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EFFICIENT REMOVAL OF DISPERSE DYE BY MIXED CULTURE OF *Ganoderma lucidum* AND *Coriolus versicolor*

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In the current study, an attempt was made to check the potential of aerobic mixed culture of two indigenous white rot fungi for the decolorization of different disperse dyes in batch culture mode and optimization of different conditions to enhance the biotransformation of dyes. Initial screening trial with six disperse dyes, viz. (Foron Yellow RD5GL, Foron Red RDRBLS, Foron Rubine RDGFL, Foron Black RD3GRN, Foron Blue RDGLN and Foron Turquoise SBLN), was carried out using mixed culture of *Ganoderma lucidum* and *Coriolus versicolor*. From all the tested dyes, the mixed culture showed better removal efficiency (93 %) with Foron Turquoise SBLN dye after 8 days of incubation period as compared to other tested dyes. Enhanced color removal (98 %) was observed when the medium was amended by ammonium tartarate, maltose, $MnSO_4$ at pH 4.5 and 30˚C with 2 mL fungal culture during 2nd day of incubation period. Enzyme profile showed that the mixed culture produced three liginolytic enzymes like lignin peroxidase (LiP), manganase peroxidase (MnP) and laccase but MnP was found to be the major enzyme. The results indicated that white rot fungi (WRF) could be used to treat wastewater containing disperse dyes.

Keywords: Biodegradation; disperse dyes; *Ganoderma lucidum*, *Coriolus versicolor*, mixed culture

INTRODUCTION

Synthetic dyes are coloring agents mainly used in textile industries which generate a huge amount of wastewater in the process of dyeing. Colored waste water from textile industry is rated as the most polluted in almost all industrial sectors (Andleeb *et al*., 2010). The presence of dyes in effluents is not only highly visible and undesirable, but also causes the environmental and health problems to human beings and aquatic animals (Eftekhari *et al*., 2010). Conventional methods for dealing with textile wastewater consist of various combinations of biological, physical and chemical methods (Mo *et al*., 2008). Unfortunately, although the conventional chemical and physical methods are versatile and useful, but only transfer the pollutant from one form to another (Konstantinou and Albanis, 2004) and end up in producing secondary waste products. Innovative technologies, such as bioremediation, are needed as alternatives to conventional methods to find inexpensive ways of removing dyes from large volumes of effluents.

Several microorganisms, including fungi, bacteria, yeasts and algae, can decolorize and even completely mineralize azo dyes under certain environmental conditions. The role of fungi in the treatment of wastewater has been extensively researched (Azmi *et al*., 1998; Coulibaly *et al*., 2003). The fungal mycelia have an additive advantage over single cell organisms by solubilising the insoluble substrates by producing extracellular enzymes (Kaushik and Malik, 2009). The capacity of ligninolytic fungi to degrade synthetic dyes is generally correlated with their ability to produce lignin degrading extracellular enzymes such as lignin peroxidase (LiP), manganese peroxidase (MnP), or laccases (Schliephake *et al*., 2000; Saparrat and Guillen, 2005). These ligninolytic enzymes are substrate non-specific, and tolerant to pollutants at high concentrations (Shin, 2004; Levin *et al*., 2008). The majority of the studies on the biotransformation of dyes have been carried out mainly by single fungal cultures with few reports on mixed cultures (Asgher *et al*., 2007). Single culture has their limited use for treatment of effluents that contain mixtures of different dyes. Therefore the present study was focused on bioremediation of dye by a mixed culture of indigenous white rot fungi *Ganoderma lucidum* and *Coriolus versicolor* and to investigate its ability to degrade disperse dye, Foron turquoise SBLN and the effect of various operational parameters on the efficiency of mixed cultures of WRF were examined. The enzyme profile of mixed culture of WRF during the decolorization of disperse dye was also investigated.

MATERIALS AND METHODS

The experimental work was conducted in the Environmental Chemistry Laboratory, Department of Chemistry and Biochemistry, University of Agriculture, Faisalabad Pakistan.

Dyes: Disperse textile dyes Foron yellow RD5GL, Foron red RDRBLS, Foron rubine RDGFL, Foron black RD3GRN, Foron Blue RDGLN and Foron Turquoise SBLN were gifted by Clariant Pakistan (Pvt) Limited, Canal Road, Faisalabad.

