# Role of lignolytic fungal enzymes in removal of industrially important carcinogenic dyes

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**ABSTRACT :** Synthetic dyes are widely used in many industries such as leather, paper printing, textiles, wool, cosmetics, and food. The major problems associated with the use of dye are that they are resistant to biodegradation, difficult to decolorize, non eco-friendly causing environmental and health hazards due to their carcinogenic nature. The use of extracellular enzyme systems from white rot fungi are now growing very fast for bioremediation and dye decolorization purposes. In view of above, present study was undertaken for decolorization of various synthetic and toxic dyes using wood rot fungal cultures. Thirty two fungal cultures were screened qualitatively for production of extracellular lignolytic enzymes. The selected fungal cultures removed the dye from the dye containing broth medium either by accumulating it in mycelia (biosorption) or by metabolizing it to some non-colored components. The cultures varied in their dye decolorizing potential, showing 47.31-97.36% and 14.18-93.63% decolorization of brilliant green and malachite green respectively in 24 d. All the selected cultures showed complete removal of congo red dye from the medium within one month. None of the fungal culture could remove/ metabolize crystal violet and fuchsin basic. The efficient strains were further evaluated for the production of various enzymes. In all the cases, maximum extracellular laccase, lignin peroxidase and Mn-dependent peroxidase activities were observed within 15 to 18 d of incubation in culture supernatant. Photomicrographs clearly revealed presence of congo red dye within the fungal mycelia/spores. Among various cultures tested, the isolate WRF15 was found as the most potential isolate for synthetic dye decolourization and was identified as *Ganoderma* sp. using phenotypic characteristics of the fruiting body.

**Key words:** Dye decolourization, laccase, lignolytic enzymes, synthetic dyes

Over 10,000 different synthetic dyes and pigments are used in many industries such as textile, cosmetic, printing, drug, and food-processing industries (Mohan *et al*., 2002). Most of them are mutagenic/ carcinogenic and belong to the most dangerous pollutants group. It is estimated that between 10 and 15% of the total dye used in the dyeing process may be found in wastewater (Shinkafi *et al*., 2015). Several of these dyes are very stable even at high temperature, as well as to microbial attack, making them recalcitrant and tough to degrade (Pagga and Brown, 1986). Among many different groups of synthetic dyes, triarylmethane dyes are also most commonly used in various industries such as nylon, cotton, wool, and silk, food, oils, fats, waxes, varnishes, cosmetics, paper, leather, and plastics as well as for staining specimens. The major drawback is also that these can be transformed to carcinogenic compounds under anaerobic conditions (Bumpus and Brock, 1988).

Various physicochemical methods, such as adsorption on activated carbon, electrocoagulation, precipitation, flocculation, froth flotation, ion exchange, membrane filtration, ozonation, and reverse osmosis have been used for decolorization of these harmful carcinogens; however, these methods possess inherent limitations such as high cost, formation of hazardous by-products, and intensive energy requirements. Contrary to it, biological processes using microorganisms provide a low-cost, environmentally benign, and efficient alternative for the treatment of dye wastewater (Elizabeth *et al*., 1998; Ali *et al*., 2009). Currently, a lot of studies have focused on wood rot fungi that seem to be more prospective organisms because of their unique oxidoreductive enzyme systems (Dey *et al*., 1994). Microbial dye decolorization may take place in two ways- either by adsorption or biosorption (entrapment of dyes into the microbial biomass) or its biodegradation using unique enzyme systems produced by microorganism (Zhou and Zimmermann, 1993). However, due to operational ease and facile adaptability

of microorganisms to a given set of conditions, the biodegradation mechanism is considered efficacious in comparison to biosorption for treatment of dye wastewater (Singh and Arora, 2011). Hence, the isolation of potent microbial species that have the capability for degradation and detoxification of these dyes is of interest in the biotechnological aspect of dye effluent treatment. In view of above, present study was undertaken for decolorization of various synthetic and toxic dyes such as congo red, crystal violet, fuchsin basic, malachite green and brilliant green using wood rot fungal cultures. Various wood rot fungi were isolated and checked for their dye decolorization ability on the basis of the presence of dye decolorizing unique enzymes namely laccase (Lac), lignin peroxidise (LiP) and Mn dependent peroxidase (MnP). The laccase enzyme that was shown to be involved in decolorization reaction was quantified along with the LiP and MnP.

