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Research Article

DECOLORIZATION OF ANTHRAQUINONE DYES BY ASPERGILLUS STRAINS AND ALSO OPTIMIZATION OF LIGNOLYTIC ENZYMES

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ABSTRACT

Isolation of *Aspergillus fumigatus strain YHYS, Aspergillus tamarii strain SRRC 1088* around the textile industry effluents for the decolorization of anthraquinone dyes such as Remazol brilliant blue-R (RBBR), Brilliant blue-R (BBR) and Acid blue-25 (AB-25). *Aspergillus fumigatus strain* YHYS show the maximum decolorization with AB-25 (86.3%), minimum with RBBR (64.7%). Similarly *Aspergillus tamarii strain* SRRC 1088 show the maximum decolorization with RBBR (78.3%) and minimum activity with BBR (46.3%) at 35°C, pH-7.0. The lignolytic enzymes were responsible for the decolorization of dyes. The high MnP enzyme activity (33.9 U/ml), low LiP (31.3 U/ml) activity was produced by *Aspergillus fumigatus strain* YHYS. Similarly *Aspergillus tamarii strain* SRRC 1088 produced high MnP (287.6 U/ml) and minimum LiP (41.6 U/ml) activity. The enzyme production was optimized with different parameters the optimum p^H for lignolytic enzymes was 3.5 at 30°C & 0.1% of Glucose and 0.05% of peptone sources.

INTRODUCTION

Due to rapid industrialization and urbanization, a lot of chemicals including dyes are manufactured and are being used in day-to-day life. About 100,000 viable dyes are manufactured including several varieties of dyes such as acidic, basic, reactive, azo, diazo, anthraquinone based meta complex dyes with an annual production of over 7 x 105 metric tons are commercially available (Campos *et al.*, 2001). Roughly 50% of the dyes are released in the industrial effluents. They are used on several substrates in food, cosmetics, paper, plastic and textile industries. Some of them are dangerous to living organisms due to their fused aromatic ring structure, toxicity and carcinogenicity.

In general dyes in wastewater frequently lead to calamities viz. the incidence of bladder tumors has been reported to be particularly higher in dye industry workers than in the normal population (Suryavathi *et al.*, 2005). Natural pigments used for coloring textiles have been replaced by "fast colors" which do not fade on exposure to light, heat and water. About 15% of the dyes used for textile dying are released into processing waters (Eichlerova *et al.*, 2006). Besides being unaesthetic, these effluents are mutagenic, carcinogenic and toxic. Commonly applied treatment methods for color removal from colored effluents consist of integrated processes involving various combinations of biological, physical and chemical decolorization methods (Robinson *et al.*, 2001; Azbar *et al.*, 2004), of these, approximately 10-15% of unused dyes enter the wastewater after dyeing and after the subsequent washing processes (Rajamohan and Karthikeyan, 2006). Chemical and physical methods for treatment of dye wastewater are not widely applied to textile industries because of exorbitant costs and disposal problems. Green technologies to deal with this problem include adsorption of dyestuffs on bacterial and fungal biomass (Fu and Viraraghavan, 2002; Yang *et al.*, 2009) or low-cost non-conventional adsorbents (Crini 2006; Ferrero, 2007).

So many scientists have already worked with many microorganisms, the imperative bacteria being *Staphylococcus arlettae* (Elisangela *et al.*, 2009); Lactic acid bacteria (Khaled *et al.*, 2010); *Pseudomonas putida* (Leebana *et al.*, 2012); *Micrococcus luteus*, *Listeria denitrificans* and *Nocardia atlantica* (Hassan *et al.*, 2013) *Bacillus megaterium* (Joshi *et al.*, 2013), and fungi viz. Basidiomycetous fungi (Machado *et al.*, 2006); *Tramates pubescens* and *Pleurotus ostreatus* (Casieri *et al.*, 2008); *Aspergillus tamarii* and *Penicillium purpurogenum* (Ramalingam *et al.*, 2010); *Aspergillus ochraceus* (Tisma *et al.*, 2012); and *Pleurotus ostreatus*

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(Siddique *et al.*, 2012); *Aspergillus niger*, *Fusarium oxysporum* and *Trichoderma lignorum* (Shahid *et al.*, 2013). Among the numerous biological treatment technologies, but most of researchers on the fungal bioremediation due to their biomass compared to the bacteria, has increased significantly for decolorization and degradation of synthetic dyes (Shahid *et al.*, 2013). Mainly the extracellular enzymes are participating in dye degradation are heme-containing lignin peroxidase, manganese peroxidase and Cu-containing laccase.

