

Optimization and Mycoremediation of Brilliant Blue by Fungus Collected from *Nerium oleander*, *Mangifera indica*, *Azadirachta indica*, *Morus nigra* and *Psidium guajava*

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Mycoremediation of majorly used Textile Dye "The Brilliant Blue" was investigated by using wood growing fungus collected from *Nerium oleander*, *Mangifera indica*, *Azadirachta indica*, *Morus nigra* and *Psidium guajava*. Three out of thirteen fungal isolates formed clear zone on agar plates when grown for 40 days with dye. Percentage concentration of dye left after degradation observed for three strains was 26% (w/v), 28% (w/v) and 32% (w/v). In the process, activity of Laccase was also observed for three strains. Optimization of the process was carried out by Response Surface Methodology. Optimization of process parameters indicated a 48.86 % degradation at pH 8, N concentration 4% (w/v), Carbon concentration of 6 % (w/v) incubation time of 16 days at temperature of 36 °C.

Key words: Wood growing fungus, Textile Dye, Response Surface Methodology.

The unscrupulous use of water soluble textile dyes has rendered a detrimental effect on the fragile ecosystems around the industries (Chung, 1983). The dyes are many a times carried by the water bodies thereby having adverse effects on aquatic ecosystems over a huge area (Jarosz-Wilkolazka et al., 2002). Textile industries use large amount of dyes by dissolving them in water and after imparting colour to the cloth, the rest of water is discarded to the water bodies. These dyes affect the water solubility, gas solubility and biological oxygen demand and are essential to eliminate from the environment. These dyes are resistant to sun light, water and chemicals and hard to degrade. For removal of these dyes various physical methods like filtration, precipitation, oxidation

reduction are used but these methods are not much effective (Robinson *et al.*, 2001a), so ability of White Rot Fungus to produce ligninases is used to degrade the lignin present in all type of Dyes (Swamy and Ramsay, 1999; Selvam *et al.*, 2003). Degradative system of WRF includes three kinds of Ligninases: Laccases, Lignin peroxidises, manganese peroxidises (Dirk Wesenberg, 2003). These enzymes collectively are known as lignin modifying enzymes (LMF). In this study only Laccases that belong to the Oxidoreductase class of enzymes was considered. Laccase are copper-containing (Thurston, 1994; Xu, 1999) and it depends upon presence of molecular oxygen for their activity (Thurston, 1994; Kiiskinen and Saloheimo, 2004). In the present investigation thirteen different strains of wood growing fungus were collected for degradation of Brilliant Blue with the aim of decolouration of textile dye by mycoremediation by selecting the best strain of fungus. For experimentation Brilliant Blue was chosen as model compound for degradation studies.

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MATERIALS AND METHODS

Microorganisms

Thirteen strains of wood growing fungus were collected from oleander tree (*Nerium oleander*), mango tree (*Mangifera indica*), neem tree (*Azadirachta indica*), mulberry tree (*Morus nigra* LINN), and guava tree (*Psidium guajava*).

Acclimatization of fungal samples

Fruiting part of fungus was collected from woody substrates. Exterior surface of the sample was sterilized by wiping it with alcohol. Small piece of this sample was taken with the help of a forced and cultured over PDA plate. These plates were incubated at 27°C and growth of mycelium was being monitored. Sub culturing was done for 4-5 times to obtain purified culture. The purified mycelium was taken and transferred to the PDA plates containing 0.5 % (w/v) dye and again incubated at 27°C for further analysis. These strains were selected on the basis of dye agar plate assay and used for further study. The strains showing sign of degradation over dye agar plates were assayed by using Liquid Broth containing 0.5% (w/v) dye and NaNO₃ as nitrogen source and dextrose as carbon source. Liquid broth was prepared by adding K₂HPO₄ (1 gm per litre), MgSO₄ (0.5 gm per litre), KCl (0.5 gm per litre), NaNO₃ (2.5 gm per litre) and dextrose (5 gm per litre). Conical flasks containing 100 ml of Broth were inoculated with 1/8th of petri plate of the strains. After incubation of certain days, cultural extract was obtained and analysed for decolouration percentage. This decolouration was analysed by determining the decrease in percentage concentration of dye. For calculating the concentration of dye during the period of incubation a standard curve was plotted between the known concentrations and their respected optical densities, measured with the help of spectrophotometer.

Spawn preparation for scale up

Spawn preparation was carried out on wheat after drying it with CaSO₄ and CaCO₃. The spawns were incubated at 30 °C.

