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EXTRACELLULAR LIGNINOLYTIC ENZYMES OF WILD *IRPEX LACTEUS* AND *TRAMETES OCHRACEA* COLLECTED FROM HIMACHAL PRADESH

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ABSTRACT

The mushroom mycelium able to grow on wide spectrum of xenobiotic waste materials which can be attributed to its ability to secrete a range of ligninolytic enzymes. Study of estimation of extracellular ligninolytic enzymes of *Irpex lacteus* and *Trametes ochracea* were carried out on nutrient rich medium (NRM) and nutrient poor medium (NPM) up to 20th day under static and shaking conditions and enzyme assay was calculated. Extracellular enzyme activity varied with the incubation conditions and media used. Both varieties of wild White-rot fungi (WRF) showed high production of aryl alcohol oxidase (AAO) under static condition of both media and manganese peroxidase (MnP) in NRM under both conditions. Both selected strains

Keywords: ***Irpex lacteus*, *Trametes ochracea*, White rot fungi, Ligninolytic enzymes**

INTRODUCTION

Agro-centric livelihood releases tremendous quantities of waste cellulosic materials which may be classified into agricultural, industrial wastes in every part of India. These solid wastes are usually dumped or discarded indiscriminately at various sites where they are burnt, buried or left for decomposition which cause enormous environmental pollution with a lot of serious health related problems (Abu et al., 2000). Organisms having the ability to utilize cellulosic materials for their carbon, nitrogen and energy source could be exploited for the conversion of these wastes into other products that are beneficial to human and nature. In recent years, there has been an enormous growing interest in fungal enzyme researches for newer and alternative fungal strains for production of value-added and efficient ligninolytic enzymes. Several works about ligninolytic enzymes have been carried out mainly in white-rot basidiomycetes (Dhouib et al., 2005).

Fungi especially white-rot fungi, belonging to the basidiomycetes, are the only organisms have the ability of mineralizing lignin efficiently. Lignin, which is one of the most widely distributed natural organic polymers, is decomposed very slowly in the environment due to its complex chemical structure (Dashtban et al., 2010). Lignin biodegradation performed by these white rot fungi is a multi-step process mediated by enzymes of the ligninolytic complex. The rate of lignin decomposition depends on the concentration of molecular oxygen. Both the nature and direction of this process can be different in different fungi (Revin et al., 2002).

Apart from white-rot fungi, some microorganisms have the ability to degrade and decolorize wide range of recalcitrant organic compounds (Gopinath et al., 2005) but at very low concentrations. Intensive study of ligninolytic fungi revealed that these organisms produce extracelluar enzymes with very low substrate specificity, enabling them to mineralize all the components of ligninocellulosic materials and a wide range of highly recalcitrant organopollutants that are structurally similar to lignin (Hofrichter, 2002).

Ligninolytic enzymes include laccase (Lac; EC 1.10.3.2), aryl alcohol oxidase (AAO; EC 1.1.3.7), lignin peroxidases (LiP; EC 1.11.1.4), manganese peroxidases (MnP; EC 1.11.1.3) and are secreted by almost all the white rot fungi. Activities of ligninolytic enzymes appear only in the culture medium after attainment of peak growth with uptake of essential nutrients—C, N, and S (Wong, 2008, Praveen *et al*., 2011). Profiles of different enzymes of ligninolytic system depend on growth conditions and vary from organism to organism.

Although the majority of earlier studies have been focused on ligninolytic enzymes of modal fungus like *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor*. There has been a growing interest in studying ligninolytic enzymes of wider range of wild white-rot fungi from the angle of comparative biology but also with expectation of finding better lignin degrading system. The present study focuses on the ligninolytic system of wild varieties of *Irpex lacteus* and *Trametes ochracea*.

MATERIALS AND METHODS

Collection and identification

Fresh fruiting bodies of wild fleshy fungi were collected from the forests of Solan district of Himachal Pradesh during rainy season. Pure cultures were obtained from the tissue of fruiting body by tissue culture method on rose bengal added (2%) malt extract agar medium. The fruiting bodies were identified classically (microscopic and macroscopic features) as well as on the basis of molecular classification and gene sequences were submitted to NCBI.