Laccases (EC 1.10.3.2) has received much attention for their superiority in degrading various recalcitrant pollutants. These oxidase enzymes widely distributed among plant, fungi and bacteria, and can catalyze a variety of aromatic compounds (Canas A.I *et al*., 2010). Some redox mediators can facilitate the catalytic activity of laccase and expand its substrates specificity to a wider range (Cambria M.T *et al*., 2008). But the Laccase mediator systems (LMS) have been extensively used in different fields such as organophosphorus compounds (OPs) degradation, insecticide, PHA, pulp bio bleaching and dye decolorization (Trovaslet-Leroy M *et al*., 2010; Kudanga T *et al*., 2011). The potential application of ligninolytic enzymes in biotechnology has stimulated the investigation of their production with the purpose of selecting the enzyme producers and increasing their yield (Kalmis *et al*., 2008). The major objective of the present study was to investigate the state of art of influence of several parameters on enzyme production by *Aspergillus fumigatus strain YHYS, Aspergillus tamarii strain SRRC 1088* and their applications in anthraquinone dye decolorization.

MATERIALS AND METHODS

Isolation and identification of dye degrading fungal strains

The effluent samples were collected in and around the textile industries of Chirala, Bapatla, Dharmavaram, Madhanapalli, Hyderabad (A.P) and Siera Silk Mills Ltd., (Bangalore). After collection of the effluents, the samples were analyzed for fungi isolation by inoculation onto Potato dextrose agar medium at 30±1°C for 2 days. Isolated cultures were identified morphologically &18SrRNA sequence analysis.

Decolorization assay

The identified strains were analyzed for their dye decolorization efficiency. Anthraquinone dyes (Reactive blue-19, Brilliant blue-R, Acid blue-25) were used in different concentrations viz 10,50 &100mg/L, incorporated in Sabouraud's dextrose broth and subsequently inoculated with the known fungal spore suspension containing $5.0x10^6$cfu/ml (colony forming unit) at 35±1°C for 8 days. After incubation the decolorization efficiency was measured by spectrophotometer at 592nm (Remazol Brilliant Blue R), 470nm (Brilliant Blue-R) and 630nm (Acid Blue-25) along with control flasks. Repeated the above experiment in triplicates & calculated their statistics (S.D & S.E) by prism software and the percentage of decolorization was calculated by using following formula:

$$\% \text{ of Decolorization} = \frac{\text{Initial OD - Final OD}}{\text{Initial OD}} \times 100.$$

Microorganisms and culture conditions for preservation

After testing of effective & efficient decolorization, the selected potential fungal strains were preserved on PDA (potato dextrose medium) or SDA (Sabouraud's agar medium). Further, the above pure fungal cultures were tested for their lignolytic enzymes production by inoculating on BHM (Bussnell Hass Medium) at 30°c for 12 days in an orbital shaker 120 rpm.

Lignolytic enzymes assay

Laccase assay

Laccase activity was measured by using Guaiacol as a substrate. The activity was assayed using 3.0 ml of 10mm sodium acetate buffer, 1ml of enzyme source and 1.0 ml Guaiacol. Reactions were initiated by the addition of Guaiacol and after mixing, the reaction mixture was incubated at 30°C for 3min. The absorbance was measured in a spectrophotometer at 450nm and the increase in absorbance was calculated. One unit activity was defined as the enzyme producing one absorption unit/min at 450 nm. Enzyme activity was expressed in international units (IU), where 1 IU is defined as amount of enzyme required to oxidize1 micromole of guaiacol per min and laccase activity was calculated by the following formula:

E.A= (Abs)/ (e*t*r),

Where E.A= enzyme activity (U/ml), Abs=Absorbance at 450 nm, e= extinction coefficient (M^{-1} cm^{-1}), t=incubation time, v=volume of enzyme.

