Guaicol (0.4 ml) and an aliquot of enzyme source (culture filtrate). The colour change was monitored at 470 nm (€= 6,740) for 3 min. Enzyme activity was expressed in terms of IU (international units) where one unit of enzyme is defined as the amount of enzyme that oxidized one micromole of substrate per min.

iv. Manganese peroxidase assay

Manganese peroxidase (MnP) activity was determined following the method of Bonnen *et al.* (1994). The assay medium contained 1 mM guaicol (0.4 ml) and 1 mM $MnSO_4$ (0.5 ml) in 10 mM citrate buffer pH 5.5 (0.5 ml). The reaction was initiated by the addition of 50 µM H_2O_2 (0.2 ml). After incubation at room temperature for 10 min the change in absorbance due to oxidation of guaicol was monitored at 460 nm for 3 min. Enzyme activity was expressed in terms of IU (International Units) where one unit of enzyme is defined as the amount of enzyme that oxidized one micromole of substrate per min.

v. Lignin peroxidase assay

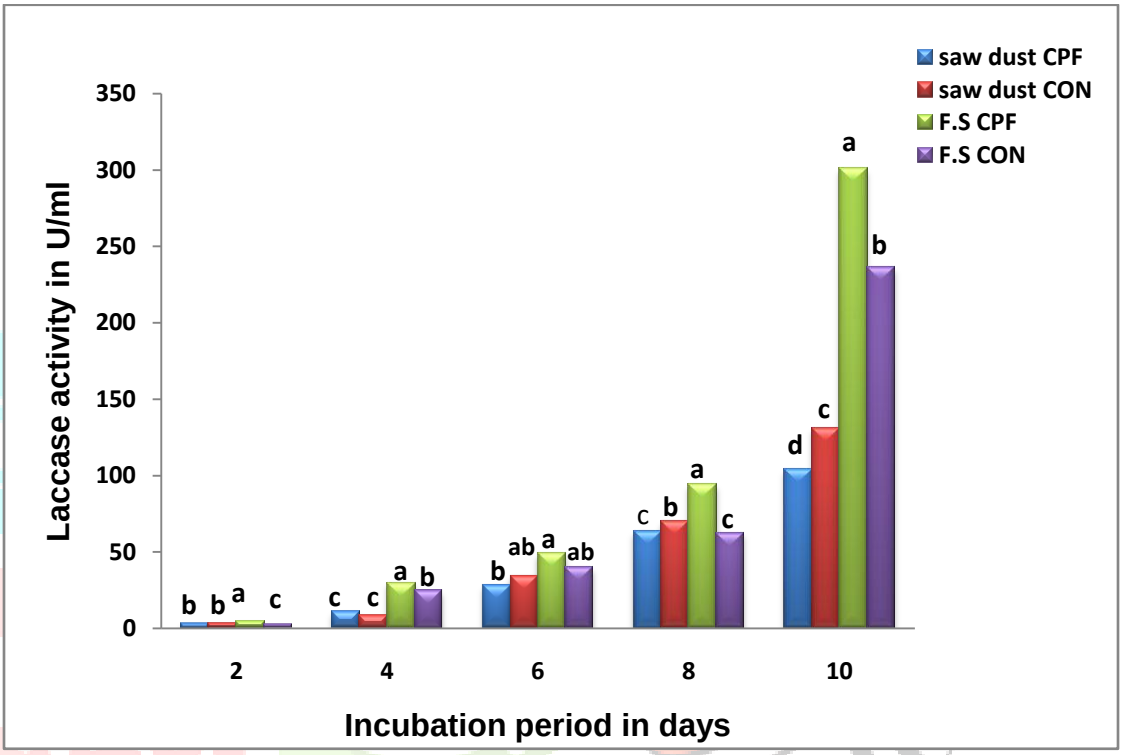
Lignin peroxidase (LiP) enzyme assay was based on the method of Tien and Kirk (1988). The assay medium contained 0.25 M tartaric acid (0.5 ml), 10 mM veratryl alcohol (0.5 ml) and 5 mM H_2O_2 (0.5 ml). Absorbance was monitored at 310 nm for 3 min after addition of enzyme source. Enzyme activity was expressed in terms of IU where one unit of enzyme is defined as the amount of enzyme that oxidized one micromole of substrate per min.

