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Full Length Research Paper

Screening and induction of laccase activity in fungal species and its application in dye decolorization

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A great deal of works has been carried out assessing dye-decolorization capabilities of fungi, but only few species were investigated. In this present study, ten fungal species were screened for laccase activity by indicator plate method, of which five species were found to be laccase-positive. Laccase activity varied during growth and maximal laccase activity was observed during the 9th day, except for *Agaricus bisporus*. *Pleurotus ostreatus* gave the highest laccase activities, showing average value around 570 U/L, which were higher than other strains. Addition of 1 mM copper sulphate induced the laccase production efficiently by 60 to 80%, while veratryl and benzyl alcohol induced laccase production in all the laccase positive species except for *A. bisporus*. Constitutive expression of laccase was observed in *F. solani* in the presence of copper sulphate. In addition, the extracellular laccase from the *P. ostreatus* could decolorize reactive dyes, which suggests the potential application of laccase in textile effluent treatment.

Key words: Dyes, decolorization, inducer, laccase, *Pleurotus*, textile.

INTRODUCTION

Laccase (EC 1.10.3.2) is an extracellular, multicopper enzyme that uses molecular oxygen to oxidize various aromatic and nonaromatic compounds by a radical-catalyzed reaction mechanism (Thurston, 1994; Polaina and MacCabe, 2007; Couto and Herrera, 2006). Laccases are widely distributed in nature in higher plants, fungi and bacteria (Mayer and Staples, 2002). Fungi that possess laccase ability are capable of degrading screening reagents with a similar structure to lignin as well (Wunch et al., 1997). The main purpose of screening is to select laccase producing fungi with desired characteristics intended for various applications, example, dye decolorization. The screening methods are based on the colorimetric measurements where color changes of synthetic chemicals in the media are associated with specific fungal laccase activities. Microbes that produce laccases have been screened for either on solid media containing colored indicator compounds that enable the visual detection of laccase production (DeJong et al., 1992) or with liquid cultivations monitored with enzyme activity measurements. The use of colored indicators is generally simpler as no sample handling and measurement is required. As laccases oxidize various types of substrates, several different compounds have been used as indicators for laccase production. Common indicators used are guaiacol (Coll et al., 1993; Kiiskinen et al., 2004; Vishwanath et al., 2008; Ang et al., 2010), 2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Floch et al., 2002; Wilkołazka et al., 2002; Hao et al., 2007), syringaldazine (Floch et al., 2002; Wilkołazka et al., 2002; Wang et al., 2010) and polymeric dyes like remazol brilliant blue-R (RBB-R) (Desouza et al., 2004).

The technical and industrial interest is to investigate whether the methods used for enhancing laccase production can be applied to basidiomycetes and deuteromycetes. In general, two approaches have been followed for the production of elevated amounts of laccases. Whereas the satisfying heterologous

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overexpression and production of ligninolytic enzymes is still a challenge (Couto and Herrera, 2006; Couto and Herrera, 2007), great advances have been made in enhancing laccase production in basidiomycetes by optimizing the culture conditions. Chiefly, laccase inducers and different nutritional conditions have been used extensively (Arora and Gill, 2001). Inducers are usually natural substrates or substrate analogues for the enzyme (Kumar, 2010). Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms (DeSouza et al., 2004; Revankar and Lele, 2006) and the nature of the compound that induces laccase activity differs greatly with the species. Inducers reported to be used for the laccase production are copper sulfate (Revankar and Lele, 2006; Baldrian, 2003), ferulic acid and veratryl alcohol (Couto et al., 2002; DelaRubia et al., 2002), 4-hydroxy-benzoic acid, 2, 5-xylidine or vanillin (Couto et al., 2002).

Laccase plays a major role in lignin degradation and their industrial and food application are increasing day by day (Couto and Herrera, 2006). Laccases are increasingly being used in a wide variety of industrial oxidative processes such as delignification, dye bleaching, pulp and paper processing, prevention of wine discoloration, production of chemicals from lignin, waste detoxification and textile dye transformation, plant fiber modification, ethanol production, biosensors, biofuel cells etc (Couto and Herrera, 2006; Rogalski et al., 1991). On account of their importance in variety of applications, this study was carried out to screen highly efficient laccase enzyme producing fungi. Hence, the present work reports the simple screening method for laccase in different fungal species and to investigate the effect of different inducers on laccase production. The present study also reports the dye decolorization abilities by extracellular liquid of *Pleurotus ostreatus*.

