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Experimental Evidence for Presence of a Growth Regulating Extracellular Laccase in Some *Pleurotus* Species

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ABSTRACT

The aim of the present study was to find out a common laccase isoenzyme in five *Pleurotus* spp. which might be responsible for regulation of mycelial growth in producer organisms. Laccase activities were assayed from the extracellular culture filtrates and optimum dates for laccase production under stationary condition were detected. Partially purified 25th days culture filtrates were analyzed for activity staining in 10% Native PAGE. The culture filtrate of all the strains had shown the oxidization of o-dianisidine, guaiacol and ABTS with different efficiencies. *P. ostreatus* showed optimum laccase activity in 25th day whereas other strains shown optimum activities in 26th day. The highest laccase activity and biomass were found in *P. ostreatus* and lowest in *P. florida*. Though different *Pleurotus* species exhibited differential laccase isoenzyme expression patterns but one isoenzyme band had been found to be common in all the species, which resulted in similar r_f value with that of a previously reported growth regulating laccase (L_2) of *P. florida*.

Key words: *Pleurotus*, oxidoreductase, extracellular enzyme, zymogram

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase; EC 1.10.3.2) is a common extracellular copper containing enzyme in white rot fungi including *Pleurotus* sp. (Mayer, 1987; Youn *et al.*, 1995; Khammuang and Sarnthima, 2007; Mukherjee and Das, 2009). Different isoenzymes of laccase have been the results of extracellular secretions in fungal species depending on the environmental conditions (Giardina *et al.*, 1999). Apart from its main role in lignin degradation (Galliano *et al.*, 1991; Thurston, 1994) various other functions of laccase has been suggested by investigators viz. rapid cell growth and formation of primordia (De Vries *et al.*, 1986), detoxification of pollutants (Eggert *et al.*, 1997; Desai and Nityanand, 2011), pathogenesis (Vaiterbo *et al.*, 1994; Galhaup *et al.*, 2002) etc. Laccase is very much stable and easy to handle enzyme so utilized in a number of biotechnological purposes (El Shora *et al.*, 2008; Gilaki, 2010).

Oyster mushroom (*Pleurotus* spp.) enjoys second position after the button mushroom (*Agaricus bisporus*) in terms of yield/ production turnover around globe (Sanchez, 2010). *Pleurotus* spp. are not only regarded as edible mushroom but produced a number of pharmaceutically important substances (Tambekar *et al.*, 2006). The yield of this very mushroom varies not only from species to species but also in between strains of the same species. There is hardly any reported marker character of this mushroom which can be helpful for the assessment of yield before cultivation. The present investigator has reported that one extracellular laccase enzyme (L_2),

produced by *P. florida,* is associated with the regulation of mycelial growth, a prerequisite for fruit-body production (Das *et al.*, 1997, 2001). In the present study, it is tried to enumerate that *P. florida* shares the similar laccase isoenzyme profile with *P. ostreatus* and irrespective of the differential laccase isoenzyme patterns in different *Pleurotus* species, a common growth regulating laccase-like isoenzyme (L_2 of *P. florida*) is present in all the species.

MATERIALS AND METHODS

Strain and culture media: The mycelial strain of *P. florida* (ITCC, 3308) was obtained from Society for Rural Industrialization, Ranchi, India. *P. ostreatus* (MTCC, 1802), *P. flabellatus* (MTCC, 1799), *P. sajorcaju* (MTCC, 1806) and *P. pulmonarius* (MTCC, 1805) were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India and maintained in Potato Dextrose Agar (PDA) (Das and Mukherjee, 2007). Mushroom strains were grown in Potato Dextrose (PD)) media (Das *et al.*, 1997) to obtain the extracellular enzymes.

Inoculum source and culture conditions: An inoculum (6 mm in diameter) was taken from the periphery of colonies growing on PDA for 10 days. The production of laccase was studied in liquid PD medium in stationary conditions at 25+1°C. Generally the culture filtrate was collected after 25 days of growth wherever not mentioned. The culture supernatant was obtained by centrifugation (10000 g×30 min) and used as the source of enzyme.