Microbial cultures: Pure cultures of two white rot fungi *G. lucidum* and *C. versicolor* were obtained from the Industrial Biotechnology Laboratory Department of Chemistry & Biochemistry, University of Agriculture Faisalabad, Pakistan. The fungi were raised on slants of Potato Dextrose Agar (PDA) medium at pH 4.5 at 28±2˚C. After having sufficient population of spores, the slants were refrigerated (4˚C) for subsequent use in decolorization studies.

Inoculum preparation: Inoculum medium was carried out in two separate Erlenmeyer flasks (500 mL) containing 100 mL of liquid Kirk's basal medium (Kirk and Farrel, 1987), brought to pH 4.5 with 0.1M NaOH/0.1M HCl and autoclaved at 121˚C. Cultures of *G. lucidum* and *C. versicolor* were added to the respective flasks and incubated in a reciprocating shaker (PA 250/25. H) at 120 rpm and under 30 °C for 6-7 days to get inoculum level with 1×10^6-10^8 spores/mL.

Screening experiment: Six sets of triplicate flasks (250 mL) each flask containing 100 mL of Kirk's nutrient medium and 0.01% dye were prepared for screening of co-culture of *G. lucidum* and *C. versicolor*. All the decolorization flasks were maintained at pH 4.5 and sterilized (121˚C) in an autoclave for 15 min. The flasks were inoculated with 2 mL inoculum of mixed cultures (1:1 ratio) of both strains in laminar air flow aseptically. The inoculated flasks were incubated for 10 days at 30˚C in shaking cultures at 120 rpm.

Determination of extent of decolorization: After every 24 h, 2 mL sample was taken out from each experimental flask and analyzed for the percent decolorization. These samples were centrifuged at 5000 rpm for 15 min at room temperature and clear supernatants were analyzed spectrophotometrically by using CECIL CE 7200 UV/Vis double beam spectrophotometer at λ_{max} of each dye in order to determine the % age decolorization of textile dye by mix-culture of *G. lucidum* and *C. versicolor*.

Percent decolorization of dye was calculated by:

$$\% \text{ decolorization} = (Abs_i - Abs_f / Abs_i) \times 100 \quad (1)$$

Optimization of process parameters: Based on the results of screening experiment, Foron turquoise SBLN was maximally decolorized dye by the mixed culture of *G. lucidum* and *C. versicolor*. Maximally decolorized dye was therefore, used for optimization of different parameters for decolorization by the mixed culture. For process optimization the classical method was adapted; varying one parameter in an experiment and maintaining the pre-optimized in subsequent study.

To find out the most suitable pH for effective decolorization by the mixed-culture, shake flask experiments were conducted at different initial pH (3–5.5) values. In the subsequent experiments, the flasks adjusted at optimum pH were incubated at different temperatures (25–45°C) to find out the tolerable temperature for effective decolorization by the co-culture of fungi. Effect of different carbon sources on dye decolorization was studied using glucose, maltose, fructose, wheat bran, sucrose and rice bran, effect of varying nitrogen sources on dye decolorization was studied by addition of ammonium tartarate, peptone, ammonium nitrate, ammonium sulphate and urea to get enhance removal of dye by mix-culture.

Enzyme profile: Supernatants of each experiment were analyzed for its lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase activities to study the mechanism of biodegradation of the dyestuff.

Manganese peroxidase (MnP) activity was measured by the oxidation of 1 mM $MnSO_4$ in 50 mM melonate buffer (pH 4.5) in the presence of 1 mM H_2O_2 (Wariishi *et al*., 1992). One unit (U) of MnP was defined as the amount of enzyme necessary to oxidize 1.0 µmol Mn(II) to Mn(III) per minute.

Laccase activity was determined by a slight modification of the method of (Shin and Lee, 2000) monitoring the oxidation of 2- 2′-azinobis (3-ethylbenthiazoline)-6-sulfonate (ABTS) at 420 nm (ε_{420} 36 000 M^{-1} cm^{-1}) in a 2 mL of the reaction mixture containing 0.1 mL of the enzyme, 0.9 mL of 0.3 mM ABTS and 1 mL of 50 mM sodium malonate buffer for 10 min. One unit (U) of laccase was defined as the amount of enzyme necessary to oxidize 1.0 µmol ABTS per minute.

LiP activity was determined by the oxidation rate of 4 mM veratryl alcohol to veratraldehyde in the presence of 10 mM tartarate buffer (pH 3) and 0.2 mM H_2O_2 (Tien and Kirk, 1988). One unit of LiP activity was defined as the amount of enzyme required to catalyze the formation of 1 µmol of veratraldehyde per min under the reaction conditions.

