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Optimization of Culture Conditions for Enhanced Decolorization of Cibacron Red FN-2BL by *Schizophyllum commune* IBL-6

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Abstract The objective of this study was to exploit the decolorization potential of a newly isolated white-rot fungus *Schizophyllum commune* IBL-6 for the biodegradation of reactive textile dye Cibacron Red FN-2BL. In the initial decolorization study of 10 days, it was observed that *S. commune* IBL-6 was a better decolorizer of Cibacron Red FN-2BL. Various process parameters like composition of basal nutrient medium, pH, temperature, additional carbon and nitrogen sources, and initial dyestuff concentration were optimized to develop an economic decolorization process. The optimum dye decolorization was achieved in basal nutrient medium II containing 0.1% Cibacron Red FN-2BL and supplemented with 1% glucose after 3 days incubation at pH 4.5 and 30 °C. All the additional carbon sources were found to enhance decolorization process, whereas most of the nitrogen supplements caused fungal-growth inhibition. The pattern of enzymes involved in the biodegradation of this dye was studied, and manganese peroxidase was found to be the major peroxidase with minor lignin peroxidase and laccase activities.

Keywords Reactive dyestuff · *Schizophyllum commune* IBL-6 · Decolorization · Optimization · Ligninase profile

Introduction

The textile finishing generates a large amount of dyes and pigments containing wastewater from dyeing and subsequent steps that forms one of the largest contributions to water pollution [1]. Color present in dye-containing effluents gives a straightforward indication of water being polluted, and discharge of this highly colored effluent can damage directly the aquatic life in receiving water [2]. Due to their chemical structures, dyes are resistant to fading on exposure to light, water, and many chemicals [3]. Reactive dyes contain chromophoric groups such as azo, anthraquinone, triarylmethane, etc., and reactive groups,

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e.g., vinyl sulfone, chlorotrizine, etc., that form covalent bond with the dye [4]. The reactive dyes pose the greatest problem in terms of color because, in both ordinary and hydrolyzed form, they are not easily biodegradable, and thus, even after extensive treatment, color may still remain in the effluent [1].

Conventional treatment methods of textile effluents are either ineffective, costly, complicated, or have sludge problems [3, 5]. The economic and safe removal of the polluting dyes is still an important issue. Among the most economically viable methods available for decolorization, the most practical in terms of manpower requirement and expenses appears to be biological system [6, 7]. Although decolorization is a challenging process to the textile industry, the great potential of microbial decolorizing can be adopted as an effective tool. In the recent past, there has been an intensive research on bioremediation of dyes, and the use of ligninolytic fungi is turning into a promising alternative to replace or supplement present treatment processes [8, 9, 10].

Lignolytic fungi can mineralize xenobiotics to CO_2 and H_2O through their highly oxidative and non-specific ligninolytic system, which is also responsible for the decolorization and degradation of a wide range of dyes [11, 12]. These ligninases including laccase, lignin peroxidase (LiP), and manganese peroxides (MnP) are able to decolorize dyes of different chemical structures [13, 14]. In continuation of our previous studies [10, 14], this study on decolorization of reactive textile dye Cibacron Red FN-2BL by *Schizophyllum commune* IBL-6 is a part of our efforts for developing indigenous technology for decolorization of textile dyes and dye-containing effluents.

Materials and Methods

Textile Dye Stuff and Chemicals

The distribution office of Ciba at Faisalabad, Pakistan very kindly provided the reactive dyestuff Cibacron Red FN-2BL. The analytical-grade chemicals and reagents were purchased mainly from Merck (Darmstad, Germany) and Fluka (Sigma-Aldrich Chemie, Germany).

Fungal Cultures

The pure culture of white-rot fungus *S. commune* IBL-6 was obtained from the culture collection of Industrial Biotechnology Laboratory, Department of Chemistry, University of Agriculture Faisalabad, Pakistan. The cultures were grown on potato dextrose agar (PDA; Oxoide, Hampshire, UK) slants for 5 days under their respective pH and temperature conditions and were preserved at 4 °C.