Manganese Peroxidase assay

Manganese peroxides was determined by the oxidation of Guaiacol at 465 nm. The reaction mixture contains 0.1M Sodium tartarate buffer (pH-5.0)-0.5ml, 0.1M $MnSo_4$-0.5ml, 0.1M Guaiacol-0.5ml and H_2O_2- 0.05 ml. The reaction was initiated by adding Guaiacol as substrate, the presence of peroxide enzyme oxidized the guaiacol to tetraguaiacol, which gives reddish brown colore. The developed colore was read at 465 nm for 5 min with 10 second time interval and the activity was expressed in enzyme units which are related to increase the optical density. The Manganese peroxidase activity was calculated at 465 nm using by the following formula:

E.A= (Abs)/ (e*t*r),

Lignin peroxidase assay

Lignin peroxidase enzyme activity was determined by the oxidation of Veratryl alcohol at 310 nm. The assay mixture consists 0.1M Sodium tartarate buffer (pH-3.0)- 0.5ml, Veratryl alcohol-0.1ml and H_2O_2 -0.05ml and culture filtrate 0.5 ml. The reaction was initiated by adding 0.5 ml of H_2O_2.The developed colore was measured at 310 nm for 3minutes with 10 seconds time intervals. The Lignin peroxidase activity was calculated by the following formula.
E.A= (Abs)/ (e*t*r),

Optimization of Lignolytic enzymes

Lignolytic enzymes activity was optimized with different parameters via temperature, pH, carbon source and nitrogen source. Lignolytic enzymes were optimized with temperatures ranged from 20°C to 70°C and the influence of pH (2.5, 3.0,

3.5, 4.0, 4.5, 5.0, & 6.0.) was assessed by using Na-acetate buffer (10mm) & Sodium tartarate buffers (0.1M) for laccase &MnP, LiP respectively. Similarly we also optimized enzyme activity with different carbon sources like Lactose, Glucose, Sucrose, Starch and with different nitrogen source like Peptone, beef extract, Yeast extract & Ammonium nitrate using Na-acetate buffer(10mm) & Sodium tartarate buffers (0.1M) for laccase & MnP, LiP respectively.

RESULTS

The isolated and characterized fungal strains (National Centre for Cell Sciences, Pune for molecular characterization by 18S rRNA gene sequencing) viz *Aspergillus fumigatus strain YHYS* (Accession number-KC986235.1), *Aspergillus tamarii strain SRRC 1088* (Accession number- AY373870.1).

These two fungal species *Aspergillus fumigatus strain YHYS, Aspergillus tamarii strain SRRC 1088* tested for anthraquinone dye decolorization using Reactive blue-19, Brilliant value-R and Acid lue-25. It was also been observed that by increasing the incubation time that prominently decrease the intensity. *Aspergillus fumigatus strain YHYS* showed maximum decolorization activity with AB-25 (86.3%) and minimum activity reported with RBBR (64.7%). Similarly *Aspergillus tamarii strain SRRC 1088* showed maximum decolorization activity with RBBR (78.3%) & low activity with BBR (46.3%) has been shown in figure-1.

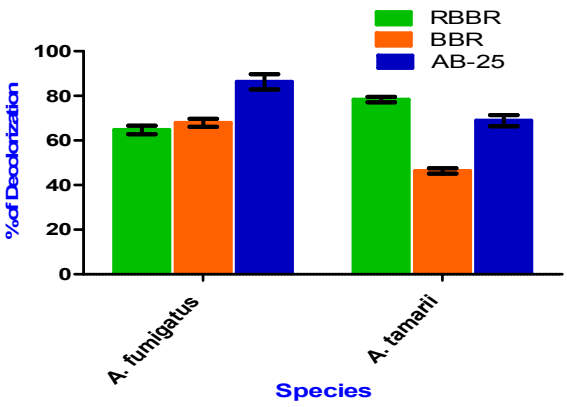


Fig 1 Decolorization of Anthraquinone dyes by *Aspergillus* strains

Lignolytic enzymes Production

To test the lignolytic enzyme activity of *Aspergillus fumigatus strain YHYS, Aspergillus tamarii strain SRRC 1088* were grown on BHM medium, the produced extracellular lignolytic enzymes are laccase, manganese peroxidase (MnP) & lignin peroxidase (LiP). Highest enzyme activity was recorded at an approximate growth conditions viz pH-3.5, temperature (30^{o}C) along with different nutritional factors, that influence the highest activity of enzymes i.e, laccase, MnP & LiP on the 12th day of incubation. *Aspergillus fumigatus strain YHYS* produced high MnP (33.9U/ml) & low LiP (31.3U/ml) and *Aspergillus tamarii strain SRRC 1088* produce the high MnP (287.6U/ml), & low LiP (41.6U/ml) activity, the complete activity of these enzyme were recorded in fig:-2.