RESULTS AND DISCUSSION

Most of the previous studies reported were focused on the biotransformation of direct and reactive dyes by single culture. Recently, there has been a growing interest in studying the lignin-modifying enzymes of a wide array of mix-culture of white rot fungi with the expectation of finding better lignin degrading systems for use in various biotechnological applications. Hence, in the present work, co-culture of *G. lucidum* and *C. versicolor* was explored for its disperse dye decolorization ability.

Screening of disperse dye: The initial screening trail experiment for the removal of disperse dye viz, Foron Yellow RD5GL, Foron Red RDRBLS, Foron Rubine RDGFL, Foron Black RD3GRN, Foron Blue RDGLN and Foron Turquoise SBLN by co- culture of *G. lucidum* and *C. versicolor* was carried out using Kirk basal media and incubated for 10 days in temperature controlled shaker, at 120 rpm. Co-culture of WRF showed variable decolorization potential for each dye and rate of color removal was gradually increased by increasing incubation time. Co-

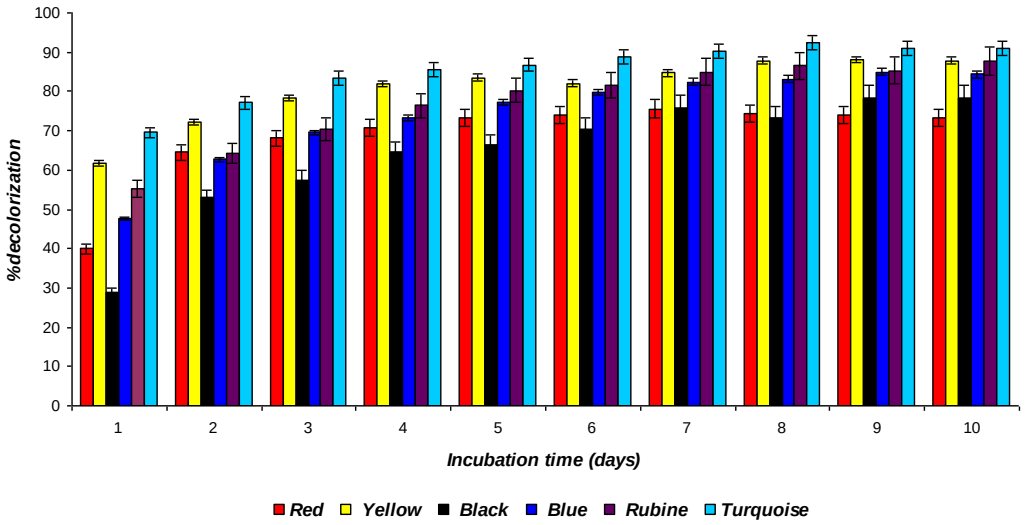

Figure 1. Screening of different disperse dyes for mixed-culture of WRF.

culture showed maximum color removal (92 %) of Foron Turquoise SBLN (λ_{max} 754 nm) on 8th day of incubation in comparison to others. While least decolorization of Foron Red RDRBLS (76 %) was achieved (Fig.1). The variation in decolorization potential of co-culture of white-rot basiodiomycete might be due to variations in structure of all subjected dyes. The decolorization ability of white rot fungi also depend on structures of dyes (Santos *et al*., 2007). Foron turquoise SBLN dye was selected for further process optimization for enhanced decolorization.

Effect of pH: Effect of pH (3-5.5) on decolorization of Foron turquoise SBLN at 0.01% initial concentration of dye with 2 mL inoculum is shown in Fig.2. The figure clearly shows that the percentage removal of dye increased with increase in pH. The maximum removal (93 %) of dye was found at pH 4.5 on 8th day of incubation period. Further increase in pH beyond 4.5 resulted in decreased percentage removal of dye since enzymes of *G. lucidum* and *C. versicolor* did not perform their proper function on highly acidic and basic pH. The optimum pH was found to be between 4.5 for maximum removal of dye. The pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 4.0 and 10.0 for most of the dyes (Tien and Kirk., 1983). MnP was the major enzyme produced in optimally decolorized pH 4.5 flasks, having maximum MnP activity of 1370 U/mL. The pH optima for a variety of WRF lies in the range of 4-6 depending upon the medium composition as well as on the nature of dye structures in the decolorization medium (Hai *et al*., 2006; Asgher *et al*., 2008; Bhatti *et al*., 2008).

