CRYSTAL VIOLET (TRIPHENYLMETHANE DYE) DECOLORIZATION POTENTIAL OF *PLEUROTUS OSTREATUS* (MTCC 142)

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The extracellular enzyme production capacity of *Pleurotus ostreatus* MTCC 142 was investigated for decolorization of crystal violet under solid and submerged conditions. Laccases are the major extracellular lignocellulolytic enzymes produced by fungus. *Pleurotus ostreatus* provided an effective decolorization of dye at 20 mg/L concentration up to 92%. Mycelial growth was observed maximum on plate for a dye concentration 20 mg/L while lowest on 200 mg/L on day 12, respectively. At all concentrations of dye studied, maximum laccase activity was observed on day 8. For 20 mg/L of dye laccase activity was 133 U/L. The decolorization was attributed to microbial action and without role of pH change; less than 0.4 pH change was observed. Manganese dependent peroxidase activity was 106 U/L, maximum on day 8 incubated with 20 mg/L dye concentration. The present study suggested that the high efficiency decolorization of crystal violet by *P. ostreatus* was assisted by laccase and manganese-dependent peroxidase activity and can be exploited as a promising in biological treatment of waste water containing crystal violet.

Key words: Pleurotus ostreatus; Crystal violet; Dye decolorization

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INTRODUCTION

Industrial waste water effluent that contains dye is a life-threatening problem. Since it contains many toxic compounds, it is hazardous for mankind and the normal flora of the affected region. During the dyeing process, approximately 10 to 15% of the dyes used are released into the wastewater. The presence of these dyes in the aqueous ecosystem is the cause of serious environmental and health concerns (Fang et al. 2004; Asad et al. 2007). Release of industrial effluent heavily contaminated with dyes into potable and domestic water reservoirs would be the main cause of the heath care burden in the Gujarat region of India. The control of water contamination is becoming increasingly important these days. Government legislation for waste water treatment is

forcing industries to remove contaminating dyes by physico-chemical methods. Such methods are often very costly, and the removal of dyes, accumulated in concentrated sludge, creates disposal problems. The occurrence of very low concentrations of dyes in effluent is highly noticeable and undesirable (Nigam et al. 1996). Synthetic dyes are chemically diverse, divided into azo, triphenylmethane or heterocyclic, and polymeric structures (Gregory 1993). They are used extensively for biochemical analysis, food, plastics, and textile industries (Vaidya and Datye 1982).

Primary studies of the biodegradation of triphenylmethane dyes are through decolorization of dyes via reduction reactions (Azmi et al. 1998). Although several triphenylmethane dye-decolorizing bacteria have been isolated, several microorganisms including bacteria and fungi can be employed (Azmi et al. 1998). White rot fungi are able to degrade a wide range of recalcitrant organic compounds, including various dyes (Wesenberg et al. 2003). Crystal violet, a triphenylmethane dye, causes cancer, eye irritation, and inflammatory responses. The cytogenetic toxicity of crystal violet in Chinese Hamster ovary cells was studied, and it was concluded that a dosage (10μg/mL) showed a significant accumulation of abnormal metaphase, such that they might act as a mitotic poison (Au et al. 1978).

The present study evaluates the extent of decolorization of crystal violet by *Pleurotus ostreatus* white rot fungus by producing different lignocellulosic enzymes such as laccase (EC 1.10.3.2), lignin peroxidase (EC 1.11.1.14), and manganese-dependent peroxidase (EC 1.11.1.13). The dynamics of these enzymes were studied during the growth of the organism during dye degradation for involvement of enzymes in the degradation pathway.

EXPERIMENTAL

Materials

Dyes, chemicals, and microbiological media

All of the chemicals were of analytical and molecular biology grade. Crystal violet dye was procured from Merck (Vadodara, India). Other fine chemicals were purchased from Sisco Research Laboratories (India). Yeast extract and glucose were obtained from Hi Media laboratory (Mumbai, India).