Discolouration Assay

Absorbance of each sample was monitored at A₅₈₀. Corresponding concentrations were determined with the help of standard curve and then percentage decrease in concentrations

was calculated.

Screening of Laccase Assay

The enzyme Laccase was estimated by Substrate Oxidation Method (Airong Li and Yue Zhu, 2008).

Optimization of the Process

Optimization of the process was done by using response surface methodology. For Decolouration assay a set of 46 tubes containing broth with different concentration of nitrogen and carbon source was prepared for each of the three strains showing positive response. These set were monitored under different temperatures after different incubation periods. In the present investigation, Minitab B^R was used for statistical analysis.

RESULTS

Acclimatization of the isolated fungal sample

To degrade the dye, cultures were acclimatized on PDA plates supplemented with 0.5 % (w/v) of dye. The cultures were subcultured at regular intervals of 9 days and the concentration of glucose and potato cubes for extract preparation were gradually reduced to 0.5 % (w/v) and 20 % (w/v) from 2% (w/v) and 30% (w/v) respectively. Figure 1 shows the purified growth of fungal strains after successive subcultures.

The gradual depletion of the easily accessible nutrients, that is, potato extract and dextrose was expected to put nutritional pressure on the fungi which would result in the production of more lignolytic enzymes. This was evident when three strains (named Fungus 1, Fungus 2 and Fungus 3) were incubated on plates containing 0.5 % (w/v) dye in nutrient deficient conditions, showed signs of degradation after a period of 40 days. Figure 2 shows comparative degradation of dye by various strains.

In the present investigation, a study was carried out to check the feasibility of using different strains of wood growing fungus for the bioremediation of waste waters contaminated with textile dyes. Hence, after acclimatization, equal volumes of fungal mycelia were inoculated in an aqueous solution containing 0.5 % (w/v) dye supplemented with essential salts, it was observed that a degradation of dye occurred after an incubation period of 40 days which was later

verified spectrophotometrically at 580 nm.

Quantification of dye degradation

After measuring the optical densities of broth inoculated with all fungal strains at certain intervals of time (in days), the respective concentration of dye was determined from standard curve, that showed the continuous decrease in concentration of dye for few strains. Figure 3 shows comparison between the three strains that show maximum decrease in percentage of concentration. The biodegradation of Brilliant Blue is related to its discoloration during the growth of the fungi. There was a decrease in concentration percentage along with increase in the incubation time.

Screening of enzyme activity using one variable at a time approach

When the supernatant from 15 days old culture from all four sets was centrifuged, followed by oxidation of ABTS, gave positive response for Laccase presence. Activity of Laccase observed by varying different conditions like pH, carbon source, surfactants and liquid to solid ratio, is shown below. The three strains showing maximum discoloration were subjected to different pH conditions and it was found that the strains show

different activity for Laccase at different pH. Figure 4 represents the comparison between the effects of pH on laccase production of different strains. Similarly the strains showing maximum discoloration were subjected to different carbon sources, different surfactants and different solid to liquid ratios and it was found that the strains show different activity for Laccase with different sources. Figure 5, 6 and 7 represent the differences in the amount of laccase production under various conditions.

Optimization using response surface methodology

Five factors namely pH, Incubation, temperature, Nitrogen source (N) and Carbon source(C) were considered at 3 levels (-1, 0, +1). The design of experiments was carried out using Box-Behnken method and a regression equation obtained which has been given in uncoded terms as:

The significance of the regression equation, linear, square and interaction effects are indicated by the ANOVA as depicted in the table below. The wireframe plots in figures 8, 9, 10, 11, 12 show the interactions between various parameters.

ANOVA (Analysis of variables) for the Regression Equation has been given below.

Source	DF	Sequence Similarity	Adj. SS	Adj. MS	F	P
Regression	20	469.290	469.2898	23.4645	683.62	<0.001
Linear	5	303.245	40.6213	8.1243	236.69	<0.001
Square	5	86.937	86.9367	17.3873	506.57	<0.001
Interaction	10	79.108	79.1077	7.9108	230.47	<0.001
Residual Error	26	0.892	0.8924	0.0343		
Lack of fit	20	0.892	0.8924	0.0446		
Pure Error	6	0	0.0000	0.0000		
Total	46	470.182				