Optimization of Lignolytic enzymes

The pH plays a key role in the production of lignolytic enzymes by *Aspergillus fumigatus strain YHYS, Aspergillus tamarii strain SRRC 1088*.

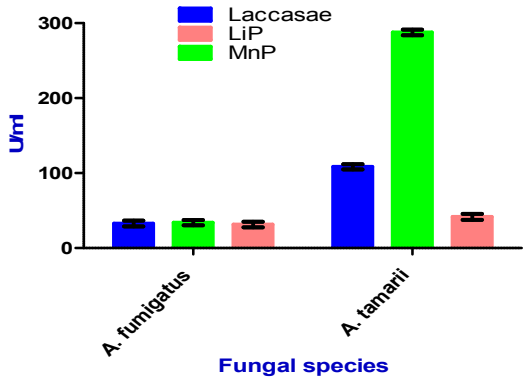


Fig 2 The lignolytic enzymes produced by the *Aspergillus* strains

The production of enzymes had been influenced by different pH ranges from 2.5, 3.0, 3.5, 4.5, 5.0 & 6.0. *Aspergillus fumigatus strain YHYS* & showed high activity of MnP (33.9U/ml) & *Aspergillus tamarii strain SRRC 1088* also showed high activity of MnP (287.6U/ml) at p^{H}-3.5. Similarly *Aspergillus fumigatus strain YHYS* showed low laccase (3.4U/ml) activity, *Aspergillus tamarii strain SRRC 1088* showed low activity of LiP (7.1U/ml) at p^{H}-6.0. From our observations the maximum activity at p^{H} -3.5, was indicated as an optimum for the laccase, MnP & LiP (fig:-3).

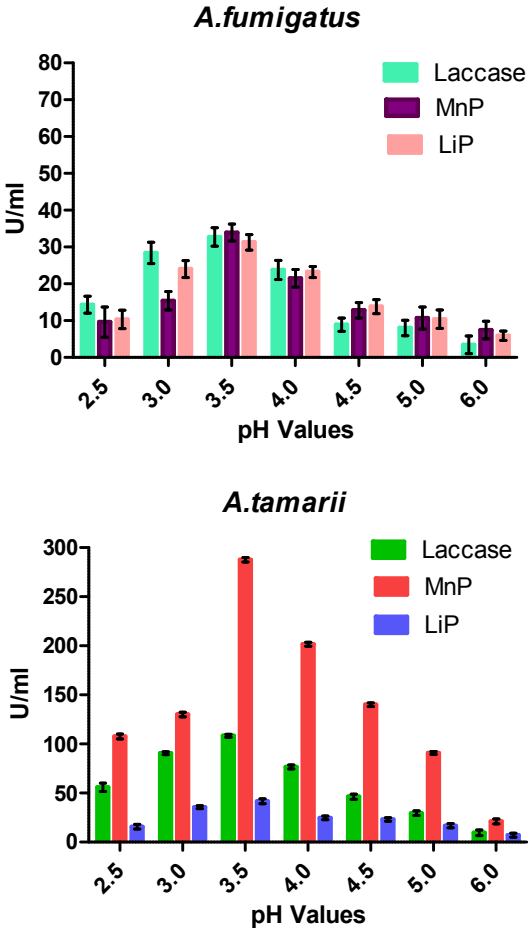


Fig 3 Effect of pH on laccase, MnP, LiP production

The temperature plays an important role for the production of lignolytic enzymes. *Aspergillus fumigatus strain YHYS* showed maximum activity of MnP (33.9U/ml) at 30°C and low activity of Laccase (1.5U/ml) at 70°C. Similarly *Aspergillus tamarii strain SRRC 1088* showed maximum activity of MnP (287.6U/ml) at 30°C & less activity of laccase (4.0U/ml) & LiP (4.7U/ml at 70°C. Form these observations it was confirmed that the 30°C would be the optimum temperature for enzyme production (fig-4).