Basal Nutrient Media

The four basal nutrient media of different composition [10, 15] were used to select the most suitable medium. Glucose (5 g/l) and dyestuff (200 mg/l) were added into each medium. pH of all media were adjusted to 4.5 by using 2,2-dimethyl succinic acid.

Meduim I. It was Kirk's basal salts medium [16].

Meduim II. It was of the same composition as that of the Kirk's basal salts medium 1, excluding veratryl alcohol and Tween-80 solution.

Meduim III. This medium had the following composition (g/l): urea, 0.036; KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.5 and $CaCl_2 \cdot 2H_2O$, 0.099. No tween-80 and veratryl alcohol was added.

Meduim IV. 0.036 g/l urea, 0.09 g/l KH_2PO_4, 0.1 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7\ H_2O$, 0.05 g/l $CaCl_2 \cdot 2H_2O$, 0.1 mg/l thiamine and 10 ml/l trace elements solution [10].

Inoculum

Aqueous suspension of conidia was prepared in 1% sterile glucose solution from maintenance medium. The suspension was passed through sterile glass wool columns to remove hyphal fragments. The concentration of the conidial suspension was adjusted to get 1×10^8 conidia/ml [17].

Decolorization Protocol

Decolorization shake-flask experiments were performed using 250 ml conical flasks in a temperature-controlled incubator/shaker (Gallenkemp). Flasks were prepared in triplicate, each containing 50 ml of Cibacron Red FN-2BL dye solution (0.02%) in basal nutrient media. The pH of the dye solution was highly basic, and it was adjusted to 4.5 using 2,2-dimethyl succinic acid. The entire test flasks were sterilized (121 °C) in autoclave for 15 min inoculated with 5 ml of the fungal conidial suspension and incubated for 10 days (120 rpm) unless otherwise stated in case of optimization studies. Triplicate flasks were removed every day (unless otherwise mentioned) and centrifuged at 10,000 rpm for 5 min, and supernatants were collected.

Process Optimization

For optimization of the decolorization process, the traditional stepwise strategy was adopted; varying one factor at a time by maintaining the previously optimized conditions. Shake flasks were incubated for 7 days at different initial pH values for selection of best pH for dye decolorization. For investigating the effect of temperature, the shake flasks were incubated at different temperatures (30–45 °C) at optimum pH for 7 days. In the next experiment, different carbon sources (1%) like glucose, fructose, sucrose, maltose, starch, and molasses were used in decolorization flasks to study their effect on dye decolorization under pre-optimized conditions. Subsequently, the decolorization medium was supplemented with different nitrogen sources such as ammonium sulphate, ammonium nitrate, peptone, yeast extract, urea, and maize seed meal, each at 1% level.

Effect of Varying Dyestuff Concentrations

To determine the maximum dyestuff concentration tolerated by *S. commune* IBL-6, the optimum decolorization medium was developed by varying the dyestuff concentration (0.01–0.1%). Decolorization flasks containing varying concentrations of Cibacron red FN-2BL, basal nutrient medium II, and glucose (1%) were processed for 3 days (final optimized incubation time) at optimum pH (4.5) and temperature (30 °C).

Enzyme Profile of *S. commune* IBL-6

To study the mechanism of biodegradation of Cibacron Red FN-2BL, the profile of lignolytic enzymes synthesized by *S. commune* IBL-6, the supernatants of the final optimum decolorization medium were analyzed for lignin peroxidase, manganese oxidase, and laccase activities.

Determination of Dyestuff Concentration

The supernatants at the end of each investigation were subjected to dyestuff analysis by a simple spectrophotometric assay [10]. Absorbance was measured by using a UV/Visible spectrophotometer (U-2001, Hitachi, Japan). Wavelength resulting in maximum absorbance (λ_{max} 510) by 0.01% Cibacron red FN-2BL was used. Dyestuff concentrations before and after biodegradation were calculated from a concentration vs absorbance calibration curve.