When comparison between nitrogen concentration and temperature (Figure 8) was done, it is evident that there is a decrease in the degradation with the increase in nitrogen concentration, whereas when temperature is considered, it is observed that there is an increase followed by a subsequent decrease in degradation with the continuous increase in temperature. A maximum degradation of >35 % (w/v) is observed when the nitrogen concentration is 1% (w/v) and temperature is 30 °C. From this observation it may be inferred that nitrogen limiting conditions are

best for dye degradation. It was also observed that there is an increase in the degradation till 30 °C followed by a subsequent decrease in the degradation when the temperature is increased further. This may be attributed to the fact that every microorganism has its optimum conditions and here it is evident that the fungus is mesophilic by nature. Comparison between incubation period and carbon concentration (Figure 9) depicted that there is an increase in concentration of dye with continuous increase in concentration of carbon. But with change in incubation time the degradation first



Fig. 1. Plates showing purified growth of fungal strains

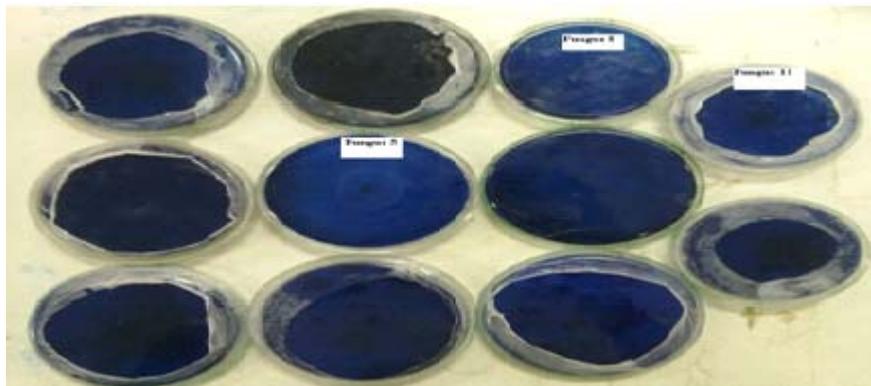


Fig. 2. Plates showing comparative degradation of dyes by certain strains

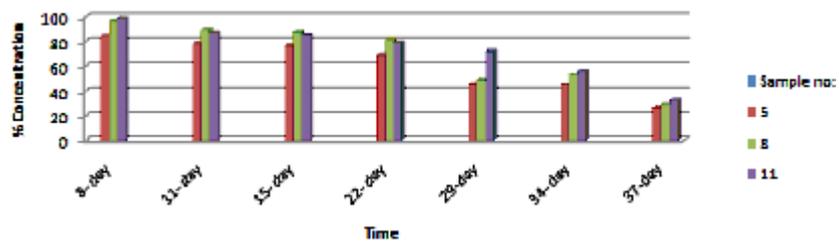


Fig. 3. Discoloration of brilliant blue in form of decrease in concentration by fungi during 40 days of incubation for three strains that show maximum degradation

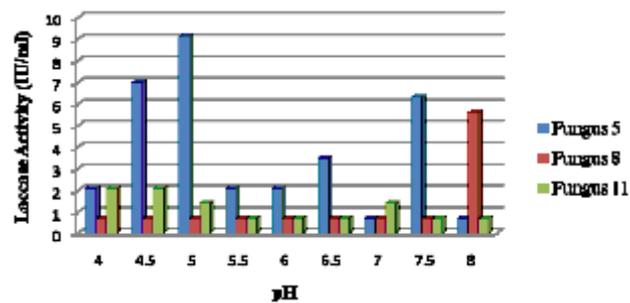


Fig. 4. Effect of pH on Laccase activity of three strains that show maximum discoloration

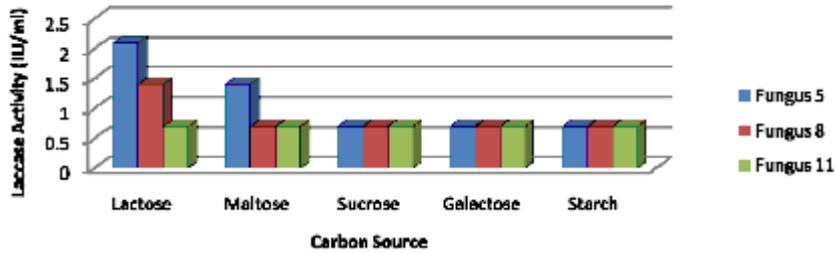


Fig. 5. Effect of carbon source on Laccase activity for three strains that show maximum discoloration