III. Results and Discussion

S. ostrea was grown on either saw dust or in free state (without saw dust) in liquid medium in the presence and absence of chlorpyrifos at 20 ppm level under shaking conditions. Growth of *S. ostrea* culture on saw dust in chlorpyrifos-amended medium resulted in lower production of three ligninolytic enzymes – Lac. MnP and LiP in comparison to production under Free State conditions (Figures 1 – 3). Higher production of Lac and MnP was recorded on chlorpyrifos-amended medium under free state conditions. Yields of LAC production by *S. ostrea* under free state conditions on chlorpyrifos-amended medium were 301.17 U/ml against 236.19 U/ml by the same culture on medium devoid of chlorpyrifos under the free state conditions on 10^{th} day of incubation. Titers of another ligninolytic enzyme – MnP to the extent of 133.14 U/ml under free state

conditions on chlorpyrifos-amended medium were obtained as against 93.82 U/ml on medium free of chlorpyrifos under the free state conditions on 10th day of incubation. Yields of Lac and MnP by *S. ostrea* cultures on saw dust in chlorpyrifos-amended medium were 103.85 and 40.82 U/ml on 10thday of incubation respectively. Corresponding values of Lac and MnP on medium with only saw dust on 10thof incubation were 130.18 and 47.04 U/ml (Figures 1 and 2)

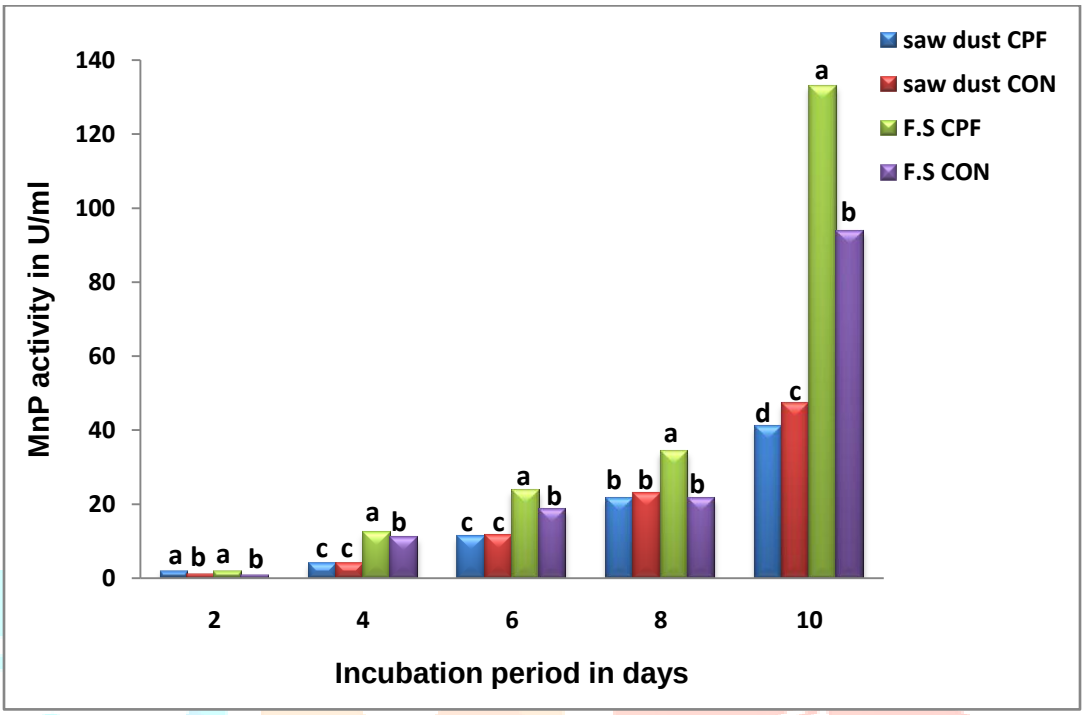
Figure 1. Secretion of laccase by *S. ostrea* on saw dust under influence of chlorpyrifos.