MATERIALS AND METHODS

Chemicals and fungi

Guaiacol, syringaldazine, RBB-R and ABTS were obtained from Himedia, Mumbai, India. All other reagents and chemicals used were of analytical grade. The fungal species used for the study were obtained from various culture collection centers from India like Microbial Type Culture Collection (MTCC), National Collection of Industrial Microorganism (NCIM), PSG Medical College (PSGMC) and Tamil Nadu Agriculture University (TNAU) and were maintained on potato dextrose agar slants.

Screening for laccase activity

Initially organisms were screened for laccase activity using guaiacol and syringaldazine as indicator compound. In the presence of guaiacol, intense reddish brown color was produced in the medium around the fungal colonies and was taken as the positive reaction for the presence of laccase enzyme activity as previously reported (Coll et al., 1993; Kiiskinen et al., 2004; Vishwanath et al., 2008; Ang et al., 2010). The pale yellow color of syringaldazine is oxidized to a purple-colored compound in the presence of fungal colonies and was taken as the positive reaction for the presence of laccase enzyme activity as previously reported (Wilkołazka et al., 2002; Floch et al., 2007; Wang et al., 2010).

Screening of laccase producing organisms was done on plates using PDA supplemented with 0.02% guaiacol and 0.1% syringaldazine (Vishwanath et al., 2008; Wang et al., 2010). Twenty different fungal strains were inoculated in sterile Petri plates containing the above media and were incubated at 30°C for a period of seven days. Cultures showing definite color changes were considered laccase producing strain and used for subsequent studies.

Laccase production

Laccase quantitative screening work was done on 500 ml Erlenmeyer flasks containing 100 ml of the culture media (pH 6.0) containing glucose, 1%; peptone, 0.3%,; KH_2PO_4, 0.06%; $ZnSO_4$, 0.0001%; K_2HPO_4, 0.04%; $FeSO_4$, 0.0005%; $MnSO_4$, 0.05% and $MgSO_4$, 0.05% as previously reported. The media were inoculated with seven day old sporulated culture suspension prepared from the cultures grown in Petri plates. For growing basidiomycetes in suspension, inoculum (250 mg) was prepared by homogenizing the six day old mycelium obtained from the Petri plates. Laccase activity was determined at three day intervals, after which the culture were centrifuged at 10,000 g for 30 min at 4°C. The supernatant was filtered through Whatman no. 1 filter paper and assayed for laccase activity.

Induction studies

Several natural and synthetic substrates have been reported to enhance laccase activity in basidiomycetes (Revankar and Lele, 2006; Baldrian, 2003; Couto et al., 2002; DelaRubia et al., 2002). Based on the previous reports the following inducers were used in the present study: Copper sulphate (1 mM), veratryl alcohol (7 mM) and benzyl alcohol (10 mM). The compounds tested as inducers were sterilized by filtration. The laccase producing fungi were cultured for fifteen days on previously mentioned media in the presence of inducers. Laccase activity was determined at three day intervals, by centrifuging the culture at 10,000×g for 30 min, and the supernatant was filtered through Whatman No. 1 filter paper and used for the assay.

Laccase assay

The laccase activity was determined using 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) as the substrate (Rasera et al., 2009). The laccase reaction mixture contained 0.5 ml of 0.45 mM ABTS, 1.2 ml of 0.1 M phosphate buffer (pH 6.0) and 0.5 ml of filtrate to give a final reaction volume of 2.2 ml. The oxidation of the substrate (ABTS) was monitored by the increase in the absorbance at 420 nm using Shimadzu UV-1800 spectrophotometer (Tokyo, Japan) over 90 s at 30°C (± 2°C), using $\varepsilon = 3.6 \times 10^4\ cm^{-1}\ M^{-1}$. Enzymatic activity was expressed as 1 U = 1 µmol of ABTS oxidized per min at 25°C (± 1). Lignin peroxidase activity was determined by measuring the production of veratraldehyde from veratryl alcohol at 310 nm in glycine-HCl buffer (pH 3.0) at 30°C, upon addition of hydrogen peroxide (1 mM concentration) (Pointing, 1999).

Further, aryl alcohol oxidase activity was assayed under the same conditions without the addition of hydrogen peroxide (Kumar and Rapheal, 2010).