Enzyme Assays

LiP activity was determined by studying the oxidation rate of 4 mM veratryl alcohol to veratraldehyde in 100 mM sodium acetate buffer (pH 3.0) in the presence of 0.2 mM of H_2O_2. Absorbance was taken at 310 nm [16]. Manganese peroxidase assay was based on the oxidation of 1 mM $MnSO_4$ in 50 mM sodium acetate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2 [18]. Absorbance was taken at 270 nm. Laccase was assayed by monitoring the oxidation of 2-2′-azinobis(3-ethylbenthiazoline)-6-sulfonato at 420 nm [19]. The reaction mixture contained 0.3 mM sodium acetate buffer (pH 4.5), and the oxidation was followed at 420 nm.

Results and Discussion

The initial time-course study was conducted for 10 days using medium I to observe the efficiency of the selected fungal strain for decolorization of Cibacron Red FN-2BL. Results of the time-course study are given in Fig. 1. Initially, the dye decolorization by *S. commune* IBL-6 was very marginal, and after lag phase of almost 3 days, the decolorization gradually increased from 2.3% on day 1 and reached a maximum of 55.9 and 48.5% on the seventh and eighth days, respectively. Further increase in incubation time gave a slower decline in

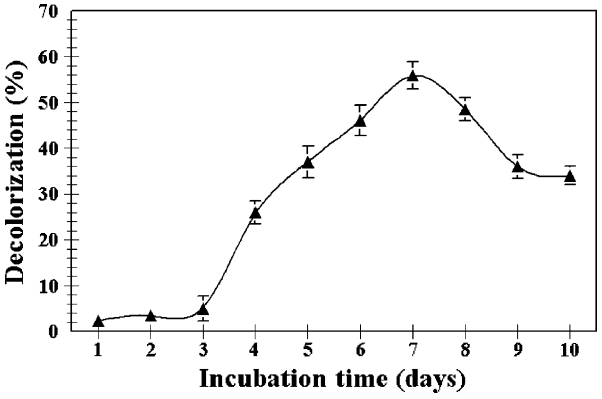

Fig. 1 Effect of incubation time on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

color removal by the fungus. This remarkable decrease in the dye decolorization on the eighth day is probably due to the formation of metabolites, and this decreasing trend continued until the tenth day. Different fungi have different decolorization potential on chemically different dyes, and screening for ligninolytic fungi for dye decolorization must be conducted under conditions as similar as possible [9, 20, 21]. The first sign of fungal mycelia growth was seen 24 h after inoculation, and that medium was completely decolorized by *Lentinula edodes* within 6 days [11].

The data on decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6 in different basal media have been presented in Fig. 2. In medium I, the decolorization on the seventh day was 56%, while it was 61% in medium Π. The decolorization was not that remarkable in media III (52%) and IV (47%), suggesting that medium Π was the best for decolorization of Cibacron Red FN-2BL. Medium I contained the components of Kirk's basal medium. Medium Π differed from medium I in composition to some extent. Medium II did not contain costly chemicals like veratryl alcohol and Tween-80 present in medium I, but it still promoted the best fungal growth and proved to be a better decolorization medium. Media III and IV contained additional unique source of urea that created a difference in the composition of these media. However, the decolorization was low in media III and 1V, indicating that these media were deficient in some essential components for growth of *S. commune* IBL-6. It has also been reported that decolorization of different dyes by different white-rot fungi is variable in different nutrient media [21].