Mean bars for each sampling interval followed by the same letter are not significantly different (P≤0.05) from each other according to the DMR test.

CON: (Control) Devoid of the pesticide; CPF-Chlorpyrifos; Saw dust of *Tectona grandis*; F.S – Free state conditions.

Figure 2. Secretion of MnP by *S.ostrea* on saw dust under influence of chlorpyrifos.

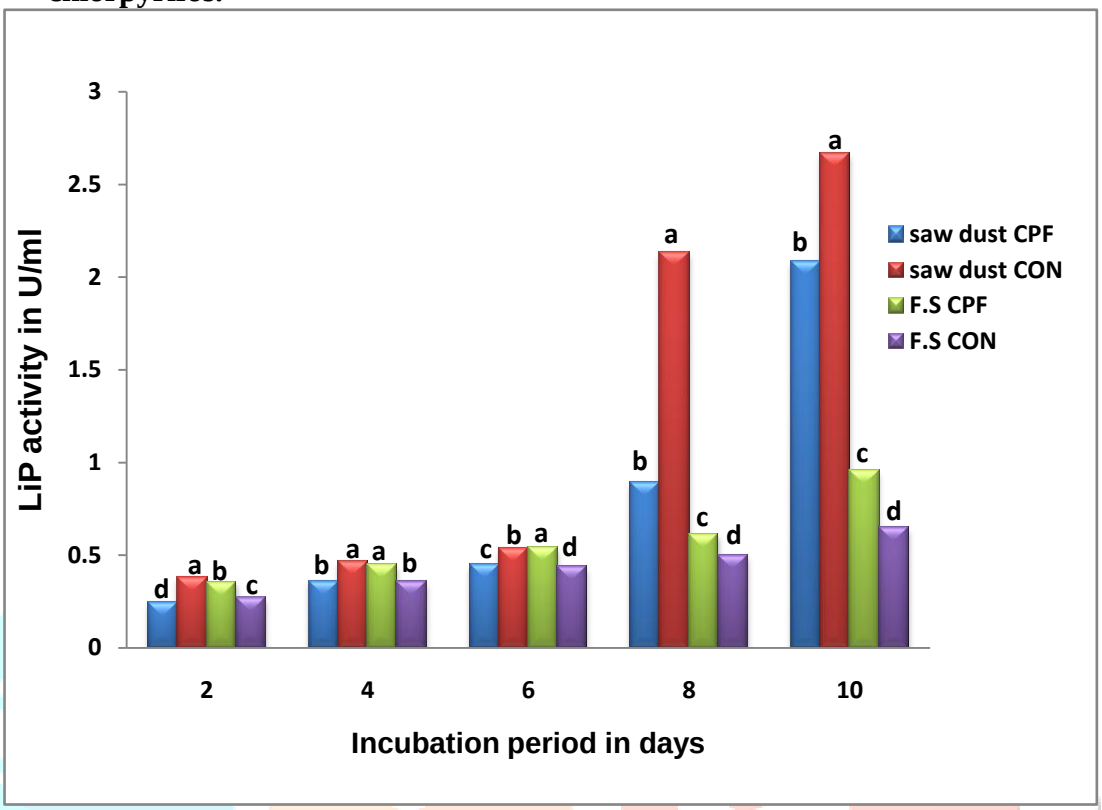


Mean bars for each sampling interval followed by the same letter are not significantly different (P≤0.05) from each other according to the DMR test.