## MATERIALS AND METHODS

### *Isolation and conservation of microbial gene pool*

Fungal cultures were isolated from the surface sterilized (70% ethanol) fruiting bodies on potato dextrose agar (PDA) plates. All the microscopic analyses were done based on Lacto phenol cotton blue (LPCB) staining. The *Phanerochaete chrysosporium* was taken as the standard culture. The fungal isolates were evaluated for their dye decolourizing activity against five synthetic industrial dyes (malachite green, brilliant green, congo red, fuchsin basic and crystal violet).

### *Screening of the cultures for Production of Lignin Degrading Enzymes*

Screening of the cultures for overall lignin modifying activity (any kind of lignolytic enzyme production) was done by inoculating the fungal disc on lignin modifying enzyme basal agar medium (LBM) (Pointing, 1999**)**. The inoculated plates were incubated at 28±1$^0$C for 5-10 d. The qualitative measure of extracellular lignin modifying activity is the presence of brown oxidation zone around the fungal colony. It is reported as the index of relative enzyme activity ($I_{LIG}$). The following formula was used for calculating the $I_{LIG}$ index.

$$I_{LIG} = \frac{Zone\ diameter}{Colony\ diameter}$$

Screening of the cultures for laccase production was done by inoculating the fungal disc on assay plates containing 15 ml of PDA amended with 0.01% guaiacol (Kiiskinen *et al*., 2004). The qualitative measure of extracellular laccase activity observed as presence of brick red zone of oxidized guaiacol around the fungal colony. It is reported as the index of relative enzyme activity ($I_{LAC}$) and calculated using the above mentioned formula. For lignin peroxidase production, fungal mycelial discs were inoculated on glucose malt extract salt agar medium (Thiyagarajan *et al*., 2008). Plates were incubated at 28°±1$^0$C for 3 d and thereafter, 3ml of 1.7mM and 2.5 mM of ABTS and hydrogen peroxide respectively were overlapped on the plate and were kept in dark at 25°C for 5 minutes. Appearance of clear bluish green zone around the fungal colony gave an indication of peroxidase production and is reported as the index of relative peroxidase enzyme activity ($I_{PER}$) and calculated using the formula as mentioned above.

### *Plate Assay for dye decolorization*

Dye degradation ability of selected fungal cultures was assayed on agar plates of low nitrogen basal medium supplemented with various textile dyes at the concentration of 100 mg/l (Murugesan *et al*., 2006). The agar plates inoculated with fungal mycelial discs were incubated at 30$^0$C under dark conditions and monitored regularly at an interval of 24 h for growth and decolorization activities.

### *Quantitative study of dye decolorization*

The selected fungal cultures were used to quantify dye decolorizing potential *in vitro*. Fungal mycelial discs (5) of active cultures were grown in low nitrogen basal broth medium supplemented with different dyes at the concentration of 100 mg/l. Samples were withdrawn periodically at an interval of 72 h and observed for colour change by measuring optical densities, spectrophotometrically. The cultures showing the bio-sorption of the dye were also checked visually and microscopically. The percent decolourization (D, %) was calculated using the following formula:

$$D(\%) = \frac{Initial\ OD\ -\ Final\ OD}{Initial\ OD} \text{X}100$$

### *Enzyme production from selected cultures*

The selected isolates were further screened for production of three extracellular lignolytic enzymes-lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase in carbon limited liquid mineral salt medium supplemented with glucose 0.3g/L and veratryl a1cohol 1mM. Growth medium (100 ml) was taken in 500 ml

Erlenmeyer flasks and inoculated with 5 fungal mycelial discs. Samples were withdrawn at regular interval of 72h and centrifuged at 10,000 rpm for 10 min, at 4°C. The cell free supernatant was used as crude enzyme.

