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Research Article

OPTIMIZATION OF LIGNOLYTIC ENZYMES PRODUCED BY ASPERGILLUS STRAINS IN ANTHRAQUINONE DYE DEGRADATION

Srinu A[1]., Lenin Kumar B[2]., Siva Prasad B.V[3]., Murali S[4]., Vijaya Lakshmi D[5] and Prasad DVR*[6]

[1,3,5,6]Dept of Microbiology, Yogi Vemana University, Kadapa-516003
[2]Centre For Biotechnology, Department of Chemical Engineering,
Andhra University College of eng (a), Andhra University, Viskhapatnam
[4]Dept of Zoology SSBN Degreee College, Ananthapur

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ABSTRACT

The present work mainly focused on the decolorization studies of anthraquinone dyes (Remazol brilliant blue-R (RBBR), Brilliant blue-R (BBR) and Acid blue-25 (AB-25)) using different fungal strains (*Aspergillus flavus strain AJ, Aspergillus terreus)* isolated from industrial effluents. The maximum decolorization activity was recorded with *Aspergillus flavus strain AJ (*AB-25-83.0%*)* and less activity showed by *Aspergillus terreus* (BBR-46.2%). These two fungal strains also tested for production of their lignolytic enzyme activity i.e Laccase, Manganese peroxidase (MnP) and Lignin Peroxidase (LiP) activity at 30°C. Among all tested MnP (196.5U/ml) enzyme produced maximum by *Aspergillus flavus strain AJ* and minimum LiP (12.9 U/ml) enzyme produced by *Aspergillus terreus.* Further these enzymes were also optimized with various parameters like temperature, pH, Carbon & Nitrogen sources.

INTRODUCTION

Synthetic dyes are widely used in the textile, paper, cosmetics, leather dyeing and food industries. Based on the composition and chemical nature these compounds are classified as azo, anthraquinone, heterocyclic, triphenylmethane or phthalocyanine dyes and they cause severe problems to the human beings and environment. Most of them are toxic, mutagenic, carcinogenic and they are resistant to degradation. Decolorization of dyes by physical or chemical methods including adsorption and precipitation methods, chemical degradation have methodological disadvantages, they also time-consuming and mostly ineffective.

Now a days number of researchers focused on bioremediation by using microorganisms which has the capacity to decolorize and biodegrade textile dyes (Cheng X *et al*.,2007, Soma S *et al*.,2004). Large quantities of dyes were used in the textile industry. The microorganisms in general, the fungi in particular are being ubiquitous in distribution, are highly victorious in endurance because of their great morphogenesis and physiology of their tremendous versatility to secrete a wide array of enzymes involved in the breakdown of complex polymers (Carlsen M *et al*., 2001). Toxic effects of dyes for fungal growth and the enzymes, responsible for decolorization in liquid culture conditions were also investigated.

Generally, the White rot fungi are well studied organism for decolorization dyes and they produce extracellular oxidative enzymes like laccase, lignin peroxidase, manganese peroxidase, horseradish peroxidase which are involved in the degradation of several dyes (Unyayar A *et al*., 2005, Deveci T *et al*.,2004, Kertsen PJ *et al*.,1990). Similarly, Phanerochaete chrysosporium (Kirk TK *et al.*, 1978) Trametes vesicolor (Swamy J *et al*., 1999), C. versicolor (Knapp JS *et al*.,1999, Kapdan I *et al*.,2000), Cunninghamella polymorpha (Sugimori D *et al*.,1999), F. trogii ATCC 200800 (Lee TH *et al*.,2000) and Rhizopus arrhizus (Aksu Z *et al*., 2000) are the major fungal trains has ability to decolorize the dyes. Trametes versicolor (Christian V *et al*.,2005) produces the LiP, pure fungal laccase used in the textile industry (Soares GMB *et al* .,2001) and T. versicolor (Peralta-Zamora P *et al*.,2003)

Corresponding author:* **Prasad DVR
Dept of Microbiology, Yogi Vemana University, Kadapa-516003

produced free and immobilized laccase were used for RBBR decolorization. With these lines we selected *Aspergillusas* as a model organism for these decolorization and degradation studies.