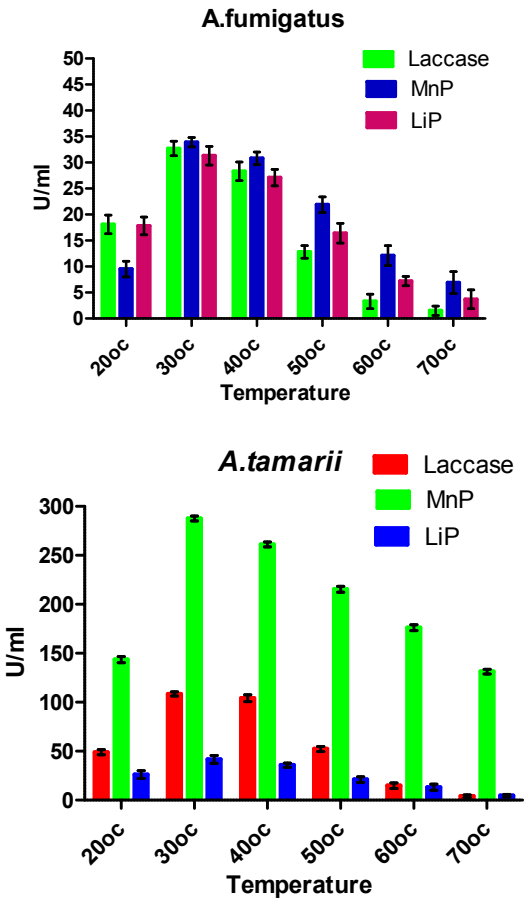
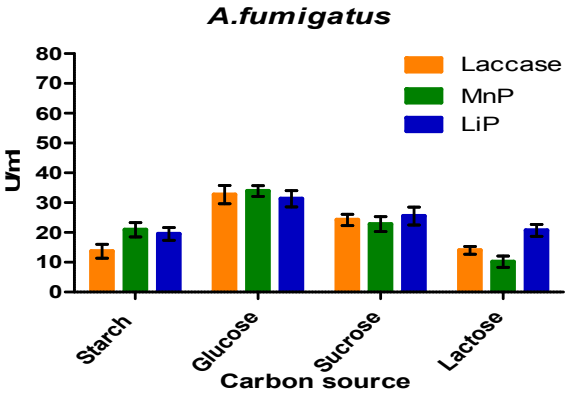


Fig 4 Effect of Temperature on laccase, MnP, LiP enzymes production

The influence of carbon sources i.e. starch, glucose, sucrose and lactose were optimized for the enzymes activity.



Aspergillus fumigatus strain YHYS produced maximum MnP activity (33.9U/ml) in the presence of glucose, but the less MnP activity (10.2U/ml) was observed in presence of lactose. Similarly *Aspergillus tamarii strain SRRC 1088* showed the maximum activity of MnP (287.6U/ml) in the presence of glucose and less MnP (10.2U/ml) activity was observed with lactose (fig:-5).

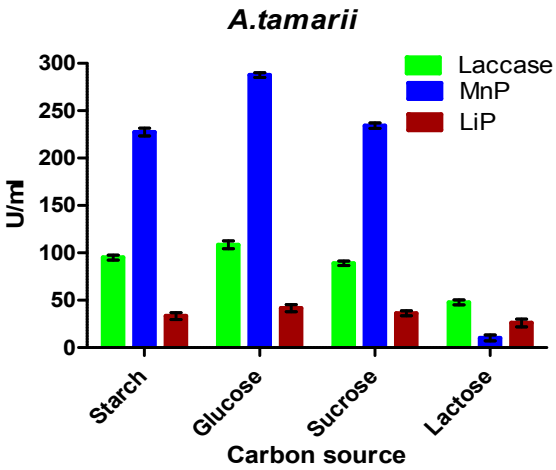


Fig 5 Effect of carbon source on lignolytic enzymes production

For the optimization of nitrogen source use peptone, yeast extract, beef extract and ammonium nitrate. MnP (33.9U/ml) enzyme produced very high in the presence of yeast extract,