Table 1. Presence of laccase activity among the different fungal species.

S/N	Species	Guaiacol oxidation	Syringaldazine oxidation
1	*Agaricus bisporus* [b]	+	+
2	*Aspergillus niger* MTCC 1344 [c]	-	-
3	*Aspergillus terrus* MTCC 2580 [c]	-	-
4	*Aspergillus flavus* [a]	-	-
5	*Beauveria bassiana* MTCC 6100 [c]	-	-
6	*Beauveria feline* MTCC 6294 [c]	-	-
7	*Fusarium solani* [b]	+	+
8	*Metarhizium anisopilae* MTCC 892 [c]	-	-
9	*Paecilomyces fumosoroseus* MTCC 6292 [c]	-	-
10	*Paecilomyces lilacinus* MTCC 1422 [c]	-	-
11	*Penicillium decumbens* [a]	-	-
12	*Penicillium chrysogenum* NCIM 709 [d]	+	+
13	*Penicillium sp* [b]	-	-
14	*Pleurotus platypus* [b]	+	+
15	*Pleurotus ostreatus* [b]	+	+
16	*Pleurotus eous* [b]	+	-
17	*Pleurotus floridanus* [b]	-	-
18	*Trichoderma harzianum* MTCC 936 [c]	-	-
19	*Trichoderma viridie* MTCC 2535 [c]	-	-
20	*Verticillium lecani* [b]	-	-

The fungal isolate were obtained and maintained as pure cultures from the following culture collection centers: [a] PSGMC = PSG Medical College, Coimbatore, India; [b] TNAU = Tamil Nadu Agriculture University, Coimbatore, India; [c] MTCC = MICROBIAL type culture collection, Punjab, India; [d] NCIM = National Collection of Industrial Microorganism, Pune, India.

Decolorization of textile dyes

Two textile dyes used in textile industry (Reactive blue 49, Reactive red 243) were selected and their solutions were prepared in the concentration range of 50 mg/L in distilled water. Each dye was incubated with laccase enzyme (0.36 U/mg protein/min) in 0.1 M phosphate buffer, pH 6.0 at 30°C. The disappearance of the color by laccase enzyme was monitored at predetermined λ_{max} of the respective dye solution. The percentage decolorization was calculated by taking the maximum absorbance of each untreated dye solution as control (100%). The optical density was measured using Shimadzu UV-1800 spectrophotometer (Tokyo, Japan).

RESULTS, DISCUSSION AND CONCLUSION

Screening of laccase positive fungi

The main purpose of the screening is to select fungi with desired characteristics intended for various applications, example, dye decolorization and lignocellulolytic enzyme production (Ang et al., 2010). The use of guaiacol and syringaldazine as an indicator of action for laccases provided for the rapid visual manifestation of laccase positives. Ten different genus of fungi, namely, *Pleurotus, Fusarium, Agaricus, Penicillium, Trichoderma, Aspergillus, Beauveria, Verticillium, Paecilomyces and Metarhizium,* were screened for their laccase enzyme activities, (Table 1). Laccase have been mainly screened and studied in wood rot fungal species of the basidiomycetes family especially in white rot fungi. Studies in other fungal families are largely lacking. Therefore in the present study we have screened laccase activity in other fungal families that are native to plant ecosystem (that is, *Agaricus, Pleurotus species, Ascomycetes* species like *Aspergillus, Penicillium, Trichoderma, Beauveria, Verticillium, Paecilomyces, Metarhizium* and *Fusarium species).* In the present study, two types of screening agents were used to screen for fungal laccase activity. The formation of reddish brown color due to oxidation of guaiacol in the presence of peroxidase (DeJong et al., 1992) and syringaldazine oxidation was essential for further confirmation of laccase (Pointing et al., 1999; Wilkołazka et al., 2002; Wang et al., 2010).

By day four, six of the strains oxidized guaiacol and began the formation of reddish brown color around the colonies on agar plates. In case of syringaldazine, five of the strains oxidized the dye on fourth day of incubation.