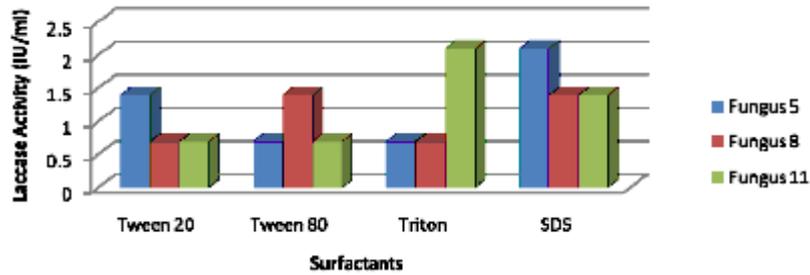


Fig. 6. Effect of surfactants on Laccase activity for three strains that show maximum discoloration

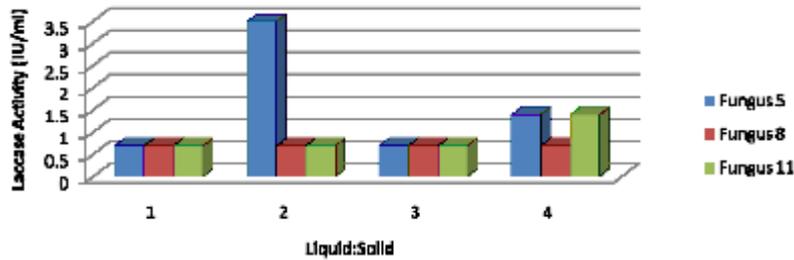


Fig. 7. Effect of liquid to solid ratio on Laccase activity for three strains that show maximum discoloration

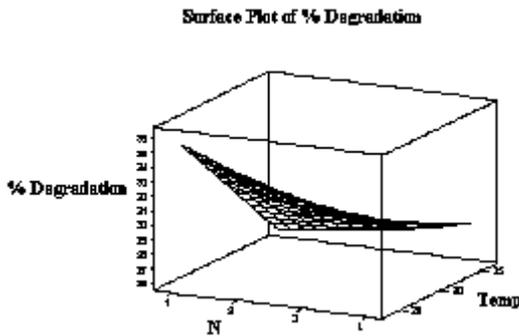


Fig. 8. Response surface plot showing the effect of temperature and nitrogen on the degradation of dye (Degradation is expressed in % and Hold Values are Incubation time 12 days; pH 7; Carbon 4 gm/l)

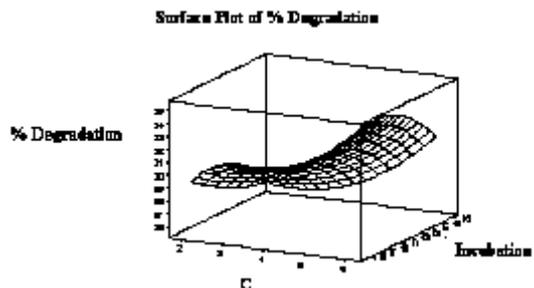


Fig. 9. Response surface plot showing the effect of incubation and carbon on the degradation of dye (Degradation is expressed in % and Hold Values are Temperature 30°C; pH 7; nitrogen 2.5 gm/l)

increases to an optimum value and afterwards start decreasing with increase in incubation time. Maximum value of degradation, that is, more than 35% is observed at 6% (w/v) concentration of carbon. From this curve, it was observed that excess carbon is required for optimum growth of microbes. On the other side, maximum degradation was observed with 8 to 12 days of incubation period and beyond 12 days degradation shows a decline. This is due to the occurrence of stationary phase of microbial growth.

Comparison between incubation period and nitrogen concentration (Figure 10) showed a continual decrease in degradation of dye with increase in concentration of nitrogen which again shows that nitrogen limiting environment is essential for degradation. Maximum degradation was observed at 1% (w/v) concentration of nitrogen. The second parameter considered here is incubation time which also showed the same effects as shown in previous graph, that is, degradation first increases then decreases with continual increase of incubation time (follow microbial growth curve). Maximum degradation was observed with incubation time of 10 days.

Comparison between incubation time and temperature (Figure 11) depicted the increase in degradation followed by decrease with increase in incubation time. Maximum degradation was observed with incubation time of 12 days. Graph also showed an increase in degradation with temperature up to 30 °C followed by decrease in degradation with increase in temperature beyond this.

Comparison between carbon concentration and temperature (Figure 12) depicted gradual increase in degradation with increase in carbon concentration and gradual decrease in degradation with increase in temperature. Value of temperature for maximum degradation falls in range of 25-30 °C and carbon concentration for maximum degradation is 6 % (w/v).