Microorganisms and culture conditions

The pure culture of *Pleurotus ostreatus* (MTCC142) was procured from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The culture was maintained on Yeast Dextrose Agar (YDA) Hi Media laboratory (Mumbai, India) at 25±2º C and stored at 4º C and sub-cultured once in a month.

Inoculum Development of P. ostreatus MTCC142

Inoculum for *Pleurotus ostreatus* was prepared on boiled wheat grains supplemented with 0.2% calcium carbonate and 1.2% calcium sulphate (Sisco Research

Laboratories, India). Cultures were incubated at 25˚ C for 15 days, and these grains with mycelium were used as inoculum.

Methods

Effect of dye concentration on growth of P. ostreatus MTCC142

A disc (2 mm) of fungal mycelium of *P. ostreatus* in YDA was inoculated into the centre of petri dishes (90 mm) containing YDA and 20, 50, 100, and 200 mg/L of the crystal violet dye, respectively. The plates were incubated at 25º C in incubator for 12 days. The diameters (cm) of the growth were determined in two perpendicular directions of the plate. Plates containing organisms without dye served as control (Weitz et al. 2001).

Decolorization of dye in liquid medium by P. ostreatus MTCC142

Discs (2 mm) of *P. ostreatus* on YDA were transferred to 250 mL flasks containing 50 mL YDB. After 6 days, the flasks were supplemented with 20, 50, 100, and 200 mg/L of crystal violet from 40 mg/mL stock solution. Corresponding experiments without dye served as the control. The flasks were incubated at 25º C shaking on 130 rpm. Biodegradation experiment was started with addition of dye in culture medium. The final concentration of dye in the medium on day 0 was considered to be 100%. Degradation of the dye was monitored by measuring O.D. at 589 nm, and the pH was monitored by a pH meter at an interval from 2 days to 12 days (Yatome et al. 1993).

Biochemical estimations and enzymes assays

The amounts of reducing sugars present in YDB medium were estimated by the dinitrosalicylic acid method (Miller 1959), and protein concentrations were estimated by the method of Lowry (Lowry et al. 1951). *P. ostreatus* grown media containing dye was collected at regular intervals of 2 days up to 10 days and immediately analyzed for activity of laccase (Setti et al. 1999; Xiao et al. 2001), lignin peroxidase, and manganese-dependent peroxidase (Tien et al. 1983). Laccase unit activity was defined as µmol of product formed per minute after the reaction with guaiacol. Lignin peroxidase unit activity was defined as the µmol of the veratraldehyde formed from veratrylcohol per minute. Manganese-dependent peroxidase unit activity was defined as µmol of Mn^{+3} formed from Mn^{+2} per minute.

RESULTS AND DISCUSSION

As shown in Fig. 1, mycelial growth was decreased with increasing dye concentration. Growth of *P. ostreatus* mycelia showed delay by 1 day in the presence of 20 and 50 mg/L of dye, while 4 and 6 day showed delay in the presence of the 100 and 200 mg/mL of dye. Mycelia were grown around 56%, 44%, 19%, and 0% in presence of 20, 50, 100, and 200 mg/L of dye, respectively, compared to the control at the end of 6th day of incubation. Figure 2 indicates that the sugar consumption was decreased with the increase in dye concentration in media. Increase in the 2.5 fold concentration of the dye from 20 mg/L to 50 mg/L showed a decrease in sugar less than 2.5 fold, which suggests

the dye partially inhibited the growth. Sugar consumption and growth patterns were not correlated, suggesting the role of other growth factors are required for the growth of fungi along with a carbon source in presence of the dye. Sugar consumption was 35% and growth was 46.87% compared to the control in 20 mg/L dye medium, while the medium containing 200 mg/L dye showed 25 % sugar consumption and 6.25% growth compared to the control. These results suggest that with increasing dye concentration, mycelia growth was inhibited and consumed more sugar for bioenergetics, rather than utilizing it for other biosynthetic pathways.