Laccase activity showed in the colonies of five strains *P. ostreatus, Fusarium solani, Pleurotus platypus, Agaricus bisporus* and *Penicillium chrysogenum* (Table 1). In case of *Pleurotus eous*, the color formation was due to the peroxidase, it was further confirmed with oxidation of polymeric dyes remazol brilliant blue (Data not shown). In our previous study, *Pleurotus eous* have the ability to produce aryl alcohol oxidase (AAO) (Kumar and Rapheal, 2010), so combined action of peroxidase

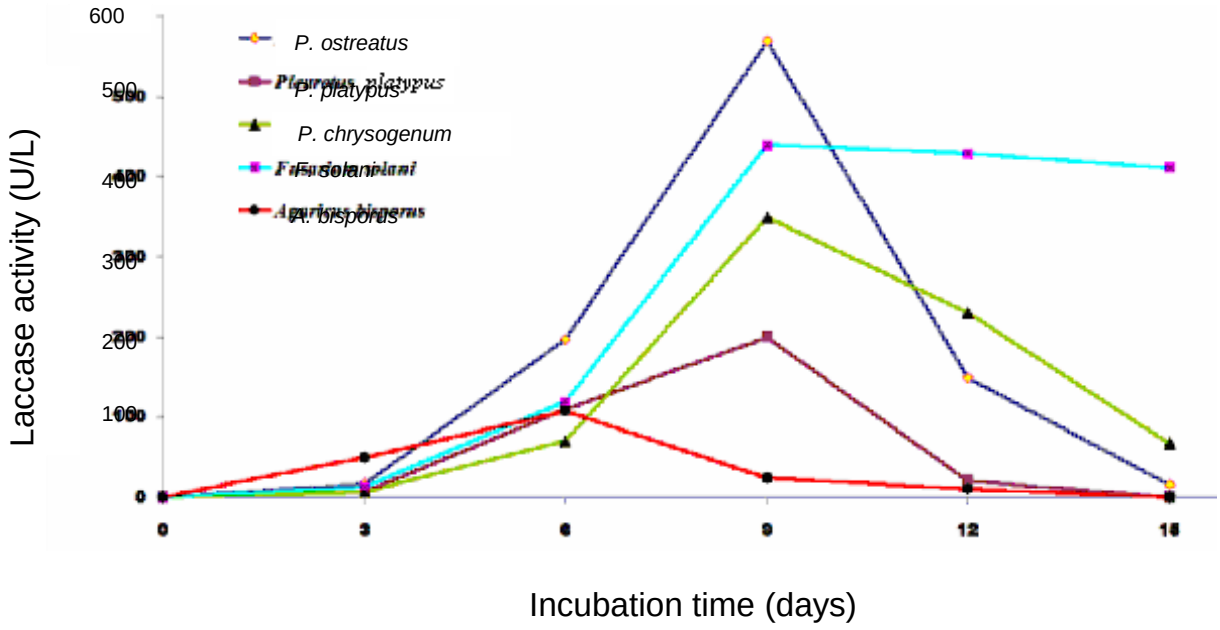

Figure 1. Laccase production during growth of fungi. Fungal cultures grown in standard conditions in defined medium at pH 6, and 30°C.

and AAO decolourize the polymeric dyes. In the earlier reports, laccase activity was incidence in *Trichoderma harzianum* (Sadhasivam et al., 2008), but we failed to detect laccase activity in this fungus.

This may be due to the fact that the composition of the culture medium is known to substantially affect the production of this enzyme. Kiiskinen et al. (2004) suggest that color reactions with guaiacol are more easily detectable for laccase positives and it is reliably used for laccase activity screening. Six laccase positive fungal species from different environmental samples were obtained using guaiacol as an indicator (Vishwanath et al., 2008). Harkin et al. (1974) used syringaldazine as an indicator to find the laccase positive wood rotting fungi.

Effect of inducers on enzyme production from laccase positive fungal species

Enzyme production was monitored during the growth of laccase positive fungal strains. Laccase production was obtained on six days by *A. bisporus* (108 U/L) followed by nine days in *P. ostreatus* (570 U/L), *P. platypus* (200 U/L), *P. chrysogenum* (350 U/L) and *Fusarium solani* (440 U/L) (Figure 1). For enhanced laccase production, the effects of different inducers were studied. The laccase positive fungi were grown separately on defined medium containing one of the following inducers: copper sulphate, benzyl alcohol and veratryl alcohol.

Compared with control, inducers increased the laccase production in *P. ostreatus* in the following order: copper sulphate > veratryl alcohol > benzyl alcohol (Figure 2).