Qualitative determination of ligninolytic enzyme activity on solid media

Laccase spot test was performed in Petri plates using ABTS as substrate (0.2 gl^{-1}) in the modified Kirk medium at pH 5 by the method provided by Saparrat et al., (2000). The MnP spot test was performed in the basic Kirk medium supplemented with $MnCl_2.4H_2O$ (0.1gl^{-1}) at pH 5 by the method provided by Steffen et al., (2000). Plates without ABTS and $MnCl_2.4H_2O$ as substrates were used as control for laccase and MnP.

For each test, 90 mm diameter Petri plates were inoculated at the center using a 5 mm in diameter cylindrical bit of mycelium and incubated at 25°C for 21 days. Fungal mycelial growth was measured in terms of diameter of fungal colony and extracellular enzyme activity was measured by the change in colour intensity of the medium.

Extracellular ligninolytic enzyme assay

The extracellular ligninolytic enzymes production were carried out in 100 ml flasks containing 50 ml liquid medium inoculated with five mycelium bits (~5 mm in diameter) at static condition in BOD incubator and shaking condition at 130 rev/min at 30°C in the nutrient poor medium (NPM) and the nutrient rich medium (NRM) both at pH 7. NRM consists of 2 g of ammonium tartrate, 10 g of glucose, 1 g of KH_2PO_4, 1 g of yeast extract, 0.5g of $MgSO_4.7H_2O$, 5g of KCl and 1 ml of solution containing trace elements per liter of medium and the NPM consists of 10 g of glucose, 2 g of KH_2PO_4, 0.2 g of yeast extract, 0.1 g of peptone and 1 ml of solution containing trace elements per liter of medium. The cultures were harvested at the 5th, 10th, 15th and 20th day of incubation, centrifuged (12,000 x g for 5 min) at 4°C and the supernatant was employed for enzyme assay (Tripathi et al., 2012).

Laccase activity was measured following the oxidation of ABTS ($\mathcal{E}_{420}$, 36,000M^{-1} cm^{-1}) and was carried out in sodium acetate buffer pH 5 (Han et al., 2005). AAO activity was measured using Veratryl alcohol as substrate and sodium phosphate buffer (pH-6) at 310nm ($\mathcal{E}_{310}$, 9,300 $M^{-1}cm^{-1}$) (Varela et al., 1999). LiP activity was measured by the oxidation of veratryl alcohol to veratraldehyde ($\mathcal{E}_{310}$, 9,300$M^{-1}cm^{-1}$) in sodium tartrate buffer (pH-3) in the presence of 30% H_2O_2 (Camarero et al.,1999) and MnP activity was determined from $MnSO_4$ in sodium tartrate buffer (pH-5) with 30% H_2O_2 and guaiacol by the production of a Mn^{3+} tartrate complex ($\mathcal{E}_{238}$, 6,500$M^{-1}cm^{-1}$; Cohen et al.,2001; Tripathi et al., 2012). The enzymatic reactions were carried out in replicate of three and determined using a double beam Systronics UV/VIS spectrophotometer. All the enzyme activities were obtained at room temperature (20 ± 2°C). The enzymatic activity of above stated enzymes was expressed as international units (U) and defined as the amount of enzyme required to produce 1 µmol product min^{-1} and expressed as Ul^{-1}.

RESULTS AND DISCUSSIONS

Collection, culturing and identification

Survey of forests of different ecological regions were carried out. Pure cultures of wild fleshy fungi were isolated using tissue culture method from the collected wild macro-fungi and identified in the basis of macroscopic characteristics, sporocarp morphology and mushroom identification keys. Two pure culture *Irpex lacteus* and *Trametes ochracea* were selected on the basis of their growth characteristics and woody origin for production of ligninolytic enzymes.