CON: (Control) Devoid of the pesticide; CPF-Chlorpyrifos; Saw dust of *Tectona grandis;* F.S – Free state conditions

Secretion of LiP by *S. ostrea* on saw dust in the presence or absence of chlorpyrifos (Figure 3) followed the pattern of secretion of Lac and MnP. LiP productions in higher titers were attained upon growth of *S. ostrea* culture on medium amended with only saw dust (control) than on saw dust in chlorpyrifos-amended medium on 10^{th} day of incubation. LiP production by *S. ostrea* was higher on medium amended with chlorpyrifos than on medium devoid of chlorpyrifos under free state conditions. Maximum LiP production (2.67 U/ml) was recorded on 10^{th} day incubation in culture of *S. ostrea* grown on medium amended with saw dust only as against 2.084 U/ml by the same culture grown on medium with both saw dust and chlorpyrifos on 10^{th} day of incubation. Under free state conditions, LiP production on CPF-amended medium was 0.9538 U/ml as against 0.6486 U/ml on control under free state conditions on 10^{th} day of incubation.

Figure 3. Secretion of LiP by *S.ostrea* on saw dust under influence of chlorpyrifos.

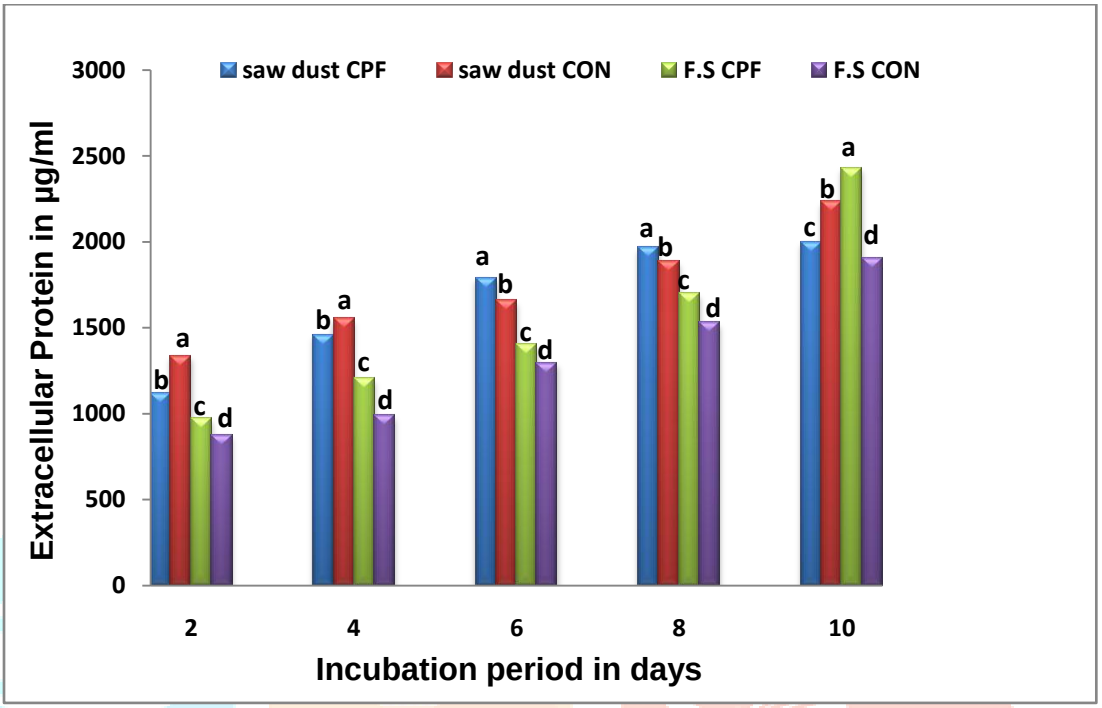


Mean bars for each sampling interval followed by the same letter are not significantly different (P≤0.05) from each other according to the DMR test.

CON: (Control) Devoid of the pesticide; CPF-Chlorpyrifos; Saw dust of *Tectona grandis;* F.S - Free state conditions.