***Enzyme assays***

The lignolytic enzyme activities were determined in culture filtrates (crude enzyme). Lignin peroxidase (LiP) activity was assayed according to Tien and Kirk (1988) with some modifications by measuring the rate of $H_2O_2$-dependent oxidation of veratryl alcohol to veratraldehyde. One unit of LiP was defined as 1 µmol of veratraldehyde formed per minute and was expressed as U/ml. Manganese peroxidase (MnP) activity was determined following the method given by Paszczynski *et al*. (1988) with slight modifications, and measured by monitoring the $H_2O_2$ dependent oxidation of $Mn^{2+}$ to $Mn^{3+}$. One unit of enzyme activity was defined as the increase in absorbance at 465 nm per minute. The laccase activity was determined according to Niku-Paavola *et al*. (1990) with some modifications by monitoring the oxidation of 500 µM ABTS (2,2' - azino - di - [3 - ethyl benzothiazoline - 6 - sulphonic acid]) buffered with 50 mM citrate buffer (pH 4.5) at 436 nm. One enzyme unit was defined as 1 µM of ABTS oxidized per minute. The completely decolorized broth cultures of selected isolates were also checked for the extracellular laccase enzyme activity as described above.

**RESULTS AND DISCUSSION**

Synthetic toxic dyes are extensively used in several industries and it is estimated that around 10-15% of the dyes are lost in the effluent during dyeing process. The use of fungi, could offer a much cheaper and efficient alternative treatment of wastewaters contaminated heavily with textile dyes (Murugesan *et al*., 2006; Rani *et al*., 2014). White rot fungi have been demonstrated for decolourization of synthetic dyes mediated by their lignolytic enzymes such as lignin peroxidase, manganese peroxidase and laccases (Ollika *et al*., 1993; Heinfling *et al*., 1998; Abadulla *et al*., 2000). To achieve the goal, a total of thirty two fungal strains were isolated from diverse sources.

***Selection of lignolytic fungal cultures based on relative enzyme activity indices***

Out of thirty two isolates, eight isolates showed overall lignin modifying activities. Out of these eight isolates, seven cultures showed laccase activities while lignin peroxidase activities were observed in only two isolates. Thus a total of eight isolates were selected on the basis of relative enzyme activity indices (Figure 1). The values of relative enzyme activity indices varied from 1.07 to 2.0 for $I_{lig}$ , from 1.36 to 3.0 for $I_{lac}$ and from 1.5 to 2.5 for $I_{per.}$. The maximum value for $I_{lig}$ (2.0) was shown by the fungal isolate WRF5, whereas maximum relative laccase activity indices (3.0) were found for the isolate WRF15. Only two fungal isolates WRF15 (2.5) and standard culture of *P. chrysosporium* (1.6) were found positive for peroxidase relative activity indices.

***Dye decolorization study***

To confirm the fungal dye decolourizing capacity, five synthetic dyes were incubated with the eight selected fungal isolates for 21 d on dye containing agar and liquid medium at 28°C during the present study. Out of the five dyes tested, all the fungal cultures were unable to grow on two dyes thereby showing more resistance towards the two dyes (crystal violet and fuchsin basic). The similar agar plate screening method for determining dye decolourizing potential of wood rot fungi *Ganoderma* sp. has also been performed in previous study (Okino *et al*., 2000; Arulmani *et al*., 2005). Broth cultures were found better as compared to solid cultures for decolourization studies because in all the cases although fungal growth was observed in the presence of dyes but decolourization began with the formation of very less intense or negligible decolorized zones. During the liquid cultivation experiments, the batch cultures turned from an initial deep coloration to a lighter colour, eventually becoming colourless in most of the cases, indicating either the dye decolorization or dye adsorption into the fungal mycelia or spores (Figure 2). The spectrophotometric