Copper is the most frequently applied substance to enhance laccase production in fungi. It is part of the active center of laccases, and is thus crucial for the synthesis of a catalytically active laccase protein (Baldrian et al., 2003). Maximum laccase production was obtained from *P. ostreatus* (910 U/L) on 9th day at 1 mM copper sulfate (Figure 2). In the presence of copper sulphate, maximum laccase production was obtained on six days by *A. bisporus* followed by nine days in *P. platypus, P. chrysogenum* and *F. solani*. Effect of different inducers on the laccase production in different fungi was given in Figure 3.

Copper sulphate at 1 mM concentration increased laccase production in *F. solani* (80%) followed by *P. ostreatus* (60%) and *A. bisporus* (54%). The finding emphasizes the importance of copper sulphate in the laccase production and in case of *F. solani* constitutive production was showed.

Addition of copper enhance the levels of gene transcription for laccase enzyme. Some of the genes are expressed constitutively and many are induced by nitrogen deficiency, by chemicals, like copper (Collins and Dobson, 2005). The promoter region of the genes encoding for laccase contain various recognition sites that are specific for xenobiotics and heavy metals (Baldrian et al., 2003). It has been demonstrated that the *Pleurotus* laccase genes *poxc* and *poxa1b* are transcriptionally induced by copper and several putative metal responsive elements found in the promoter region of these genes (Calmieri et al., 2000). In addition to copper, distinct organic inducers with structural similarities or relationships with lignin are often applied,