Irpex lacteus: Fruiting body is choclate brown in color having leathery appearance, pileus diameter 4.2-6 cm non hygrophagus attached to wood. Pileus bears gills of brown color in sets. Elliptical spores were observed under light microscope (Fig 1A).

Trametes ochracea: Fruiting body is grayish green attached to wood appears slightly corky having pileus diameter 3.6-4.5 cm semicircular with irregular margins. Surface of pileus is finely hairy, bears small pores and gives white spore print. Spores appears cylindrical under microscope (Fig 1B).

The two strains used in the study were also identified on the basis molecular taxonomy. Genomic DNA was isolated followed by PCR amplification and finally sequenced by Sanger sequencing technique. The gene sequences obtained were submitted to NCBI gene bank under accession no **KU955431** and **KX147237** respectively.

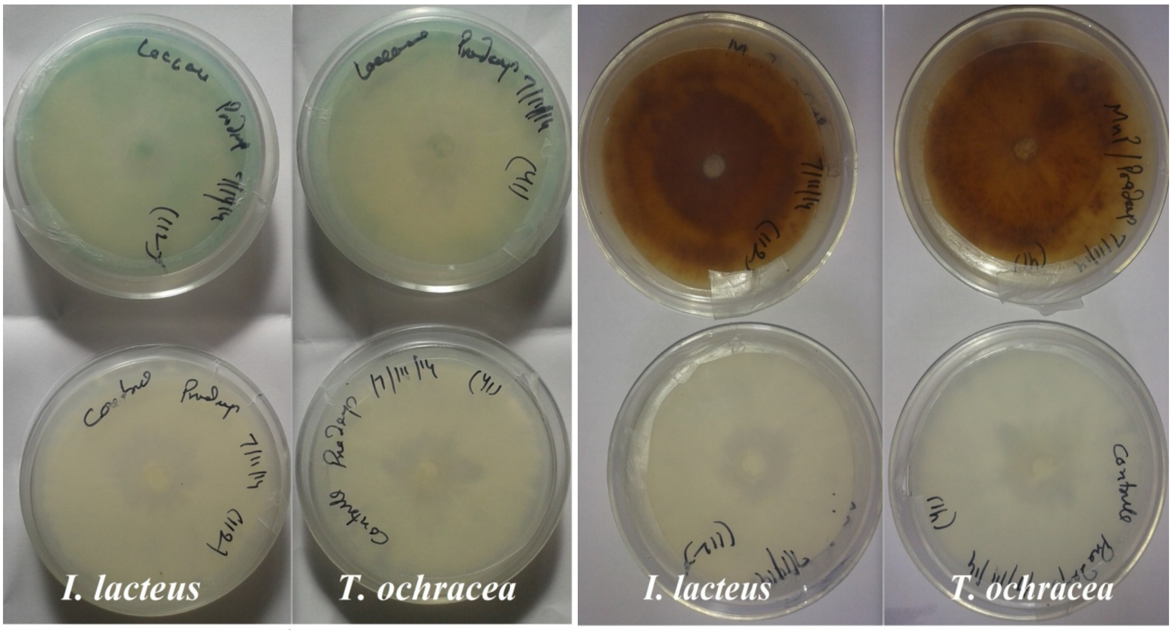
Qualitative determination of ligninolytic enzyme activity on solid media

Both wild *Irpex lacteus* and *Trametes ochracea* grows slower on spot test agar medium as compared to MEA.

Laccase:

Laccase enzyme oxidizes a variety of natural as well as synthetic substrates therefore several different compounds have been employed as indicators for laccase screening such as guaiacol, syringaldazine and ABTS (2,2'-azino-bis 3-thylbenzothiazoline-6-sulphonic acid; Kalmis et al., 2008). ABTS, being a very sensitive substrate, allows a quick screening of fungal strains producing extracellular ABTS-oxidising laccase enzymes through a color reaction (Stajic et al., 2006). Both *Irpex lacteus* and *Trametes ochracea* showed green color formation in spot test agar medium containing ABTS on 3^{rd} day onwords respectively (Fig. 2 A). *Irpex lacteus* shows less green color production as compared to *Trametes ochracea.*

Fig 1: Natural view of wild *Irpex lacteus* (A) and *Trametes ochracea* (B).