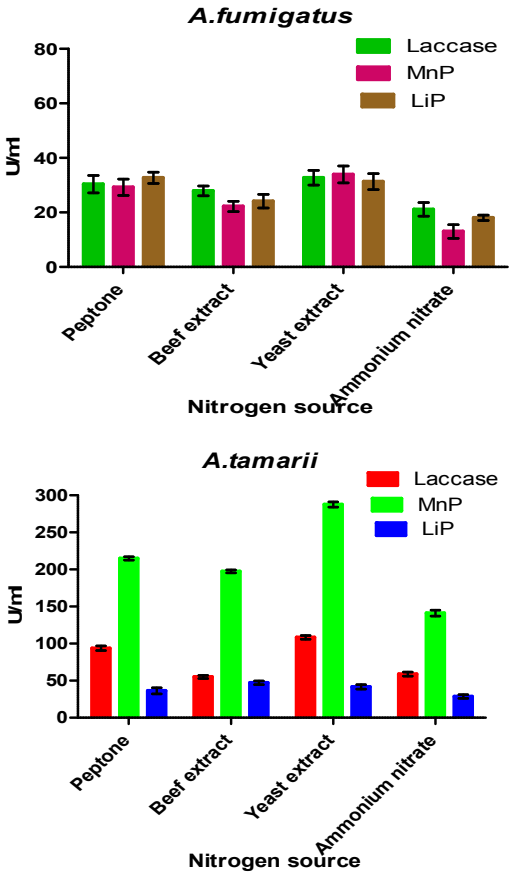


Fig 6 Effect of nitrogen source on Laccase, MnP and LiP enzyme production

low MnP (13.0 U/ml) produced in the presence of ammonium nitrate by *Aspergillus fumigatus strain YHYS.* Similarly *Aspergillus tamarii strain SRRC 1088* produced maximum activity of MnP (287.6U/ml) in the presence of yeast extract and less LiP (28.7U/ml) activity recorded with ammonium nitrate. The present study was noticed that high MnP activity was obtained in the presence of yeast extract when compared to LiP and Laccase (fig:-6).

DISCUSSION

Decolorization of several textile dyes was obtained by incubating the fungal culture containing laccase, MnP & LiP enzyme was investigated. The soil fungal strains also showed promising decol-orization activities against tested dyes. Malachite green was readily degraded in liquid culture by *A. flavus*, *A. solani* and some white rot fungi within six days up to 96%, was suggested by Ali *et al.* 2009 and Vasudev 2011. Results of the dye biodegradation by soil fungi in this study using spectrophotometric analysis were even comparable with the percent dye decolorization exhibited by the white rot fungus trametes versicolor and Pleurotus ostreatus (Yao *et al*., 2009). Soil fungi possess ligninolytic enzymes and play an important role in the degradation of lignocellulose in soil ecosystems. These lignin-degrading enzymes are directly involved not only in the degradation of lignin in their natural lingo cellulosic substrates but also in the degradation of various xenobiotic compounds, including dyes. Moreover, ligninolytic enzymes have been reported to oxidize many recalcitrant substances such as chlorophenols, polycyclic aromatic hydrocarbons (PAHs), organophosphorus compounds, and phenols (Wesenberg *et al.,* 2003). Enhancement of laccase production, by modifying the nutritional and physiologic conditions during cultivation of promising fungi, is a prerequisite for their optimum utilization at industrial scale (Dhakar and Pandey, 2013). Laboratory studies have found that increased concentrations of inorganic N can suppress the transcription of fungal genes required for the metabolism of lignin and lignocellulose (Eisenlord *et al*., 2013).

Similarly to the present study the optimal temperature for laccase production is between 25°C and 30°C, when cultivated fungi at temperatures higher than 30°C the activity of lignolytic enzymes was reduced (Pointing *et al*., 2000). Lignin peroxidases and laccases have great potential for the application in various industrial processes, because of their high redox potentials and enlarged substrate range in the presence of specific mediators (Piontek *et al*., 2001; Liers, Bobeth *et al*., 2010; Riley, Salamov *et al.,* 2014). With reference to the recent contribution of the researchers, it was observed that the temperature optima of lignolytic enzymes for the *Aspergillus terreus* (Kanayama, Tohru *et al.* 2002; Cheng, Jia *et al.* 2007; Franciscon., Zille, *et al*. 2009). It has been clarified that the rapid loss of activity in the lignolytic enzymes of a variety of other organisms i.e. with high temperature resulted the inactivation of the enzyme specificity by a denaturation process (Taboada-Puig, Lú-Chau *et al*. 2011; Saratale, *et al*. 2011). Use of 0.2 mM sodium succinate buffer (pH-4.5) enhanced the enzymatic activity and the temperatures in different ranges. Carbon and nitrogen are critical nutritional factors for the production of laccase, LiP and MnP. Excess carbon and nitrogen repress ligninolytic activities, at low C: N ratios, the fungi are carbon starved and under high-nitrogen conditions, the ligninase production is considerably reduced (Xiaoping and Xin, 2008). On the other hand, at higher C: N ratios, an imbalance between very high carbon and very low nitrogen content of the medium, leads to fungal growth inhibition.

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