Partial purification of laccase: The laccase activity present in the culture filtrate was partially purified after 80% ammonium sulphate precipitation, followed by extensive dialysis against 0.01 mM acetate buffer (pH 5.0).

Assay for laccase activity: Laccase activity was assayed spectrophotometrically as described by Das *et al.* (1997) with guaiacol or o-dianisidine as the substrates. The ABTS activity was assayed according to Bose *et al.* (2007), Enzyme activity was expressed in International Unit.

Protein estimation: Protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

Activity staining: Activity staining of enzyme after native Poly Acrylamide Gel Electrophoresis (PAGE) of the 25th days partially purified culture filtrate was done with solution of o-dianisidine in acetate buffer, pH 5.0 as reported earlier (Das *et al.*, 1997; Bose *et al.*, 2007).

Statistical analysis: All experiments were carried out using 9 replicates (3 sets×3 batches) and best values were determined by a linear least-square regression analysis using MINITAB Version 6.

RESULTS AND DISCUSSION

In the present study, all the tested species of *Pleurotus* i.e., *P. florida* (ITCC, 3308), *P. ostreatus* (MTCC, 1802), *P. flabellatus* (MTCC, 1799), *P. sajorcaju* (MTCC, 1806) and *P. pulmonarius* (MTCC, 1805) had been found to produce laccase enzyme in PD medium (Table 1). The culture filtrate of all the strains could effectively oxidize o-dianisidine, guaiacol and ABTS. ABTS oxidizing

Table 1: Production of laccase (±SE) in different *Pleurotus* spp. in PD medium using 25th day's culture filtrate

	Activity ($U\ mL^{-1}$)			Specific activity[1]	
Strain	o-dianisidine	Guaiacol	ABTS	$U\ mg^{-1}$ protein	$U\ mg^{-1}$ mycelia
1799	126±2.24	57±1.97	463±3.43	300±7.72	327±3.47
1802	346±8.03	146±4.38	514±3.84	665±3.47	375±1.59
1805	162±4.03	86±1.92	613±3.51	395±2.60	352±2.69
1806	147±4.83	79±1.31	447±4.26	320±2.76	318±1.38
3308	60±2.87	10±0.71	463±2.32	109±2.24	155±0.94

[1] Specific activity was measured in terms of o-dianisidine oxidizing activity

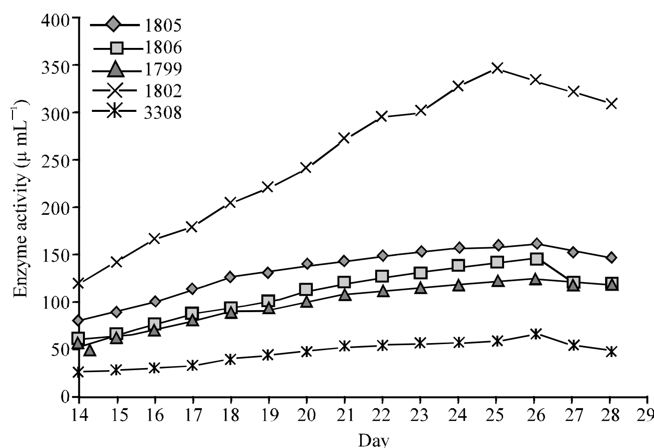

Fig. 1: Day wise (14th-28th) laccase activity of different *Pleurotus* species. Enzyme activity was measured by o-dianisidine

ability of all the strains was found to be much higher over the other two substrates (Table 1). The efficiency for extracellular laccase production was found to vary not only from species to species but also in different cultural condition. Heterogeneous laccase production patterns had been reported in several times in various fungi depending on the media compositions and/other influencing conditions (Youn *et al.*, 1995). Reports are available where *P. ostreatus* had been found to be the topper in laccase production among the different members of oyster mushrooms. Interestingly, in the present study, *P. ostreatus* (1802) was able to produce highest quantity of laccase in the 25th day of culture, whereas the other species could even effectively exhibit their optimum laccase activities in the 26th day of culture (Fig. 1). *P. florida* (3308) had been found to demonstrate the lowest enzyme activity using o-dianisidine or guaiacol as the substrates (Table 1, Fig. 1).