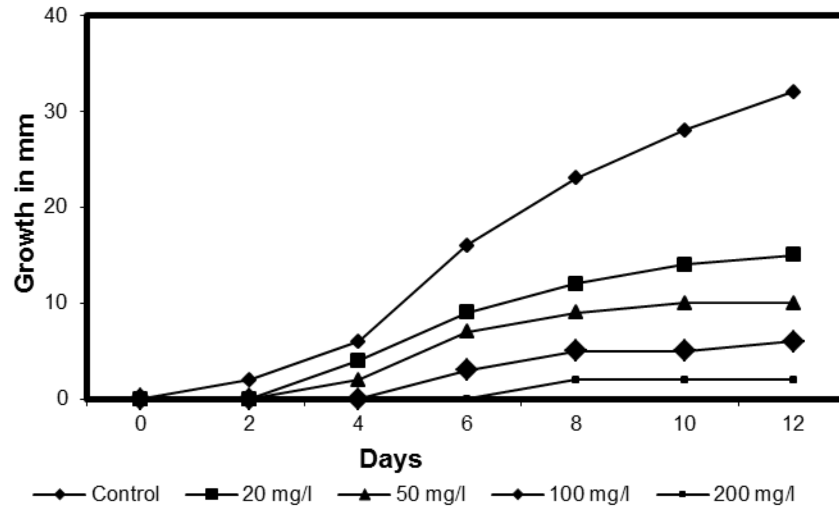

Fig. 1. Mycelial growth pattern on YDA medium containing 20, 50, 100, and 200 mg/L crystal violet

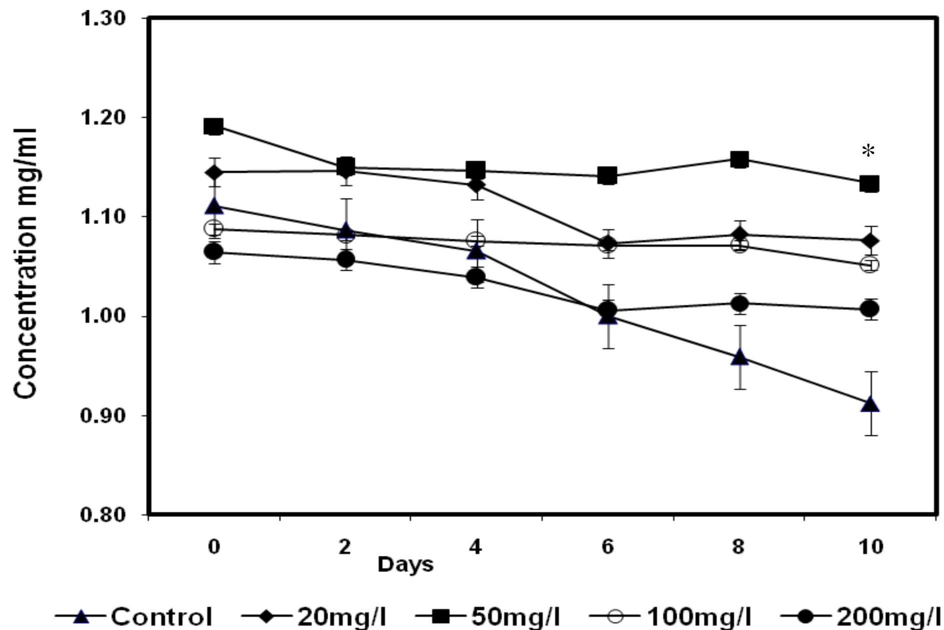

Fig. 2. Total sugar consumption pattern YDB medium containing 20, 50, 100, and 200 mg/L crystal violet; *p<0.05 compared to control group