A B

Fig. 2: Extracellular ABTS oxidising (A) and MnP (B) activity of *Irpex lacteus* and *Trametes ochracea* on agar plate

Manganese peroxidase:

Both strains showed positive reaction of extracellular MnP enzyme production and formed brownish colour in medium plate (Fig 2 B). Petri plates were evaluated everyday for 5 consecutive weeks of incubation for the emergence of brownish flecks of manganese di-oxide (MnO_2) induced by the action of MnP. *Trametes ochracea* shows fast results as compare to *Irpex lacteus* producing dark brown coloration. It was assumed that MnP plays an important role in mineralization of lignin by primarily attack on lignin polymer

Extracellular ligninolytic enzyme assay

Quantitative protein determination

The Bradford assay assay is protein determination method involves the binding of coomassie brilliant blue G-250 dye to protein. The Bradford reagent, the anionic form of dye, when binds to protein changed to a stable unprotonated blue form. In *I. lacteus* extracellular protein was not observed on 5th day in both NRM and NPM static conditions. Maximum protein was observed on 15th day in NRM shaking and static conditions respectively (91.66, 72.67 μgml^{-1}). The present finding shoes that *I. lacteus* produces more proteins in agitated conditions than static conditions. In *T. ochracea* static conditions of both media shows best results on 20th day in comparison to shaking conditions respectively 52.66, 49.15 µgml^{-1}. Both strains shows good production of extracellular protein but *I. lacteus* shows higher protein production than *T. ochracea* in both media and conditions. *I. lacteus* shows good amount of extracellular crude proteins which was higher thanother polypores like *B. abdusta,* and *L. squarrosulus* (Tripathi et al., 2012) as well as *L. tigrinis* (Adejumo and Awosanya 2005)

Laccase

Laccase enzyme activity first time was observed in Japanese lac tree *Toxicodendron verniciflua* and later detected in other plants, fungi, yeast and bacteria. Laccase is particularly widespread in many ligninolytic basidiomycetes such as *Phanerochaete, Pycnoporus, Nematoloma, Sporotrichum, Ganoderma lucidum, Stropharia* etc. and more than 125 different basidiomycetous (Dashtban et al., 2010). In *Irpex lacteus* no laccase enzyme activity was observed in NPM shaking and static conditions where as NRM shaking condition shows best laccase production (53.66 UL^{-1}) on 15th day and in NRM static condition up to 10th day no laccase was observed.

In *T.ocracea* laccase activity was best in NRM at both shaking and static condition on 10th and 15th day, respectively (53.27; 146.66UL^{-1}) followed by NRM static (34.44 UL^{-1} and 96.66 UL^{-1}). *T.ocracea* exhibit poor laccase activity in NPM shaking condition on 20th day (8.88UL^{-1}). Laccase is particularly widespread in many ligninolytic basidiomycetes such as *Phanerochaete, Pycnoporus, Nematoloma, Sporotrichum, Ganoderma lucidum, Stropharia* etc. and more than 125 different basidiomycetous (Dashtban et al., 2010). Laccase is the most renowned lignin-modifying enzyme produced by the white rot fungi belonging to the Polyporaceae (Pelaez et al., 1995) and many species secrete considerable levels of this enzyme in liquid cultures. Laccase activity in both strains observed best in NRM shaking condition and it indicates that laccase activity is inducible in these strains at nutrient rich condition for better laccase secretion. It was witnessed that supplementation and incubation conditions also affected the enzymatic activity. A few WRF gave good laccase activity under high nitrogen condition and medium supplemented with Cu but in the absence of Cu activity was not found and under low nitrogen like *Volvariella volvacea, Piloderma byssinum* (Chen et al., 2003).