Several microorganisms have been reported to produce ligninases, among all the fungal strains white rot fungi, belonging to the basidiomycetes these are the capable and broad lignin degraders (Gold M H *et al*., 1993, Wu J *et al*.,2005). They have a powerful extracellular enzymatic efficiency able to depolymerize the aromatic polymer into lower molecular compounds (Bajpai P *et al*.,2004). Particularly Phanerochaete chrysosporium and Pleurotus sp. are also said to be good for the lignolytic enzyme production (Kerem Z *et al*.,1999, Howard R L *et al*.,2003). In addition to these organisms some Ascomycetes members produced to be excel in biodegradation is repeatedly investigated as a model organism for the study of ligninolytic enzymes since it was the first fungus found to produce lignin peroxidase and manganese peroxidase.

The laccases are multicopper phenol oxidases, which oxidize phenolic compounds to phenoxyl radicals with oxygen as final electron acceptor. In the presence of a mediator such as ABTS or 1-hydroxybenzotriazole, laccases are capable of oxidation of non phenolic compounds (Eggert C *et al*.,1994).The presence of manganese peroxidase has also been demonstrated in the lignolytic system of S. ostrea (Praveen, K *et al*.,2011). MnP catalyzes the H2O2- dependent oxidation of Mn(II) to Mn(III), which, in turn, acts as a nonspecific oxidant that attacks phenolic lignin structures by one electron oxidation (Guerra, G *et al.*,2008). MnP activity has been observed in Earliella scabrosa (Silva, E *et al*.,2007), Lentinula edodes (Cheng, X *et al.*,2007), Schizophyllum sp. (Hoshino F *et al* .,2002), Lenzites betulinus (Kanayama N *et al*.,2007), Aspergillus terreus (Fakoussa RM *et al*.,1999) and Trametes versicolor. A pattern of lignolytic enzymes are not common in all fungal cultures. For instance, lignin peroxidase is the dominant enzyme in the lignolytic system of P. chrysosporium, whereas laccase is the prominent enzyme in the lignolytic system of S. ostrea. Lignin peroxidases (LiPs) are heme-containing peroxidases, they catalyzes C-C bond cleavage in the propyl side chains of two dimeric model compounds. This cleavage is prominent in the fungal degradation of lignin and is the first metabolic reaction.
Lignolytic enzymes have potential applications in a large number of fields, including chemical, fuel, food, agricultural, paper, textile and cosmetic industries (Sette LD *et al*.,2002). The major industrial application of laccase are used for many industrial purposes such as paper processing, prevention of wine discoloration and detoxification of environmental pollutants, oxidation of dye, production of chemicals from lignin. Laccases can degrade several dye structures (Buddolla Viswanath *et al*.,2008). Present study was aimed to isolate, characterize and screen out the more efficient ligninolytic fungi from textile effluents for the production of laccase, manganese peroxidase and lignin peroxidase under shaking conditions which was involved in the degradation of textiles dyes.

MATERIALS & METHODS

The soil and effluent samples were collected from the textile industry in & around the Andhra Pradesh, Telangana and Siera Silk Mills Ltd., (Bangalore).The collected samples were tested for isolation of microorganisms (fungi) by inoculation on Potato dextrose agar at 30±1°C for 2 days. Isolated fungal cultures were identified by morphology & by 18S rRNA sequence analysis. The selected & identified fungus was tested for their dye decolorization efficiency. Anthraquinone dyes (Reactive blue-19, Brilliant blue-R and Acid blue-25) at 100mg/L concentration, dissolved in to 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. The decolorization efficiency was measured by spectrophotometer at 592nm (Remazol Brilliant Blue R), 470nm (Brilliant Blue-R) and 630nm (Acid Blue-25) along with respective 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:

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

The effective potential fungal cultures identified with decolorization efficiency were preserved on PDA (potato dextrose medium) or SDA (Sabouraud's agar medium) for further analysis. The above pure fungal cultures were tested for their lignolytic enzyme production by inoculating on BHM (Bussnell Hass Medium) observed at 30^oc for 12 days in an orbital shaker.