Figure 3 shows that 92% of the dye was decolorized from the 20 mg/L dye containing medium after 10 days of incubation. 32% of dye decolorization was observed in the presence of 50 mg/L concentration of dye, while in the case of 100 and 200 mg/L concentration of dye, less than 10% of decolorization was monitored after 10 days. This indicates the inhibitory effect of high concentrations of dye on growth of fungi and ultimately decreased activity of enzymes, which results in the variation in time required for decolorization as well as percentage decolorization of dye (Parshetti et al. 2006; Jadhav et al. 2007). Decolorization of crystal violet was achieved at a level of 100% under static anoxic conditions by *Shewanella* sp. NTOU1 (Chen et al. 2008). Azo dyes are aerobically biotransformed or mineralized by the white rot fungi *Phanerochaete chrysosporium*, *Trametes versicolor*, *Pleurotus ostreatus*, etc. (Galindo and Kalt 1999; Robinson et al. 2001; Balan and Monteiro 2001; Eichlerova et al. 2006). According to Yesilada *et al.* (2003), *P. ostreatus* shows 97, 89, and 84% decolorization for azo dyes (264 mg/L), namely, astrazone red, astrazone blue, and astrazone black, respectively. The white rot basidiomycetes *Phanerochaete chrysosporium* is able decolorize various textile dye effluents. Different decolorization levels (40 to 73%) were achieved for eight textile dyes in Kirk's basal medium by this organism.

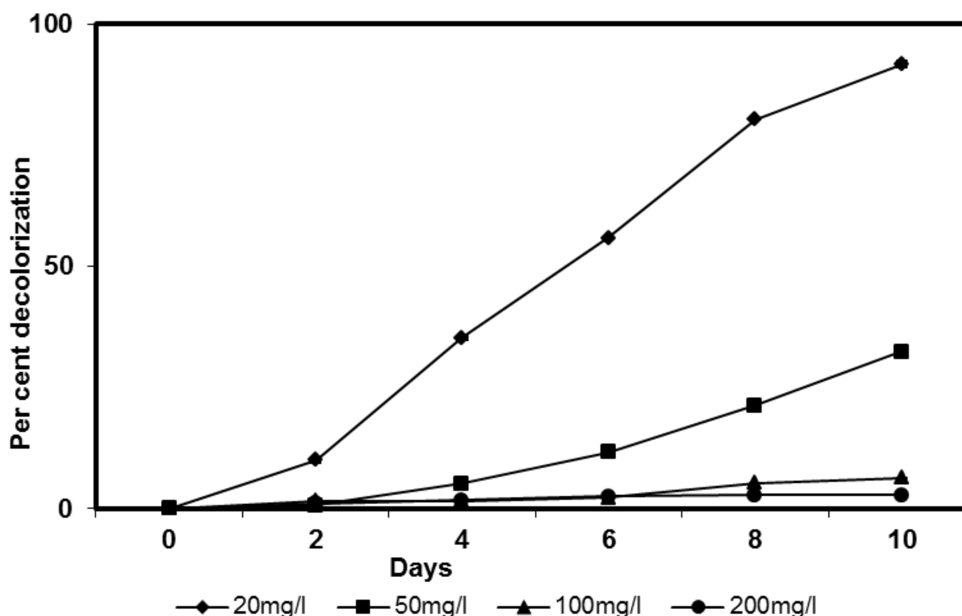

Fig. 3. Percent decolorization of crystal violet measured at 589 nm in a liquid culture containing YDB by *P. ostreatus*

To confirm that the observed dye decolorization was as a result of microbial action, we used autoclaved mycelia to decolorize dye in the presence of crystal violet; no evidence of dye decoloration was observed (not shown). Secondly, to confirm a role of change in pH in decolorization, the change in pH was recorded from 0 to 12 days at an interval of every 2 days, which was in the range of 0.05 to 0.41 pH unit (Fig. 4). Here, maximum decolorization of the crystal violet was observed in the case of 20 mg/L and the pH change was 0.18 pH unit simultaneously. In the presence of other dye concentra-

tions, very small pH change was found, which suggests no role of pH change in dye degradation.