Aryl alcohol oxidase

AAO activity for the first time was described in the fungus *Polystictus versicolor* (a synonym of *Trametes versicolor*) in 1960 (Farmer et al., 1960). Preliminary structure analysis revealed that AAO belongs to the GMC (glucose/methanol/choline) oxidoreductase family of flavoenzymes (Ferreira et al, 2005). AAO activity in *I. lacteus* was better under NPM shaking and NRM static condition and NPM shaking condition gave best activity on 15th and 20th day respectively (491.40, 332.25 Ul^{-1}). AAO activity continuously increased up to 20th day in NPM both conditions and suggests that AAO activity is directly related to incubation and nutritional conditions.

In comparison to *I. lacteus*, *T.ocracea* gave less AAO activity. Maximum activity was observed on 15th and 20th day in NPM shaking respectively (165.59; 246.23 Ul^{-1}) and in NPM static condition on 20th day (123.65 Ul^{-1}). AAO appears as main oxidative enzyme in *I. lacteus* whereas laccase laccase plays important role in *T. ochracea*. AAO has been detected and characterized in many other white-rot basidiomycetes including *Pleurotus* species, *Bjerkandera adusta* and few ascomycetous fungi (Kim et al., 2001).

Lignin peroxidase

After the discovery of the first "ligninase" (lignin peroxidase, LiP) in *Phanerochaete chrysosporium* (Tien and Kirk 1983), enzymatic degradation of lignin has been thoroughly investigated due to the biotechnological interest to gain access to lignocellulose carbohydrates in industrial applications (Martinez et al., 2009). LiP is catalytically the most powerful class II fungal peroxidase with the ability to directly oxidize the nonphenolic β-O-4 linkage type aryl glycerol-aryl ethers like dimeric lignin model compounds (Hofrichter et al., 2010). In *I. lacteus* LiP activity was maximum on 15th day in NRM shaking condition (54.4Ul^{-1}) and NPM static (48.23 Ul^{-1}). In *T.ochracea* no LiP activity was detected in NRM shaking condition up to 10th day but gives maximum Lip production on 20th (59.13Ul-1). *T. ochracea* gives batter Lip production than *I. lacteus*. The occurrence of LiP is not so often in many fungi; however, the fungi capable of producing LiP are efficient lignin degraders, for example the *Phanerochaete chrysosporium*, *Phlebia radiata* and *Phlebia tremellosa* as well as the polypore *Trametes versicolor* (Tripathi et el., 2012). LiP being a heme peroxidase is secreted extra-cellularly at the onset of secondary metabolism and triggered by nitrogen limiting conditions in some WRF like *P. chrysosporium*, but it can also be produced in nitrogen-sufficient to nitrogen rich conditions by other WRF (Collins et al.,1997).

Manganese peroxidase

Among ligninocellulose and wood-decaying macro-fungi especially basidiomycetous fungi, MnPs are the most commonly occurring class II peroxidases so far (Hatakka and Hammel 2010, Hofrichter 2010). In *I. lacteus* MnP activity was higher on 15th day (388.72Ul^{-1}) in NRM shaking whereas MnP Activity continuously increases in NPM static and was maximum on 20th day (379.49Ul^{-1}). Baborova et al (2006) reported MnP as the dominant ligninolytic enzyme produced in liquid cultures of *I. lacteus*. In *T. ochracea* MnP activity comparitively was very poor than *I.lacteus*. Best enzyme activity was observed on 15th day in NRM both static and shaking conditions, respectively (116.92, 163.07Ul^{-1}) and least enzyme activity was found in NPM shaking condition. MnP encoding genes are apparently more widespread than those of LiP between polyporoid and corticioid as well as agaric (cap forming) fungi belonging all to the subphylum Agaricomycotina (Morgenstern et al., 2008). It ranged among the smallest MnP, with its molecular weight similar to *Abortiporus biennis* and *Trametes* spp. (Hofrichter 2002). MnP was observed in most of Polyporoid fungi and other WRF (Steffen 2003). Manganese peroxidase from *I. lacteus* was appeared as the major ligninolytic enzyme produced in liquid culture.