Lignolytic enzymes assay

Laccase activity

Laccase activity was determined by the oxidation of Guaiacol used as substrate. The brown colour was observed at 450 nm due to oxidation of Guaiacol. The reaction mixture contains Guaiacol (0.1M) in sodium acetate buffer (10mm) of 1ml used as a substrate, 1ml culture filtrate as enzyme source, 3ml acetate buffer and blanks were maintained by using distilled water instead of enzyme source. The test tube contain reaction mixture was incubated at 30^oc for 3min and absorbance was recorded at 450 nm (23). Enzyme activity was expressed in international units(IU), where 1 IU is defined as amount of enzyme required to oxidize 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 activity

Manganese peroxides was determined by the oxidation of Guaiacol as a substrate at 465 nm. The reaction mixture contains Sodium tartarate buffer-0.1M (0.5ml), $MnSo_4$-0.1M (0.5ml), Guaiacol-0.1M (0.5ml) and H_2O_2-0.05M (0.05 ml). The presence of peroxide enzyme oxidized the guaiacol to tetraguaiacol, which gives reddish brown colore. The developed colore was read at 465nm for 5min with 10 second time interval and controls were maintained. The Manganese peroxidase activity was calculated at 465 nm using 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.

Lignin peroxidase activity

The action of Lignin peroxidase was determined by the oxidation of Veratryl alcohol as a substrate at 310 nm. The assay mixture consists Sodium tartarate buffer-0.1M (0.5ml), Veratryl alcohol-0.1ml and H_2O_2 -0.05ml and culture filtrate 0.5 ml as enzyme source. 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).

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.

Optimization of Lignolytic enzymes

Lignolytic enzymes activity was measured the influence of pH (2.5, 3.0, 3.5, 4.0, 4.5, 5.0, & 6.0.) and different temperatures ranges from 20°C to 70°C and also optimized with different carbon sources like Lactose, Glucose, Sucrose, Starch & different nitrogen sources such as Peptone, beef extract, Yeast extract & Ammonium nitrate. High Laccase, MnP and LiP activity was observed p^H-3.5 at 30°C and also optimized glucose as carbon source, yeast extract as a nitrogen source. The total experiment was conducted in triplicates and also maintains the respective controls. Na-acetate buffer (10mm) used for the laccase enzyme and Na-tartarate buffer (0.1M) for MnP & LiP enzymes.

Statistics

The total results pertaining to the analysis and also represented them in the for form of mean, SD, and SE.

RESULTS

The isolated fungal strains were tested for their morphological & physiological identification, and obtained molecular characterization of the identified fungal species from National Centre for Cell Sciences, Pune by 18S rRNA gene sequencing. As per the reports isolated fungal species were confirmed as *Aspergillus flavus strain AJ* (Accession number-HQ324118.1), *Aspergillus terreus* (Accession number-HQ219673.1).

Decolorization activity was measured using the characterized organisms of the anthraquinone dyes such as Reactive blue-19, Brilliant value-R and Acid lue-25 with the potential fungal strains *Aspergillus flavus strain AJ, Aspergillus terreus.* These identified *Aspergillus flavus strain AJ* and *Aspergillus terreus* tested for their decolorization activity by incorporating anthraquinone dyes, the test organisms on to the Sabouraud's dextrose media and after 8 days of incubation, it showed that potential decolorization activity. It was also been observed that there was a prominent decrease in the optical density by increasing the incubation time. *Aspergillus flavus strain AJ* showed maximum decolorization activity with AB-25 (83.0%) and minimum activity with RBBR (66.4%). Similarly *Aspergillus terreus* showed maximum decolorization activity with RBBR (53.1%) & low activity with BBR (46.2%) recorded in figure-1.