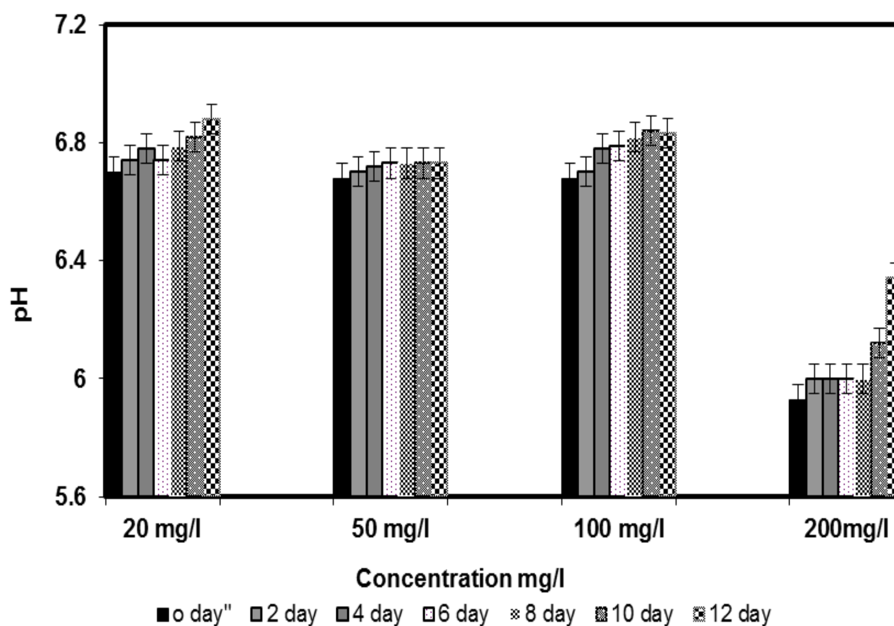

Fig. 4. Change in the pH of liquid culture YDB during growth of *P. ostreatus*

Many processes have been developed based on laccases due to their potential in degrading dyes of diverse chemical structures (Rodríguez et al. 2006; Wesenberg et al. 2003). Several studies have demonstrated the ability of fungal biomass and purified enzymes to decolorize dye (Wesenberg et al. 2003). The dyes that acted as inducers of enzyme production in the culture medium were, in turn, decolorized by the enzyme produced. Brilliant green was decolorized to the maximum by day 4, whereas RO 176 was the least decolorized. This is because brilliant green was a high inducer of the production of the enzyme in the medium, while RO 176 was a low inducer of the production of the enzyme in the medium (D'Souza et al. 2006).

Laccases are copper-containing enzymes that catalyze the oxidation of electron-rich substrates such as phenols. Laccase alone has a limited effect on bioremediation due to its specificity for phenolic subunits in lignin. However, earlier Camarero et al. (2005) demonstrated that phenolic aldehydes, ketones, acids, and esters related to the three lignin units were among the best mediators, including p-coumaric acid, vanillin, acetovanillone, methyl vanillate, and above all, syringaldehyde and acetosyringone. Phenolic compounds were shown to be efficient laccase mediators (Camarero et al. 2005). These profiles suggest that as the percent decolorization increases, the enzymes activity also increases, and later it decreases after reaching the peak.