Though all the strains of *Pleurotus* were found to produce laccase from the very early phase of growth (data not shown) but the optimum laccase activity of different strains could only be observed during 25 to 26 days (Fig. 1). The time period for laccase production also reported to vary in different species, owing to several influencing factors prevalent in cultural conditions, like temperature, pH, media compositions, stationary or shaking conditions etc. Different studies have already reported that ligninolytic enzyme activities get triggered as a result of depletion of nutritional factors and the notable enzymes like laccase was found to be produced at the stationary period of growth phase (Kirk and Farrel, 1987; Higuchi, 1990; Guillen *et al.*, 1992).

In the present study, most of the *Pleurotus* strains had been showing a positive correlation between laccase production and mycelial growth as evidenced by comparative laccase activity and

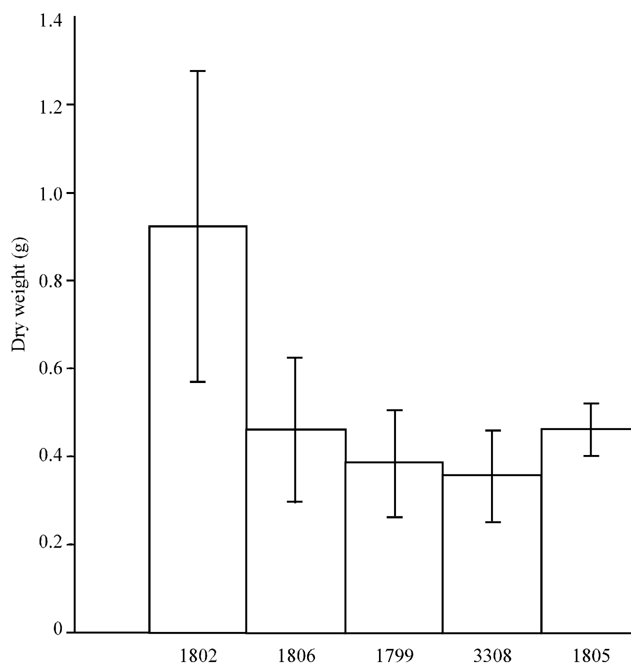

Fig. 2: Dry weight of 25 day's grown mycelium of different *Pleurotus* spp. The bar showed the standard error

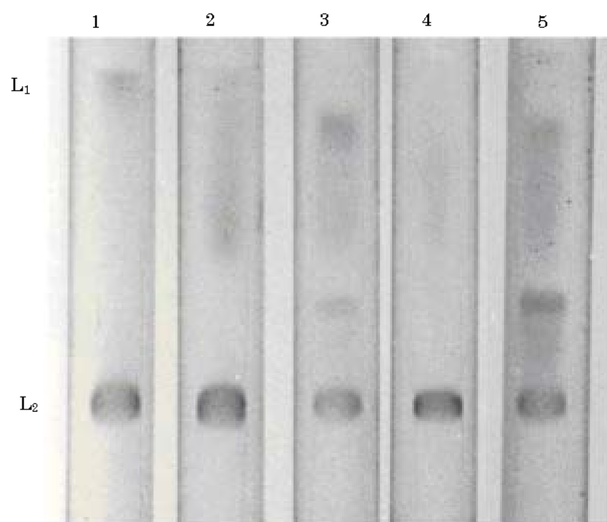

Fig. 3: Zymogram of different *Pleurotus* species (developed by o-dianisidine) from 25th day's partially purified culture filtrate. 1-3308, 2-1802, 3-1805, 4-1799, 5-1806

mycelial dry wt. (Table 1, Fig. 2). When the specific activities were measured in terms of mycelia or protein, strain 1802 (*P. ostreatus*) could exhibit the maximum activity whereas strain 3308 (*P. florida*) was the weakest in laccase enzymatic potentials (Table 1). The mycelial dry weight was