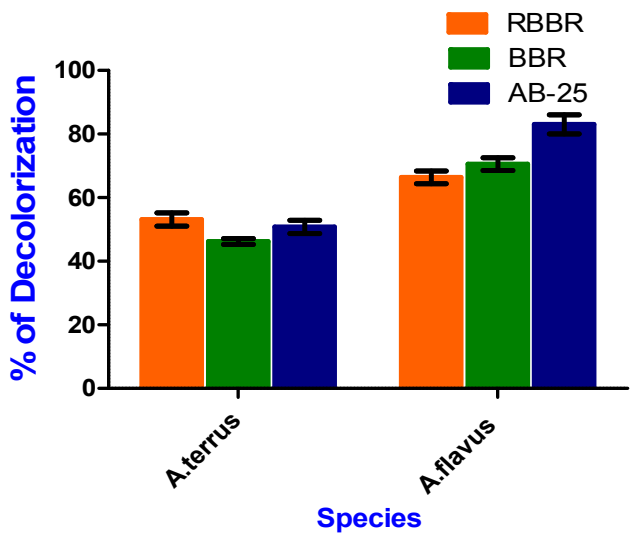


Fig 1 Decolorization of Anthraquinone dyes by fungal strains.

Lignolytic enzymes Production

To characterize the lignolytic enzyme activity of *Aspergillus flavus* and *Aspergillus terreus* were grown on BHM medium in which thus produced the extracellular lignolytic enzymes like laccase, manganese peroxidase (MnP) & lignin peroxidase (LiP). Highest enzyme activity was recorded at an approximate growth conditions viz pH-3.5, temperature (30°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 flavus strain AJ* produced high MnP (196.5U/ml) & low LiP (71.4U/ml) and *Aspergillus terreus* produce the high laccase (16.3U/ml), & low LiP (12.9U/ml) respectively, the complete activity of these enzyme were recorded in fig:-2.

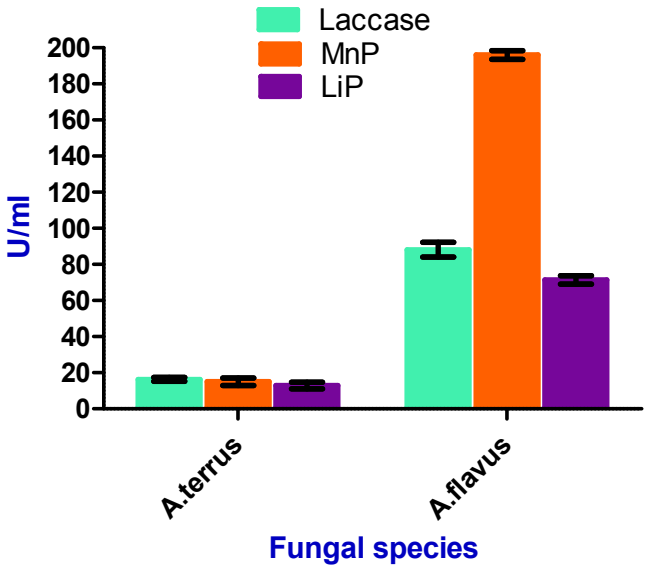


Fig 2 Potential fungal strains with their lignolytic enzyme activity

Optimization of Lignolytic enzymes

The pH plays a key role in the production of lignolytic enzymes by *Aspergillus flavus* and *Aspergillus terreus*. The production of enzymes had been influenced by pH range of 2.5, 3.0, 3.5, 4.5, 5.0 & 6.0. *Aspergillus flavus strain AJ* showed

high activity of MnP (196.5U/ml) & *Aspergillus terreus* showed high activity of laccase (16.3U/ml) at p^H -3.5. Similarly *Aspergillus flavus strain AJ* showed low LiP (8.7 U/ml) enzyme activity, *Aspergillus terreus* showed low activity of LiP (2.7 U/ml) at p^H - 6.0. We observed the maximum activity at p^H -3.5, it indicated that p^H-3.5 is the optimum for the laccase, MnP & LiP and observed that either side of the optimum p^H decrease the enzymes activity(fig:-3).