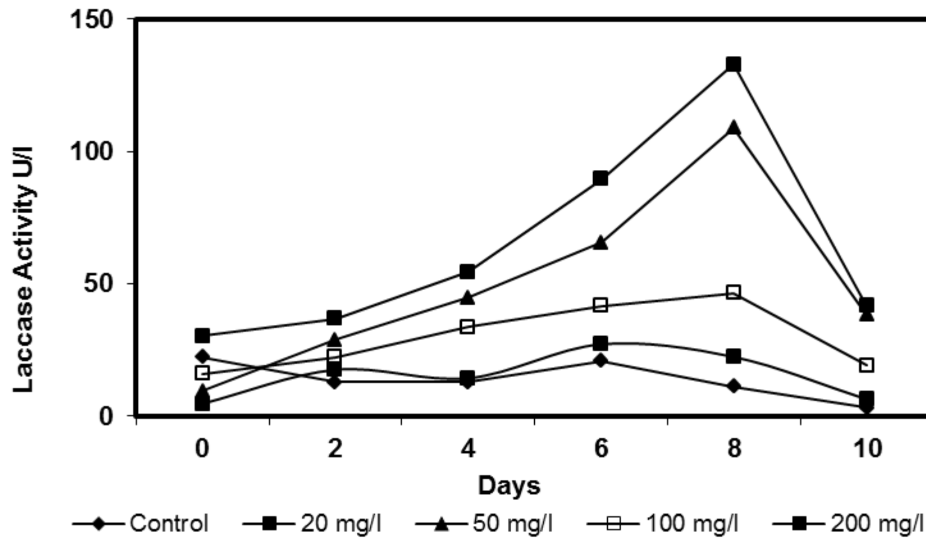

Fig. 5. Production of Laccase in liquid culture containing YDB by *P. ostreatus*

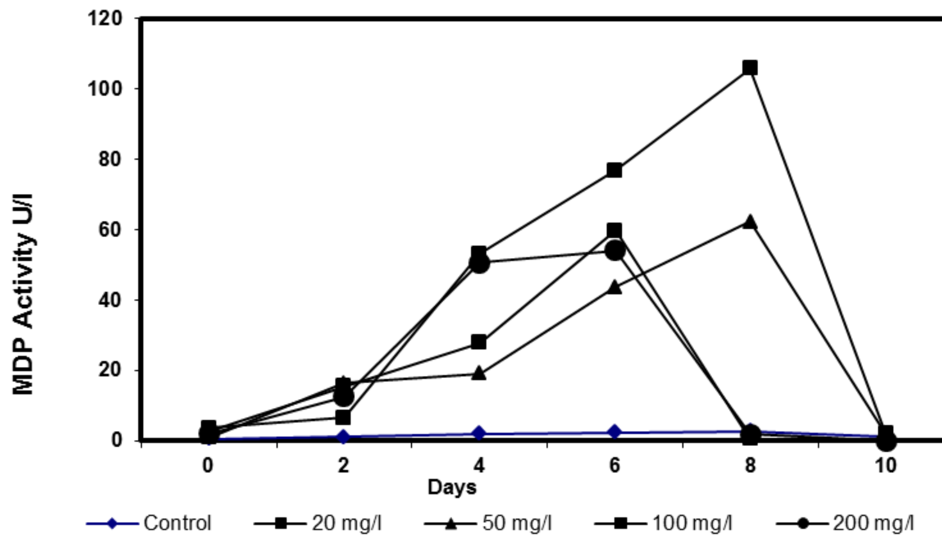

Fig. 6. Production of Manganese-dependent peroxidase in liquid culture containing YDB by *P. ostreatus*

Unprocessed dyeing effluents may cause serious ecological problems and health hazards. They are being released into the water basin, and this water could be used for agricultural practice. Thus, it is of immediate concern to analyze the phytotoxicity of the effluent before and after degradation. Kalyani *et al.* (2008) carried out one of the most common phytotoxic assays to evaluate the phytotoxicity of plant growing media based on the percent germination (%G) of seeds. The percent germination combines measurements of comparative root and shoot elongation, as both are sensitive to the presence of phytotoxic compounds. Although several species have been traditionally used for evaluating phytotoxicity, there are no standardized seed species in use worldwide (Osma et al. 2010).