Results on decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6 in basal nutrient medium II maintained at varying pH levels [3–6] are presented in Fig. 3. The decolorization of the dye by *S. commune* IBL-6 was found to be better in the acidic media, and optimum decolorization (63%) was noted in medium II maintained at pH 4.5. A higher pH value [5–6] caused a decrease in decolorization efficiency of the fungus. A gradual increase in dye decolorization by *S. commune* IBL-6 was observed with an initial increase in medium pH to 4.5. However, a further increase in pH caused a slowly decreasing effect. The pH value of the medium plays an important role in decolorization of dyes by white-rot fungi. White-rot fungi have been reported to show maximum growth and dye decolorization in acidic pH range [14, 21]. In a recent study [22], the growth and decolorization properties of *S. commune* on Acid Orange 7, Acid Red 18, and Reactive Black 5 were studied in pH varying from 1 to 6, and optimum was found to be pH 2 for both growth and color removal of these azo dyes. At higher pH values, reactive dye solutions are more negatively charged, and dye removal efficiency of white-rot fungi is decreased [23].

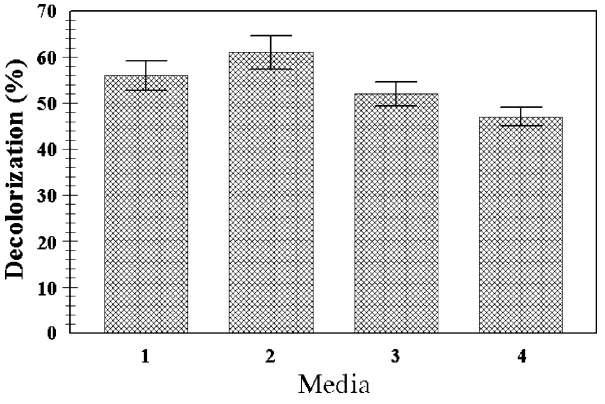

Fig. 2 Effect of basal media composition on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

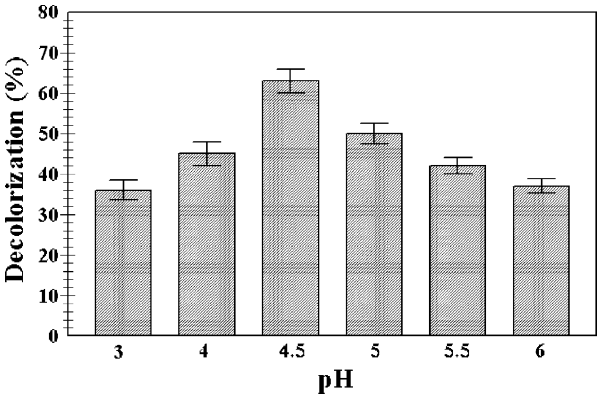

Fig. 3 Effect of pH on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

The effect of varying incubation temperatures on decolorization of Cibacron Red FN-2BL has been shown in Fig. 4. The fungus exhibited better dye decolorization in the low–medium temperature range (25–35 °C). The maximum decolorization (71%) was observed in the shake flasks incubated at 30 °C for 7 days under optimum conditions. The decreasing trend of dye decolorization was observed at higher temperatures (40–45 °C). White-rot fungi show better growth under medium temperature conditions as compared to at higher temperatures [24]. Temperature optima of 30–37 °C have also previously been reported [10, 11] for different white-rot fungi for decolorization of chemically diverse dyestuffs.

All the carbon sources added at 1% level stimulated fungal growth, and enhanced dye decolorization was noted in all flasks (Fig. 5). However, the results were very exciting in case of glucose, and maximum color removal (94%) was achieved on day 3 in the medium supplemented with glucose. Addition of fructose gave only 5% increase in decolorization as compared to control. Increase in percent decolorization of media supplemented with fructose, sucrose, maltose, starch, and molasses was also lower as compared to glucose; complete color loss was never achieved with these carbon sources even after the expiry of 7 days incubation period. Glucose was used by the fungus as readily consumable carbon source, and it caused a significant shortening of lag phase. Addition of glucose to the dye decolorization medium provides easily metabolizable energy source to the fungus and