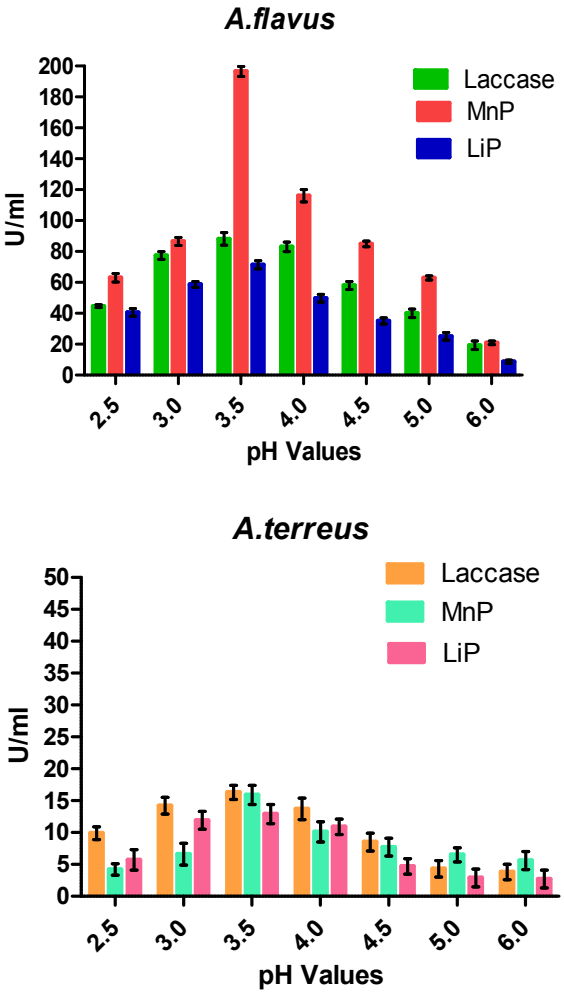


Fig 3 Effect of pH on laccase, MnP, LiP activity

The temperature plays an important role for the production of lignolytic enzymes. *Aspergillus flavus strain AJ* showed maximum activity of MnP (196.5U/ml) at 30°C and low activity of Laccase (9.0U/ml) at 70°C. Similarly *Aspergillus terreus* showed maximum activity of (16.3U/ml) at 30°C & less activity of MnP (2.5U/ml) & LiP (2.5U/ml at 70°C. We noticed the maximum activity at 30°C where enzymes indicated that 30°C is the optimal temperature for the laccase, MnP & LiP activity showed in fig 4.

The carbon sources also play a crucial role in the activity of lignolytic enzymes. The influence of carbon source is starch, glucose, sucrose and lactose were optimized for the enzymes activity of *Aspergillus flavus strain AJ* and *Aspergillus terreus*. *Aspergillus flavus strain AJ* produced maximum of MnP (196.5U/ml) presence of glucose, but the less activity of laccase (4.1U/ml) was observed in presence of lactose. Similarly *Aspergillus terreus* showed the maximum activity of (16.3U/ml) in the presence of glucose and less MnP (2.9 U/ml) activity was observed with lactose (fig:-5).