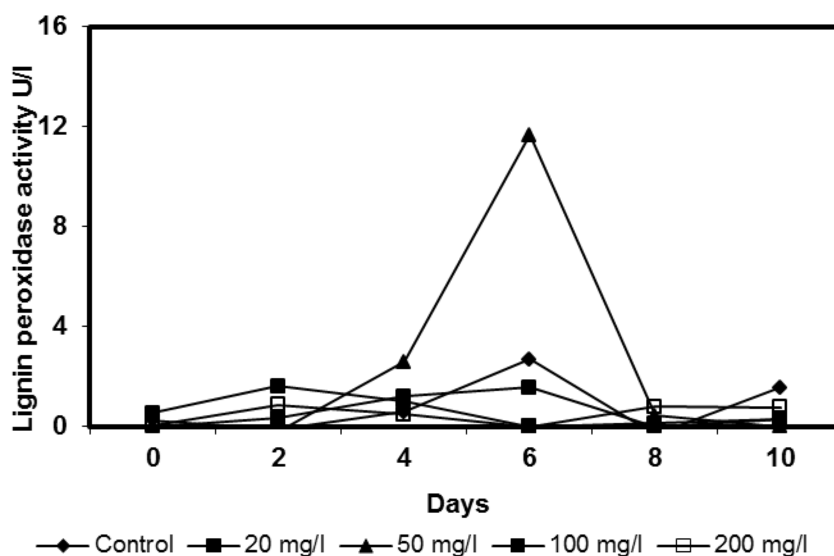

Fig. 7. Production of Lignin peroxidase in liquid culture containing YDB by *P. ostreatus*

Enzyme profiles for laccase, manganese-dependent peroxidase, and lignin peroxidase were monitored for up to 10 days in the presence of 20, 50, 100, and 200 mg/L crystal violet dye (Fig. 5-7). The highest laccase and manganese-dependent peroxidase production was 133 and 106 U/L, respectively, observed after 8 days in the presence of 20 mg/L dye. The synthetic dye acts as manganese-dependent peroxidase, and laccase inducers and dye decolorization was done by enzyme activity. High concentration of dye inhibits the growth of the fungus strain. Percent decolorization of the dye on the 8th day was around 80, which is highly correlated for decolorization of dye by increased activity of these two enzymes. However, from the 8th day to the 10th day, laccase and manganese-dependent peroxidase activity was decreased to 42 and 2 U/L, respectively. On day 10, activity of both enzymes decreased; this may be related to the physiology of the *P. ostreatus*. 109 and 62 U/L were reported for the laccase and manganese-dependent peroxidase on the 8th day, respectively, followed by 38 and 2 U/L for both enzymes on the 10th day. For the dye concentration of 100 mg/L, laccase activity was 42 U/L on day 8, while the dye concentration of 200 mg/L was at a maximum of 27 U/L on day 6. These results suggest that at a higher concentration of dye growth, inhibition leads to less production of the enzymes required for the decolorization of the dye for *P. ostreatus*. Lignin peroxidase activity remained low throughout the incubation period from 0 to 10 days; therefore the dye acts as a poor inducer for lignin peroxidase of *P. ostreatus.* Numerous reports dealing with lignolytic microorganisms have shown that the presence of microbial peroxidases and laccases seems to be correlated with their ability to decolourise certain dyes (Glenn and Gold 1983; Paszczynski et al. 1986; Cripps et al. 1990; Paszczynski and Crawford 1991; Shin et al. 1997). *Pleurotus ostreatus* secreted a wide range of enzymes lignin peroxidase, manganese peroxidase and laccase, proving that the lignolytic system is active and as white rot fungi.

Isikhuemhen and Mikiashvili (2009) reported solid waste increased the nutritional composition of mushrooms grown on a combination of wheat straw, and solid waste with millet significantly improved mushroom yield. Lignocellulolytic enzyme activities were

affected significantly by changes in the solid waste content of substrate combinations tested; increasing solid waste content resulted in increased laccase and peroxidases enzymes production. Our results are specifically with dye not on solid waste in a combination of wheat straw and millet. Both solid waste and dye show equal potential for production of laccase.

CONCLUSIONS

1. The fungus was grown and produced laccase and manganese-dependent peroxidase at various concentrations of crystal violet dye in yeast dextrose broth.
2. Activity of laccase and manganese-dependent peroxidase was induced with no change in lignin peroxidase in the degradation of crystal violet.
3. A high level of dye concentration down regulates the growth of *P. ostreatus* and mycelia production.
4. The fungus appears to be a good natural system for biological methods for industrial applications in bioremediation of colored wastewaters, and it can be used in further research related to the possibility of bioconversion.

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