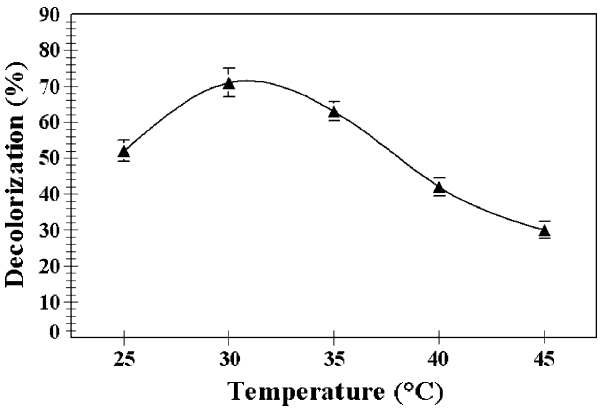

Fig. 4 Effect of temperature on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

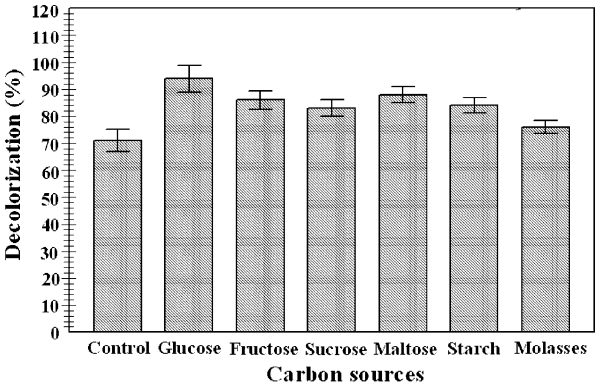

Fig. 5 Effect of different carbon sources on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

creates an environment to enhance decolorization rate of dyes. Decolorization of Poly R 478 dye by ten white-rot fungi was also reported to vary in response to different carbon regimes, and fastest decolorization rates were achieved with monomers (glucose, xylose) as carbon source [25]. In line with our findings, Kapdan et al. [21] reported the enhancing effect of glucose on decolorization of Everzol turquoise blue G by white-rot fungi. Everzol turquoise blue G was optimally (77%) decolorized by *Coriolus versicolor* in presence of 0.5% glucose supplement as compared to only 65% color removal with 0.2% glucose [26].

Different nitrogen sources were used in the optimum medium Π to study their effect on decolorization efficiency of *S. commune* IBL-6. The various nitrogen sources used were ammonium nitrate, ammonium sulphate, peptone, yeast extract, maize seed meal, and urea. It was noted that all the nitrogen sources caused a decrease in Cibacron Red decolorization by *S. commune* except urea (Fig. 6). Highest decolorization of 97% was observed with urea. However, this increase was not significant. However, the maximum value of decolorization after 7 days with urea addition was significantly lower as compared to the medium having no added nitrogen. Lignolytic enzyme production and dye decolorization by white-rot fungi is strongly dependent on nitrogen levels, with high nitrogen conditions generally suppressing enzyme production [25]. Lee et al. [27] has also reported the enhancing effect of nitrogen supplementation on the efficiency of color removal by *S. commune*. The difference in our results can be explained on the basis of different basal nutrient media used in our study that already contained ammonium tartarate as the nitrogen source. Difference

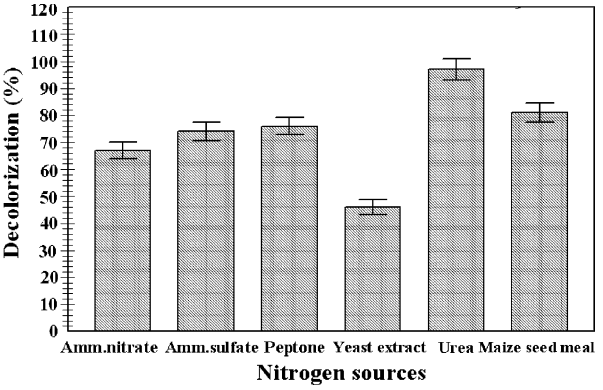

Fig. 6 Effect of different nitrogen sources on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

of *S. commune* strains and chemical nature of dyes used in this study may also account for their variable behavior.