G. lucidum has been found to maximally decolorize the textile dye at pH4.5 (Bibi *et al.*, 2009). Most of the WRF show their optimum activity on pH 4.5, at this pH ligninolytic enzymes show maximum activity (Kapdan *et al*., 1999; Toh *et al*., 2003; Asgher *et al*., 2006; Hafiz *et al*., 2008). Since at different pH, the behavior of fungus to decolorize the dye is different.

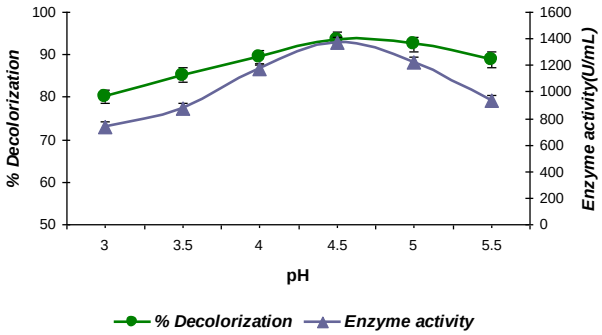

Figure 2. Effect of pH on % decolorization and enzyme profile of mixed-culture of WRF for Foron turquoise SBLN.

Effect of temperature: Effect of temperature (25–45 ºC) on decolorization of selected dye (Foron Turquoise SBLN) is shown in Fig.3 The results plotted as % decolorization and MnP activity versus incubation temperature clearly show that different dyes were decolorized at different rates at different temperatures. Maximum decolorization (95 %) and MnP activity (1390 U/mL) was obtained at 30 °C. An optimum temperature is a crucial factor for the growth of white rot fungi and activity of ligninases enzyme. For a variety of WRF cultures optimum temperatures were found to vary between 25-37˚C (Hai *et al*., 2006; Asgher *et al*., 2008; Bhatti *et al*., 2008). Maximum decolorization of different dyes by *G. lucidum* was found to be at 30 °C

(Kapdan *et al*., 1999; Toh *et al*., 2003; Asgher *et al*., 2006; Hafiz *et al*., 2008; Bibi *et al*., 2009).

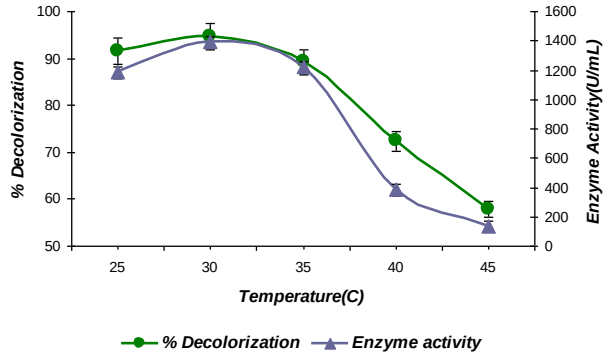

Figure 3. Effect of temperature on % decolorization and enzyme profile of mixed-culture of WRF for Foron turquoise SBLN.

These results indicate that the rate of MnP catalyzed decolorization of the dye increased with the increase of temperature up to certain degree above which the dye decolorization decreased or did not take place at all due to the fact that the rate of enzyme inactivation became faster than the enzymatic catalysis rate at high temperature.

Effect of carbon sources: To select the most effective combination of carbon and nitrogen source, different carbon (1%) sources were used in the dye decolorization media, under optimum conditions of pH and temperature. Dye decolorization was found to be enhanced to varying extents by the addition of all the tested carbon sources. Fig.4 shows the effect of different carbon sources on decolorization of Foron turquoise SBLN dye. In all the carbon sources tested, lowest dye decolorization was observed with sucrose. Maximum color removal (97.2%) was shown by the addition of maltose at pre-optimized conditions in 4 days co-related with maximum MnP activity (1498 U/mL). The decrease in dye decolorization by addition of sucrose as carbon source is also reported in literature (Hafiz *et al*., 2008). Additional carbon sources have been reported to enhance the fungal growth and enzyme activities to get maximum dye decolorization (Sanghi *et al*., 2006).

Effect of nitrogen sources: Different nitrogen sources were added to the medium under optimized conditions of pH, temperature and carbon source to get the maximum degradation of dye. The nitrogen sources (0.02 g) added to the medium was ammonium sulphate, ammonium nitrate, ammonium tartrate, peptone and urea. Addition of nitrogen sources has different effects on different strains of WRF.

The maximum decolorization (96.1 %) was observed by ammonium tartrate (Fig. 5). Peptone and ammonium nitrate showed 85.7 % and 83.5 % decolorization. Urea and ammonium sulphate showed poor decolorization. Minimum decolorization was observed by urea 48.3 %. MnP was found to be the major enzyme with enzyme activity of 1413.66 U/mL.