In the present study, pattern of secretion of extracellular protein by *S. ostrea* in free state and immobilized state on saw dust under influence of chlorpyrifos followed the trend of secretion of ligninolytic enzymes by the same culture under respective conditions. The culture broth of *S. ostrea* grown on chlorpyrifos-amended medium under free state conditions yielded highest extracellular protein content (Figure 4). Maximum protein content (2432 µg/ml) under free state conditions from chlorpyrifos-amended medium was recovered as against 1904 µg/ml of control under free state conditions on 10^{th} day of incubation. Extracellular protein content, liberated upon growth of fungal culture on medium with only saw dust and medium amended with saw dust and chlorpyrifos on 10^{th} day of incubation, were 2231.42 and 2000 µg/ml, respectively.

Figure 4. Secretion of extracellular protein content by *S. ostrea* on saw dust under influence of chlorpyrifos.



Mean bars for each sampling interval followed by the same letter are not significantly different (P≤0.05) from each other according to the DMR test.
CON: (Control) Devoid of the pesticide; CPF: Chlorpyrifos; Saw dust of *Tectona grandis*; F.S: Free state conditions.

pH changes occurred in the culture broth of *S. ostrea* grown on sawdust and under free state conditions in the presence and absence of chlorpyrifos (Table 1). There was an increase in pH from an initial set value of 5.0 within 4 days of incubation followed by drop in pH at later intervals.

Inclusion of saw dust (powder form) in the medium in the present study did not improve yields of ligninolytic enzymes and extracellular protein by *S. ostrea.* Similar observation of poor growth of *Antharcophyllum discolor* along with less production of MnP on wood chips in the absence of xenobiotics was reported (Rubilar *et al.*, 2011). All six fungal basidiomycetes grew well in form of pellets in both media (glycerol-based and mandarin peel based) producing 3.2–4.3 g mycelial biomass/l in the presence of glycerol (Kachlishvili *et al.*, 2016). Six white rot basidiomycete fungi with the exclusion of *Pycnoporus coccineus*, usually expressed appreciable levels of the laccase and MnP activities on both media with more pronouncement in the mandarin peels-based medium (Elisashvili and Kachlishvili, 2009). These cultures exhibited differential response on both media when supplemented with 0.2 mM of xenobiotic - 2, 4, 6-Trinitrotoluene (Kachlishvili *et al.*, 2016).

Table 1. pH changes in culture broth of *S. ostrea* grown on saw dust

Ligninocellulosic materials	pH changes				
	Incubation period in days				
	2	4	6	8	10
Saw dust CPF	5.95 [a]	5.88 [a]	5.78 [a]	5.6 [a]	5.38 [a]
Saw dust CON	5.91 [a]	5.84 [a]	5.77 [a]	5.52 [b]	5.19 [b]
F.S CPF	5.42 [b]	5.25 [b]	4.80 [b]	4.67 [c]	3.48 [c]
F.S CON	5.32 [c]	4.8 [c]	4.68 [c]	4.45 [d]	3.35 [d]

Means in each column followed by the same superscript letter (a, b, and c) are not significantly different (P≤0.05) from each other according to the DMR test
Control: Devoid of amendment pesticide chlorpyrifos
Saw dust of *Tectona grandis*
F.S – Free state conditions
CPF-Chlorpyrifos
CON- control

Supplementation of TNT to media resulted in more than 2-fold increase in laccase activity in the cultures of *Cerena unicolor* and *Trametes versicolor*. However, the stimulation of the laccase production by TNT was not observed when *Fomes fomentarius* and *Funalia trogii* were cultivated in presence of this compound in the synthetic medium. The differential response of cultures may be attributed to the composition of lignocellulosic materials and inherent capacities of fungal organisms and concentration of xenobiotic.

IV. Conclusions:

All three ligninolytic enzymes – Lac, MnP and LiP were secreted by *S. ostrea* upon growth on medium with/without saw dust in the presence and absence of chlorpyrifos at 20 ppm level. However, productions of Lac and MnP were higher in medium without saw dust in the presence of chlorpyrifos than in medium with saw dust either in the presence or absence of chlorpyrifos. Reverse trend in the pattern of secretions of LiP was recorded with maximum yields in respect of medium with saw dust in the absence of chlorpyrifos.

V. Acknowledgement:

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