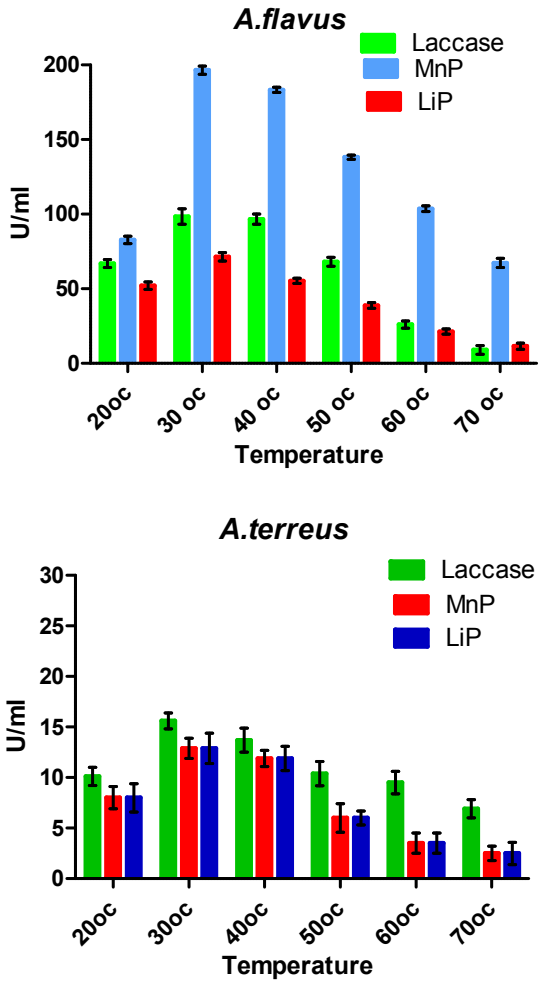
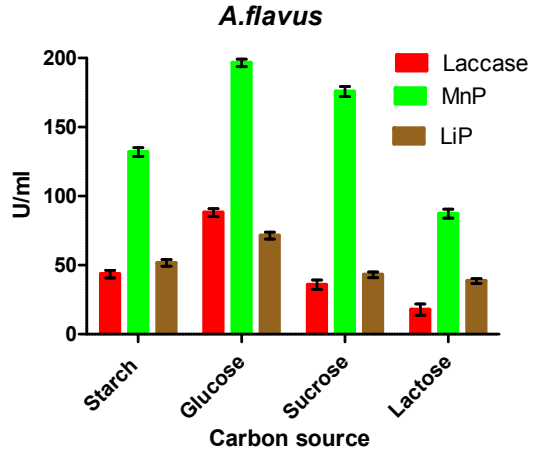


Fig 4 Effect of Temperature on laccase, MnP, LiP enzymes activity

The nitrogen is very important element for the growth of organism as well as production of enzymes. For the optimization of nitrogen source use peptone, yeast extract, beef extract and ammonium nitrate. MnP (196.5U/ml) enzyme produced very high in the presence of yeast extract, low LiP (43.4U/ml) produced in the presence of ammonium nitrate by *Aspergillus flavus strain AJ.*



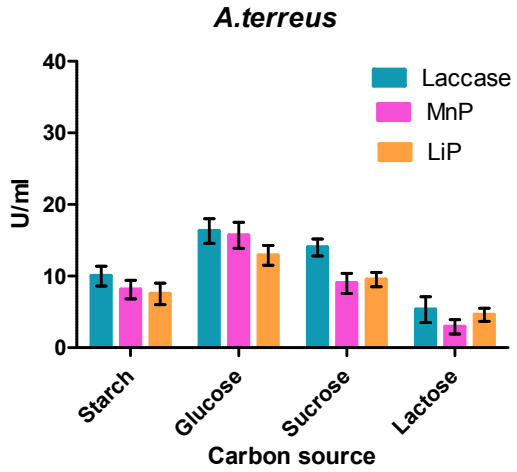


Fig 5 Effect of carbon source on lignolytic enzymes activity

Similarly *Aspergillus terreus* produced maximum activity of laccase (16.3U/ml) in the presence of yeast extract and less LiP (3.9U/ml) activity recorded with ammonium nitrate. The present study clearly indicating that high MnP activity was obtained in the presence of yeast extract when compared to LiP and Laccase (fig:-6).