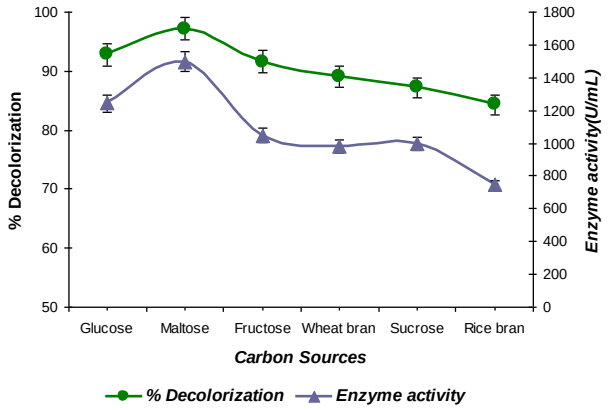

Figure 4. Effect of different carbon sources on % decolorization and enzyme profile of mixed-culture of WRF for Foron turquoise SBLN

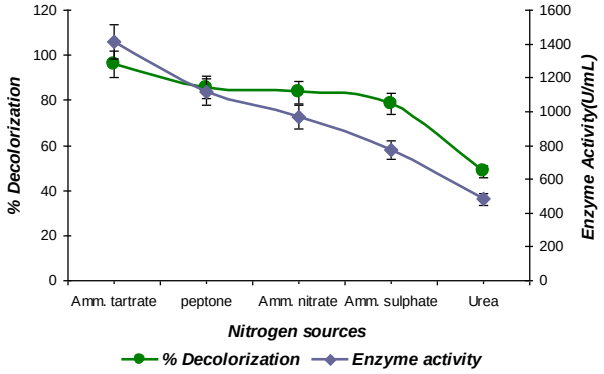

Figure 5. Effect of different nitrogen sources on % decolorization and enzyme profile of mixed-culture of WRF for Foron turquoise SBLN

Effect of mediators: To study the effect of different mediators on the biotransformation of Foron turquoise SBLN by mixed culture of *G. lucidum* and *C. versicolor*, the flasks were supplemented with 1 mL of 1 mM solutions of ABTS, veratryl alcohol, $MnSO_4$ and guaicol and incubated for 4 days under optimum conditions. The tested mediators showed remarkable differences in their abilities to enhance dye decolorization by the MnP (Fig.6). Maximum decolorization (98.5 %) and MnP activity (1720 U/mL) was achieved in only 2 days by addition of $MnSO_4$. The rates and extents of decolorization of dyes are significantly enhanced by the presence of different types of redox mediators (Matto and Husain, 2007). $MnSO_4$ is mediator of MnP and increase

in MnP production and activity by adding $MnSO_4$ has been reported in a number of studies (Kamitsuji *et al.*, 2005).

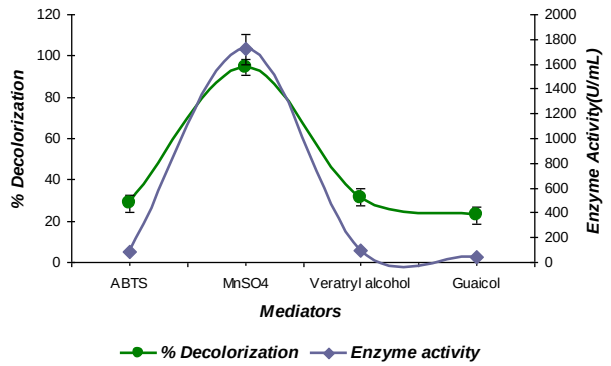

Figure 6. Effect of mediators on % decolorization and enzyme profile of mixed-culture of WRF for Foron turquoise SBLN.

Conclusions: The present study showed that mixed culture of *G. lucidum* and *C. versicolor* possessed a promising biodegradation potential for disperse dyes. However, better biodegradation was observed for Foron turquoise SBLN dye. The biodegradation potential of mixed culture was enhanced by using suitable nutrient growth medium under the acidic conditions of pH and at lower temperature. Maximum decolorization (94.7 %) of Foron turquoise SBLN dye was observed at pH 4.5 and temperature, 30 °C. Addition of mediators like $MnSO_4$ further enhanced the biodegradation of Foron turquoise SBLN dye. MnP was found to be the major enzyme involved in Foron turquoise SBLN dye biodegradation.

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