also found to be highest in strain 1802 and lowest in 3308 (Fig. 2). Das *et al.* (1997) had propounded a connection on the involvement of a particular laccase isoenzyme (L_2) of *P. florida* during the vegetative growth phase and recommended that highly productive strains were also found to produce large amounts of laccase in to the extra cellular medium. Mansur *et al.* (1997) reported that higher amount of extracellular laccase might help in efficient substrate invasion which could promote the growth and productivity effectively. Tlecuitl-Beristain *et al.* (2008) reported the positive correlation being existed between laccase activity and growth phase of the fungus *Pleurotus ostreatus*.

In present study, all the tested strains have shown two or more laccase isoenzymes (evidenced from non-denaturing zymogram profile) present in extracellular culture filtrate (Fig. 3). Interestingly, two laccase isoenzymes were found to be present in strain 3308, 1802 and 1799 while three in strain 1805 and four in 1806 in native PAGE. In the zymogram, a particular laccase band, which had been found to have similar r_f value with L_2-laccase of *P. florida* (3308) was found to be present prominently in all the lanes(Fig. 3). Different workers reported that most of the fungal members could produce more than one laccase isoforms although the number might vary from experiment to experiment (Mayer, 1987; Mukherjee and Das, 2009; Thurston, 1994). De Souza *et al.* (2004) reported at least three laccase isoforms, of which two could be produced in a non-induced culture condition and one could be the resultant in a induced culture of *P. pulmonarius*. Tellez-Tellez *et al.* (2005) had reported about two isoforms of intracellular laccase in two different strains of *P. pulmonarius*. Stajic *et al.* (2006) had reported the presence of three isoenzymes of *P. pulmonarius*, whereas Soden and Dobson (2001) could trace four isoenzymes of laccase in *P. sajorcaju*. The number of laccase isoenzymes reported by different workers in *P. ostreatus* had not been found to be same. It was reported that six strains of *P. ostreatus* could produce two different isoforms of intracelluler laccases. Stajic *et al.* (2006) have reported three isoenzymes in *P. ostreatus*. Mansur *et al.* (2003) had reported four laccase isoenzymes in *P. ostreatus* but they could locate only two isoenzymes during guaiacol staining throughout the culture period. Interestingly, during purification after ultrafiltration and ammonium sulphate precipitation, two new laccase isoforms were noticed to appear in the native gel, only when ABTS was used as substrate. Das *et al.* (1997) reported two distinct laccase isoforms in *P. florida* and suggested that within the two laccase isoforms, the L_2-laccase of *P. florida* was supposed to be actively responsible for mycelial growth. It has been found that in native PAGE both *P. florida* (3308) and *P. ostreatus* (1802) have produced two prominent laccase isozyme bands (Fig. 3).

Tellez-Tellez *et al.* (2005) have suggested that laccase isoenzyme zymogram profile could be considered as a biochemical parameter for differentiating different species among the genus *Pleurotus*. They had also reported similar laccase isoenzyme patterns in *P. ostreatus* and *P. florida* and been able to comment and highlight on their functional role in elucidating a probable phylogenetic relationships.

The most important observation in this work is that irrespective of differential laccase activity and isoenzyme patterns, a growth regulating laccase like-enzyme (like L_2-laccase of *P. florida*) has been found to be dominantly present in all the tested *Pleurotus* spp. as evidenced by their relative movement (r_f) in native PAGE (Fig. 3).

From the results, it can be concluded that laccase is an important enzyme in all the tested strains of *Pleurotus* sp. The number and position of laccase band in a species is its unique property. Present observation suggest that irrespective of divergence in laccase isoenzyme patterns in different *Pleurotus* spp., all of them possessed a unique laccase isoenzyme (as evidenced by

zymogram) which might be correlated to be actively involved in mycelial growth of all the strains, that could also be a prerequisite for sporophore (fruiting body) development. So, this particular laccase can be effectively exploited as a marker enzyme for strain differentiation, improvement and assessment of fruiting efficiency, prior to commercial cultivation of the oyster mushrooms.

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