The results regarding the effect of dye initial concentrations have been reported in Fig. 7. It was observed that at lower concentrations, the rate of decolorization was quite efficient, and it reached up to 91% with lowest concentration of 0.01%. When the concentration was gradually increased to 0.03% and 0.1%, the decolorization of the dye was decreased to 65% and 85%, respectively. The dye was well tolerated by the fungus in concentrations from 0.01% to 0.1%. Increasing concentrations of dye caused significant fungal growth inhibition. Our findings favorably compare to those reported earlier [22] about decolorization properties of *S. commune* with respect to initial dye concentration (10–100 mg/l) and reported that increasing the concentrations of azo dyes inhibited the growth of *S. commune*. *S. commune* was capable of optimally removing Acid Orange 7, Acid Red 18, and Reactive Black 5 with an initial concentration of 0.01% of the dyes. Higher decolorization was observed at lower concentrations for all the dyes. As reported earlier [21] that dyestuff concentration should be lower than 0.05% for complete decolorization of Everzol turquoise blue G by *C. versicolor*; only partial decolorization could be achieved for dyestuff concentrations above 0.05%. *Phanerochaete chrysosporium* showed a color removal efficiency of 95–100% on Direct Blue 15 in repeated batches [28] following first-order kinetics with respect to initial concentrations of dye.

Among the three ligninolytic enzymes studied, MnP activity (580 U/ml) was found be the major enzyme involved in degradation of Cibacron red FN-2BL by *S. commune* IBL-6. Lower activities of LiP (79.2 U/ml) and laccase (66.4 U/ml) were also detected in the culture supernatant of optimal decolorized medium. Lignolytic enzyme profile showed that MnP was the major lignolytic activity involved in dye degradation with minor LiP and laccase activities. This is consistent with our initial finding on selection of basal nutrient medium. The selected medium II did not contain varatryl alcohol, but it gave better decolorization results as compared to medium I containing varatryl alcohol. Varatryl alcohol is a fungal secondary metabolite that plays an important role in stimulating LiP oxidation of a wide range of recalcitrant substrates [29]. Ligninase profiles and pattern of their expression vary among different white-rot fungi cultures, and this appears to be of most interest for practical applications for dye degradation [11, 12, 30]. MnP has also previously been reported as major enzyme involved in degradation of different dyes by white-rot fungi [26, 31].

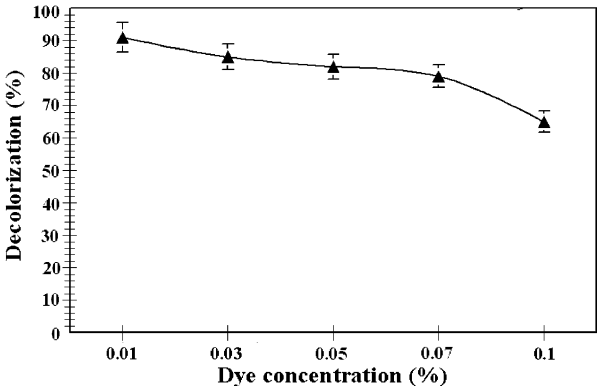

Fig. 7 Effect of dye concentration on the decolorization of Cibacron Red FN-2BL by *S. commune* IBL-6

Conclusions

White-rot fungus *S. commune* IBL-6 had excellent potential for degradation of reactive dye Cibacron Red FN-2BL. It can efficiently decolorize the dye by using suitable nutrient growth medium under the acidic conditions of pH and at lower temperature. Decolorization potential can also be enhanced by the additional of glucose at its optimum concentration. Furthermore, the MnP played an active part in biodegradation, while LiP and laccase showed lower activities.

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