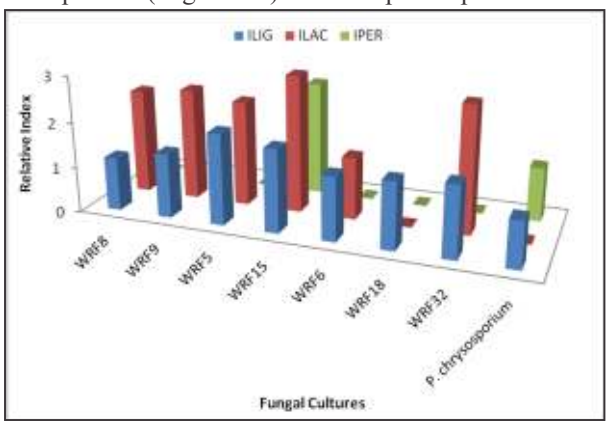


**Fig.1:** Comparative evaluation of selected fungal cultures for relative enzyme activity indices

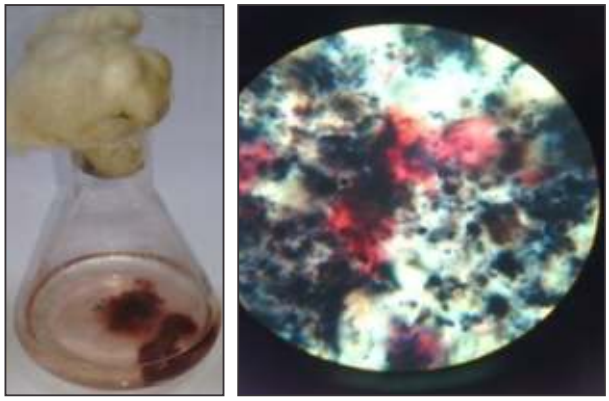

**Fig. 2:** Bio-absorption of congo red by fungal cultures.

quantification results revealed clearly the high decolourization potential of fungal cultures towards brilliant green, malachite green, and congo red dyes (Table 1). The degree of maximum percent decolourization of brilliant green dye using various fungal isolates varied from 47.31 (WRF5) to 97.36 (WRF 15). Maximum percent decolourization of malachite green dye by various fungal cultures varied from 14.18 to 93.63%. Congo red dye was removed efficiently by all selected cultures and percent decolourization of the broth varied from 92.63 (WRF 32) to 99.89 (WRF 15) within 9 d. In case of crystal violet and fuchsin basic, the dyes were not efficiently absorbed or decolorized by any culture, however, only the isolates WRF9 and WRF6 were found to slightly absorb the dye crystal violet in liquid culture within 40-50 d of incubation (data not shown, observed visually). Previously only 40% decolourization of anthraquinone dye, Remazol Brilliant Blue R (RBBR) by *Ganoderma* sp. was reported which could be increased up to 92.4% upon addition of HBT as redox mediator (Murugesan *et al*., 2006). However, contrary to previous findings, we could get much higher decolourization rate for the two dyes congo red, and brilliant green by the fungal isolates used. Thus on the basis of above data, the culture WRF15, showing maximum percent decolourization of brilliant green and congo red was selected as a potential wood rotting fungal culture for further experiments. Further, culture showed the decolorization of dyes higher than that of the standard culture of *P. chrysosporium*.

Thus on the basis of relative enzyme activity indices and dye decolourization potential, the four fungal isolates viz. WRF9, WRF32, WRF15 and the standard culture of *P. chrysosporium* were selected for enzyme production and further studies.

***Enzyme assay***

The selected cultures were grown in enzyme production liquid medium amended with varatryl alcohol as inducer. Laccase enzyme was synthesized with peak laccase activity of 164.63 U $ml^{-1}$ by fungal isolate WRF15 on $18^{th}$ d of incubation (Figure 3), peak lignin peroxidase activity of 0.34 U $ml^{-1}$ was observed with fungal isolate WRF32 within 21 d of incubation (figure 4) and maximum manganese dependent peroxidase activity of 0.0022 U $ml^{-1}$ was also given by fungal culture WRF15 on the 21 d of incubation (data not shown). In this way, the fungal cultures were able to secrete all the three extracellular oxidative and reductive enzymes. All selected isolates showed a very high laccase enzyme activity comparative to standard culture of *P. chrysosporium*. The cultures showed maximum ability for synthesizing laccase enzyme while all the cultures had very low LiP and MnP biosynthetic abilities. The enzymatic decolourization by potential fungal isolate WRF15 was also confirmed by checking laccase enzyme

**Table 1: Percent decolorization of malachite green, brilliant green and congo red dyes within 21d of incubation period using lignolytic fungal cultures**

| S.No. | Fungal Cultures | Decolourization (%) of synthetic dyes | | |
|---|---|---|---|---|
| | | Brilliant Green | Malachite Green | Congo Red |
| 1 | WRF8 | 92.66 | 58.6 | 97.89 |
| 2 | WRF9 | 96.59 | 14.18 | 97.89 |
| 3 | WRF5 | 47.31 | 14.9 | 94.74 |
| 4 | WRF15 | 97.36 | 88.85 | 99.89 |
| 5 | WRF6 | 96.33 | 43.84 | 96.84 |
| 6 | WRF18 | 89.56 | 93.63 | 98.95 |
| 7 | WRF32 | 95.19 | 92.32 | 92.63 |
| 8 | *P. chrysosporium* | 29.8 | 21.27 | 46.89 |
| | | SEm ± 0.145 | SEm ± 0.134 | SEm ± 0.074 |
| | | cd at 5% = 0.506 | cd at 5% = 0.42 | cd at 5% = 0.231 |