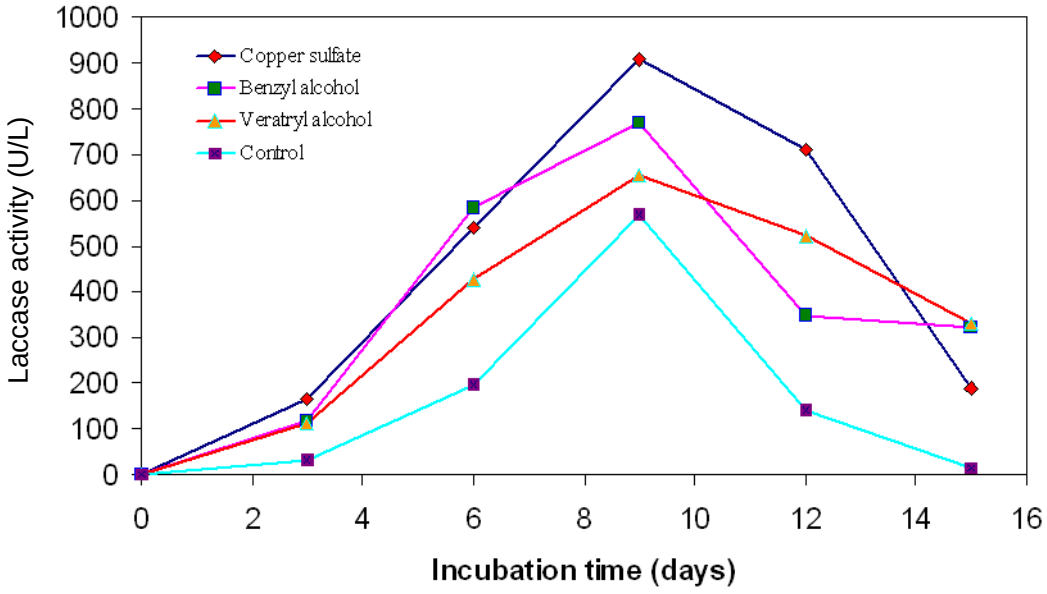

Figure 2. Effect of inducers in laccase production by *P. ostreatus.*

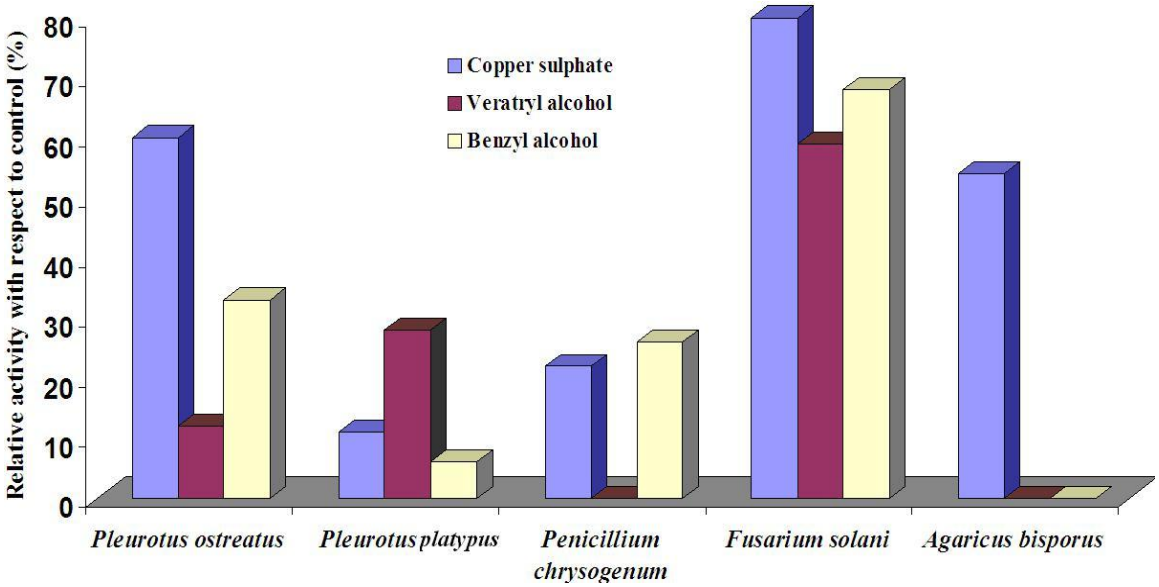

Figure 3. Effect of different inducers in laccase production by various fungi. Control activities are as follows *P. ostreatus* (570 U/l), *P. platypus* (200 U/l), *P. chrysogenum* (350 U/L), *F. solani* (440 U/L) *and A. bisporus* (108 U/L).

example, 4-hydroxy-benzoic acid, ferulic acid, veratryl alcohol, 2, 5-xylidine or vanillin (Vishwanath et al., 2008; Couto et al., 2002). In *Heterobasidion annosum*, phenolics significantly induced the laccase production (Haars and Hüttermann, 2003).

Aryl alcohols were less efficient in inducing the laccase compared to copper sulphate in most species except in *F. solani*, whereas about 68% increases were seen. In *P. chrysogenum* benzyl alcohol was effective, whereas veratryl alcohol does not have any effect on laccase production. Notable both the alcohols did not have any influence on laccase production in *A. bisporus*. This result was contrast with others, where veratryl alcohol induces the laccase production in immobilized cell of *A. bisporus* (Kaluskar et al., 1999). Exogenously added veratryl alcohol induced laccase in synthetic liquid cultures of white-rot fungi (Rogalski et al., 1991) and in an ascomycete *Botryosphaeria* sp (Barbosa et al., 1996). In

Pleurotus eryngii, veratryl alcohol did not exert any significance effect on laccase synthesis (Munoz et al., 1997).

Decolorization efficiency of extracellular laccase

The extracellular liquid of *P. ostreatus* without any inducers showed activity of laccase (0.57 U/ml), aryl alcohol oxidase (0.012 U/ml) and lignin peroxidase (0.16 U/ml). Manganese peroxidase activity was found to be negligible in this species. However, in the presence of inducers the activity of crude laccase was still higher (0.91 U/ml). On the basis of the enzyme production, *P. ostreatus* may be a suitable fungus for the decolorization of textile dyes. In order to study the degrading ability of the extracellular liquid produced, the decolorization of two reactive dyes was performed. Extracellular liquid from *P. ostreatus* degraded the two reactive dyes (50 µg/ml each) namely reactive blue 49 (66%) and reactive red 243 (55%) within 12 h. *P. ostreatus* secrete acid stable laccase, which are very useful for their application to the removal of dyes from acidic textile effluents such as those resulting from wool and polyester processing. Degradation from this study and those in literature was found to be high at acidic pH to our results, some workers have showed that the degradation of industrially important dyes by lignin peroxidase (Jadhav et al., 2008a; Jadhav et al., 2008b) and laccase (Wang et al., 2010; Hao et al., 2007; Couto and Herrera, 2006) was also more in the buffer of lower pH values. The above results show that the individual dye structures influence the decolorization extent obtained by laccase, indicating the specificity of laccase towards different dye structures. This reveals the high potential of laccase from *P. ostreatus* for dye decolorization.

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