The white-rot fungi *Irpex lacteus* and *Trametes ochracea* produces a complete lignolytic system Lcc, AAO, LiP, and MnP during their vegetative growth. Extracellular enzyme activities of both varieties were heterogonous with medium used and their incubation conditions. Among the four ligninolytic enzymes, laccase in *T. ochracea*, AAO and MnP in *I. lacteus*, LiP in both varieties, appeared to be main extracellular enzymes (fig.3, Table-1).

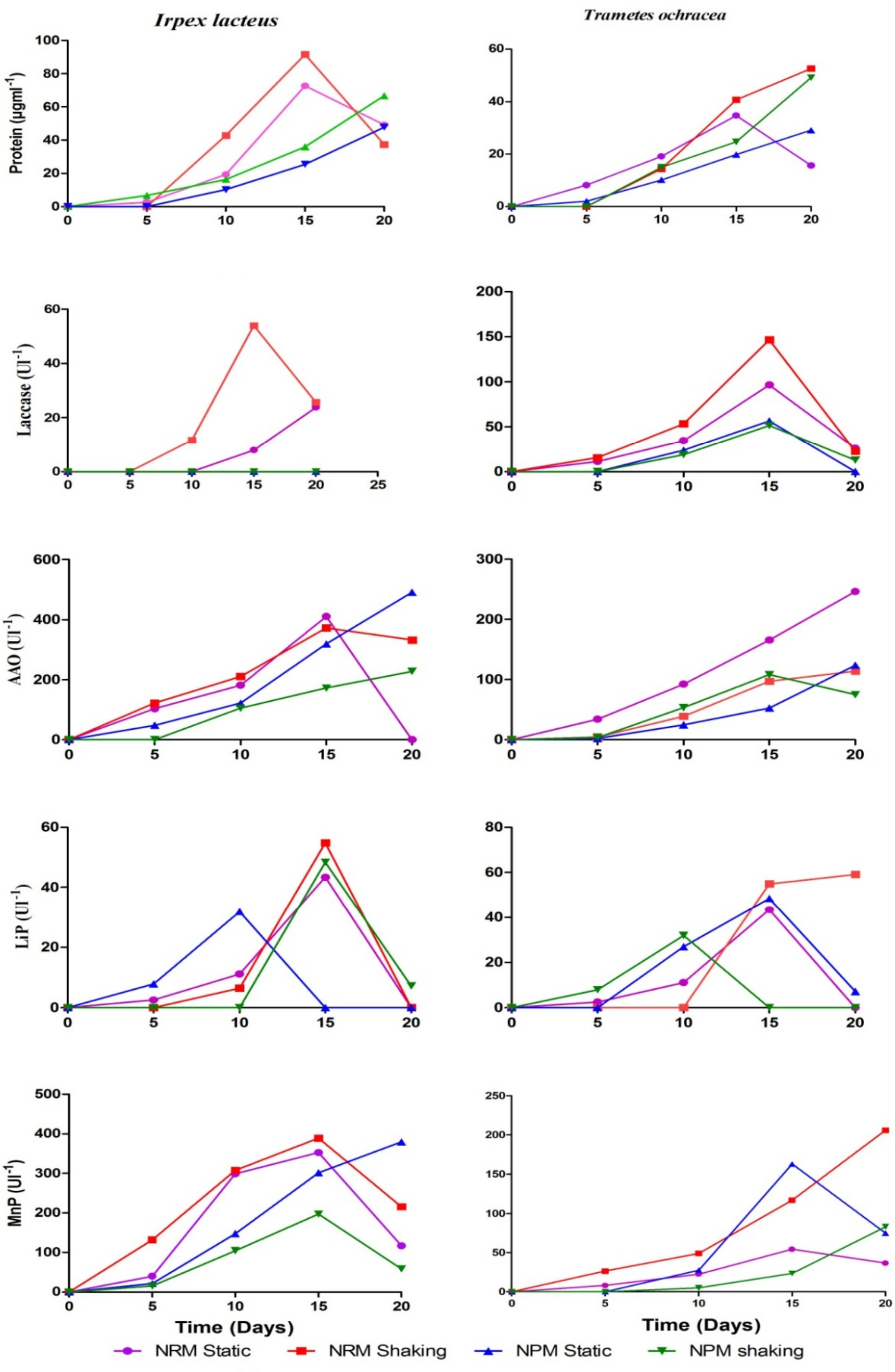


Fig. 3: Extracellular ligninolytic enzymes (UL^{-1}) in *Irpex lacteus* and *Trametes ochracea*

Table1: Extracellular protein (μgml^{-1}) and ligninolytic enzymes (Ul^{-1}) in *Irpex lacteus* and *Trametes ochracea* (mean ± SD; n = 3)

S.No.	Day Int.	*Irpex lacteus*				*Trametes ochracea*			
		NRM		NPM		NRM		NPM	
		Shaking	Static	Shaking	Static	Shaking	Static	Shaking	Static
Protein	5	3.15±1.62	nd	6.74±4.25	nd	8.16±2.54	nd	2.00±0.65	nd
	10	19.33±0.29	42.75±1.50	16.41±2.59	30.20±1.06	19.16±1.85	14.44±0.85	10.16±1.88	15.58±0.82
	15	72.67±0.94	91.66±3.00	35.99±1.6	25.49±2.15	34.80±1.62	40.73±1.53	19.83±0.94	24.66±2.59
	20	49.17±1.30	37.49±0.59	66.83±1.5	47.83±1.54	15.66±0.86	52.66±1.64	29.15±2.1	49.15±3.53
Laccase	5	nd	nd	nd	nd	11.29±0.64	15.34±1.15	nd	nd