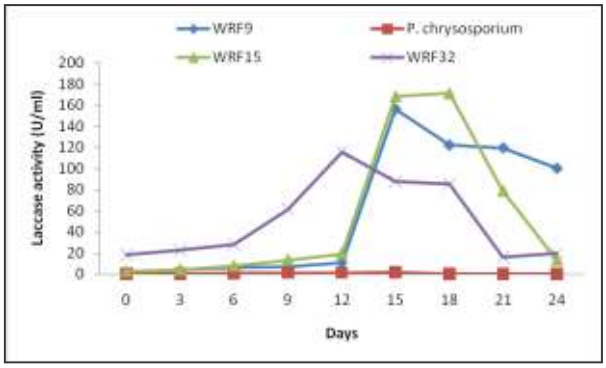


**Fig. 3:** Laccase production during growth of selected fungal cultures.

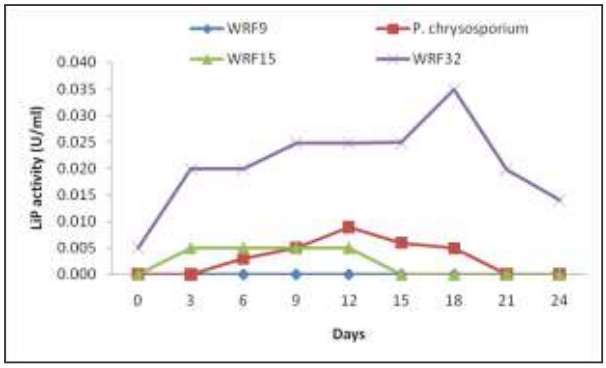


**Fig. 4:** Lignin peroxidase production during growth of selected fungal cultures.

activities in completely decolorized (40 d old) fungal culture filtrates grown in presence of congo red, malachite green and brilliant green dyes (data not shown). The enzyme activity was determined in completely decolourized culture filtrates because due to the intense colour of the dye in medium one could not calculate the activity in dye containing broth. The very low laccase activity in congo red containing culture filtrate of WRF 15 (1U/ml) may be partially correlated with the maximum bio-sorption of the dye by fungal mycelium/spores and partially due to ageing of the culture that might have had lead to the degradation of the enzyme protein. Maximum laccase activity of 28.9 U/ml, followed by 25.5 U/ml were observed in completely decolourized culture filtrates (40 d old) of brilliant green and malachite green dyes respectively. No laccase activity was observed in any case for the standard culture of *P. chrysosporium*. This induction of enzymes may be correlated with their involvement in dye decolourization process. Significant roles of lignin peroxidase, Mn-dependent peroxidase, and laccase in dye degradation by wood rotting fungi have been well documented (McMullan *et al*., 2001). Further, among these three enzymes, direct involvement of laccase in decolourization of synthetic dyes has also been demonstrated by previous workers (Novotny *et al*., 2004). Thus, our results are in agreement with the previous findings that have also revealed the vital role shown by laccase enzyme in bioremediation of synthetic toxic dyes.

The most potential fungal isolate, WRF15, was selected for further studies based on its maximum ability to synthesize laccase enzyme and dye decolourizing ability. The molecular characterization of the selected isolates is under progress, however, the potential isolate WRF15, was identified as *Ganoderma* sp. on the basis on phenotypic characteristics.

## CONCLUSION

It may be concluded from the present investigation that mycoremediation employing wood rotting fungi has a vast potential for decolourization or removal of toxic carcinogenic synthetic dyes from the industrial effluents. Moreover, biological removal of dye from industrial effluents may be more economic and ecofriendly approach. It is also apparent from the study that most of the lignolytic fungi are having very high biosorption potential towards certain synthetic dyes, indicating its efficiency for utilization in effluent treatment processes in future.

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