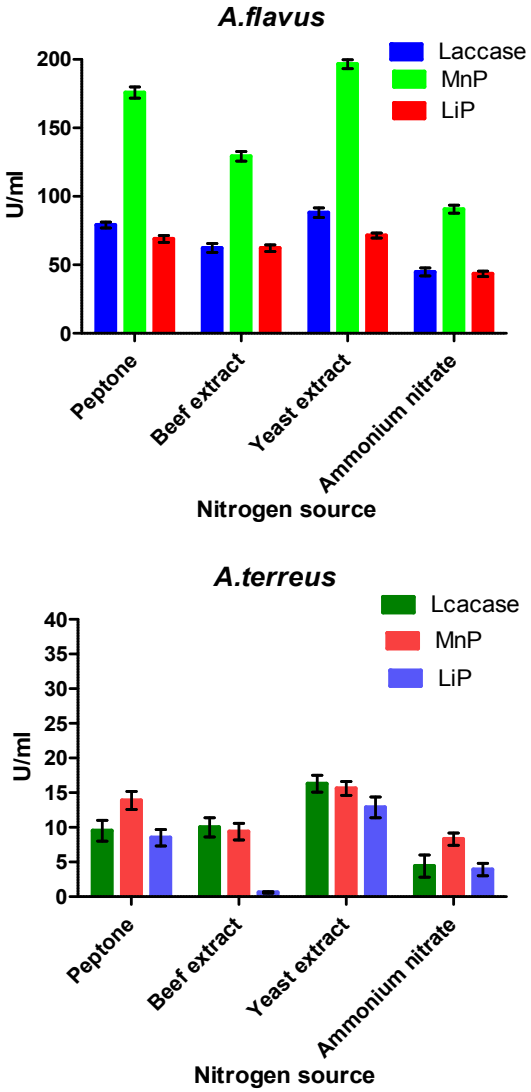


Fig 6 Effect of nitrogen source on Laccase, MnP and LiP activity

DISCUSSION

Decolorization of several textile dyes was obtained by incubating the culture filtrate containing laccase, MnP & LiP enzyme from Aspergillus species was investigated. Similar observations were made in Pleurotus ostreatus which has been recorded in various percentage levels of decolorization of industrial dyes (Soma S *et al.*,2004). Present study was highly supported by (Eggert C *et al.*,1996) who showed the decolorization ability of laccase in Pycnoporus cinnabarinus, Phlebia tremmellosa (Robinson T *et al.*,2001) and Pleurotus sojarcaju (Chagas EP *et al.*,2001). Decolorization of textile dye industrial effluents by white-rot fungus these investigation enamored by producing laccase as the major lignin-degrading enzyme was reported in white-rot fungus, Clitocybula dusenii (Wesenberg D *et al.*,2002).

Phanerochaetae chrysosporium 1, Tramates versicolor, Pleurotus sp., Pleurotus ostreatus and Chaetomium globosum are more effective fungal species compared to the other species for the production of laccase and lignin peroxidase enzymes, these enzymes associated with the lignin- degrading ability viz lignin peroxidase, manganese peroxidase and laccase (Xavier AMRB *et al.*,2007). Tramates versicolor is a basidiomycete that produces three ligninolytic enzymes in a huge amount and it has an efficient degradation capacity of lignin compounds, polycyclic aromatic hydrocarbons, a polychlorinated biphenyl mixture and a number of synthetic dyes used in various industries in general textile dying in particular (Tanaka H *et al.*,1996).

However, the Laccases has wide range of industrial applications because of having potential phenol oxidases as a biological alternative compound for chemical oxidation. e.g. waste water, pulp bleaching and ethanol production etc (Novotny C *et al.*,2004). Enhancement of laccase production, by modifying the nutritional status and physiological conditions during cultivation of these promising fungi, is a prerequisite for their optimum utilization at industrial scale (hakar K *et al.*,2013). Several laboratory studies confirmed that the increased concentrations of inorganic N can suppress the transcription of fungal genes required for the metabolism of lignin and lignocellulose (Eisenlord SD *et al.*,2013).