	10	nd	nd	nd	nd	34.44±1.24	53.27±1.46	23.80±1.39	18.88±1.9
	15	7.95±0.84	54±2.5	nd	nd	96.66±1.11	146.66±2.24	56.66±±2.4	51.53±1.39
	20	23.83±2.25	25.73±1.7	nd	nd	26.11±0.84	22.66±0.62	12.77±1.39	8.88±1.6
AAO	5	103.93±1.24	121.5±1.9	48.39±2.23	nd	3.58±0.63	4.3±0.62	33.87±4.68	2.15±2.85
	10	181.72±1.85	210.75±2.23	121.5+3.1	105.38±1.86	53.4±1.64	38.7±2.23	92.47±2.7	24.73±3.87
	15	410.75±1.67	372.89±2.4	319.35+1.14	172.04±2.23	107.88±2.7	96.77±2.15	165.59±2.23	52.69±0.62
	20	nd	332.25±1.62	491.4±1.24	227.95±0.62	74.91±3.28	113.97±5.5	246.23±2.15	123.65±2.23
LiP	5	2.5±2.7	nd	7.89±1.86	nd	2.5±0.62	nd	nd	7.89±5.4
	10	11.11±1.8	6.45±0.62	31.89±1.0	nd	11.11±2.23	nd	26.88±1.2	31.89±1.64
	15	43.37±1.67	54.84±2.8	nd	48.39±2.23	43.73±1.24	53.84±3.22	48.39±0.62	nd
	20	nd	nd	nd	7.17±1.24	nd	59.13±4.9	7.17±2.15	nd
MnP	5	41.02±4.7	132.3±3.5	21.53±1.77	15.38±11	8.2±1.77	26.66±.1.6	nd	nd
	10	298.46±15.4	307.69±11	147.69±4.6	104.61±9.4	22.56±4.7	49.23±5.3	27.7±1.77	5.12±6.4
	15	352.82±8.14	388.72±7.7	301.53±5.3	196.92	54.35±3.5	116.92±7.6	163.07±1.16	23.58±4.7
	20	116.92±3.5	215.38±6.4	379.49±6.4	58.46±1.77±5.3	36.92±2.4	206.15±3.5	74.87±1.5	83.07±5

nd- not detected

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