Optimization of process parameters indicated a 48.86 % degradation at pH 8, N concentration 4% (w/v), Carbon concentration of 6 % (w/v), incubation time of 16 days and a temperature of 36 °C. The positive effect on the biodegradation of the dye due to additional carbon and nitrogen may be attributed to the better growth of fungi due to the fulfilment of its nutritional

requirements. Additionally, an investigation on the other limiting factors responsible for growth must be carried out for further insights into the process.

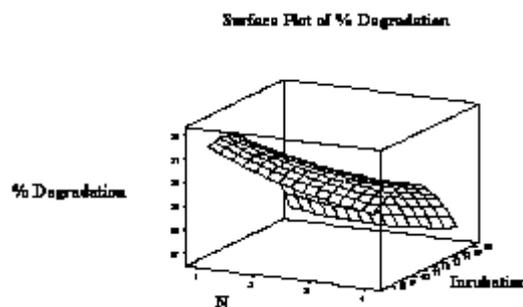


Fig. 10. Response surface plot showing the effect of incubation and nitrogen on the degradation of dye (Degradation is expressed in % and Hold Values are Temperature 30°C; pH 7; Carbon 4 gm/l)

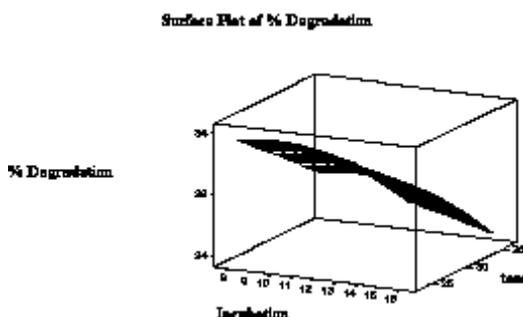


Fig. 11. Response surface plot showing the effect of incubation and temperature on the degradation of dye (Degradation is expressed in % and Hold Values are pH 7; Nitrogen 2.5 gm/l; Carbon 4 gm/l)

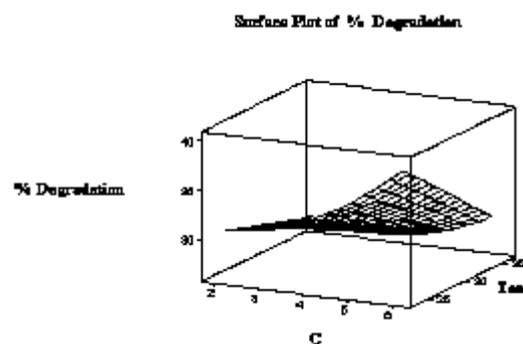


Fig. 12. Response surface plot showing the effect of carbon and temperature on the degradation of dye (Degradation is expressed in % and Hold Values are pH 7; Nitrogen 2.5 gm/l; Incubation 12 days)

DISCUSSION

Results revealed that the three strains were very effective in dye decolourisation showing 26% (w/v), 28% (w/v) and 32 % (w/v) concentration left of brilliant blue after 40 days of incubation. The role of lignolytic enzymes is useful for decolourisation of the dye. The enzyme systems of the wood growing fungi include a group of non-specific extra cellular enzymes, which catalyze degradation of several aromatic and halogenated dye compounds. Out of the three fungal strains showing maximum degradation, one strain show maximum activity of laccase at pH 5 with Lactose as sugar source with 2:1 liquid : solid ratio and in the presence of SDS. Other two strains also showed the presence of laccase under different parameters. The enzymatic systems of *Phanerochaete chrysosporium* have also been considered responsible for azo dye degradation (Paszczyński *et al.*, 1986, Cripps *et al.*, 1990, Paszczyński & Crawford, 1991). Shin *et al.*, (1997) identified a new enzyme remazol brilliant blue – R (RBBR) decolourising peroxidase produced by *Pleurotus ostreatus* and showed that the enzyme was important in decolourisation of majority of the xenobiotic dyes. *Polyporus elegans*, *Trametes versicolor* and *Lengites betulina* showed maximum production of lignin peroxidase, manganese peroxidase and laccase. Our results supported the previous reports indicating that these enzymatic productions are responsible for the maximum decolourisation activities of brilliant blue.

CONCLUSION

It is concluded that the wood growing fungus may be considered to be the best tool since they are resistant and found to be justifiably acclimatized to dye contaminated water and also regarded as natural mutant to survive in the dye containing water. A number of organic pollutants pose risks to humans, animals and ecosystem due to their toxic effects. Mycoremediation being an environment friendly technique emerges as a soft technology. The cost-effectiveness of mycoremediation also makes this technology an attractive approach. The recommendation for future work is in the line to screen potential fungus pure or in consortium to attain maximum biodegradation.

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