The present study mainly focused on the optimization enzyme production from potential strains and similar studies were emphasized by their investigation that the optimal temperature for laccase production is between 25°C and 30°C, when cultivated them at temperatures higher than 30°C, the activity of lignolytic enzymes was reduced (Pointing SB *et al.*,2010). Peroxidase and laccase catalyzes the substrate oxidation by a mechanism involving free radicals and showing low substrate specificity. These ligninolytic enzymes are directly involved in the degradation of a variety of xenobiotic compounds, including industrial dyes, polycyclic aromatic hydrocarbons, pesticides, dioxins, chlorophenols, explosives and kraft pulp bleaching (Urek, R O *et al.*,2005)

The highest MnP activity was recorded with S. ostrea the present study was comparable, or better than (0.2-1.4 U/ml) the yield obtained with other cultures such as Phanerochaete chrysosporium (Bonnarme P *et al.*,1997), P. ostreatus (Urek, R O *et al.*,2007), and Phlebia radiata [48] under different growth conditions in liquid media. It has been previously shown that

solid cultures of the basidiomycetous fungus Nematoloma frowardii on wheat straw and Phlebia radiata[49] on chopped wheat straw, produced MnP as the predominant lignolytic enzyme. MnP expression in fungal cultures appears to be controlled by Mn2+ concentrations in the media, and the inclusion of Mn2+ levels in a range of 16-40 ppm ensures the adequate expression for detection of MnP by the different cultures (Hofrichter M *et al.*,1999). *Rhizoctonia sp*. and Schizophyllum F17, exhibited extensive enzyme activity over a pH range of 2.0-6.0. By contrast, the MnP of Irpex lacteus (Machado KMG *et al*.,2005) and Phanerochaete chrysosporium exhibited at pH 2-4 the activity was high than the usual range of most MnP of various other fungi (Cai Y *et al*.,2010).

It is known that the rapid loss of activity in the MnPs of a variety of other organisms at high temperatures occurs as a result of the inactivation of the enzyme by a denaturation process (Cheng X *et al*.,2007). With these limitations, other researchers used mainly on the temperature stability of purified MnPs also differed widely where the enzyme from P. chrysosporium lost activity completely after 15 min at 60^{o}C (Taboada-Puig R, *et al*.,2010), whereas the MnP from Lentinula edodes 62% of activity after 1 h of similar treatment (Couto SR *et al*.,2006).

Shanmugam et al. (2008) reported that they would be the peptone best nitrogen source for laccase production when compared with yeast extract and tryptone. Among different complex nitrogen sources tested. But ultimately the urea was stimulated higher biomass yield and laccase production in addition to the inorganic nitrogen sources stimulated fungi biomass yield as well as laccase production.

CONCLUSION

The present investigation focused mainly on the decolorization and degradation activity of textile dyes (RBBR, BBR &AB-15) by microorganisms particularly fungi. These identified potential fungal strains has been used for the potential activity of Anthraquinone dyes (RB-19, BBR & AB-25) decolorization & dergdation. *Aspergillus flavus* and *Aspergillus terreus* showed remarkable activity of decolorization (90%). In this regard it is very fascinating to note that these fungal species were also has the capacity to produce lignolytic enzymes. Mainly there are three lignolytic enzymes (laccase, MnP & LiP) production was significantly noticed and optimized with various physical parameters and recorded their kinetic properties. The decolorization activity was obviously related to the differences in the dye chemical structure. Finally, from the present investigation focused on the lignolytic enzymes production and optimized with different parameters and obtained very interesting results because much work has already been exploited by bacteria, but the potential implementation of fungi is required for exposition, for better applications in biodegradation.

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