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Laccase-Mediated Textile Dye Decolorization by Cladosporium*oxysporum*

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Abstract: ***Water polluted by textile industry contain non-biodegradable dyes and various chemical pollutants which when treated by conventional methods still lead to the formation of sludge. In the present study laccase enzymes from Cladosporiumoxysporum were used to decolorize Reactive Blue, Reactive Yellow and Reactive Red dyes through adsorption. Immobilized treatment was tried to minimize the cost of enzyme treatment. Also the effect of pH and temperature on dye decolorization was studied. The enzyme was stable at pH 5.0 and retained 90.34% activity after 240 min. The enzyme was stable at 40°C and retained 92.87% activity after1 hour. Decolorization for free enzyme was 41.90, 34.18 and 27.87% respectively for Reactive Blue, Reactive Yellow and Reactive Red while for immobilized enzymes was 61.20, 50.51 and 39.34% . The increased level of decolorization by immobilized enzyme was attributed to initial adsorption on solid material and enzyme degradation in the later phase. The present study revealed that Cladosporiumoxysporumat optimum conditions could be effectively used for treatment of dye industry effluent.***

Keywords: Laccase, *Cladosporiumoxysporum*, Immobilization, Decolorization, Synthetic dye

1. Introduction

Textile industries contribute to 70% of water pollution due to their effluent discharge consisting of highly coloured, carcinogenic dye stuff, high BOD, COD, TDS, heavy metals, auxiliary chemicals and much more complex materials which pose a major problem in waste water treatment [1].

Decolourization of azo dyes is possible with conventional physicochemical methods but they lead to the accumulation of concentrated sludge and emission of toxic substances, which still remains as a disposal problem. Microbial decolourization has been proposed as a cost effective and environmentally friendly alternative [2]. A large number of microorganisms among bacteria, yeast and fungi have the abilities to biodegrade these dyes, through the use of various enzymes namely the oxidoreductases or phenoloxidase such as laccase, manganese peroxidase and lignin peroxidase [3].

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) produced mainly by fungal strains are multi-copper oxidases which can degrade several dye structures and transform toxic compounds into safer mechanisms [4]. The high production cost, structural instability and short lifetime of using free enzymes can be overcome by immobilization of the enzyme. Among the different methods of immobilization, the entrapment in biopolymeric matrices, such as alginates offers many advantages, including: biocompatibility, lower occurrence of denaturation, low cost and ecofriendliness [5]. The aims of this study were (i) screening, partial purification and characterization of laccase, (ii) its optimization of culture conditions for laccase production and (iii) to study the influences of conditional parameters such as enzyme activity, temperature, and pH on dye decolorization between free enzyme and immobilized enzyme.

2. Materials and Methods

2.1 Fungal strain, media and culture conditions

Samples were collected from effluent polluted soil around CEPT from 6 cm below the surface layer. The fungi were isolated and maintained on Malt extract Agar medium for 6 days and further subcultured on Czapek-Dox agar medium to obtain pure cultures [6].

2.2 Screening for dye decolorization

The isolated fungi were screened for their ability to decolorize various dyes. Mycelial pellets were prepared by inoculating fungal spore suspension into Czapek-Dox broth and incubated at 27 C for 5 days. For screening, the adsorbates – Reactive Blue, Reactive Yellow and Reactive Red- were taken and added to mycelia pellets in flasks and biomass was separated by centrifugation and OD (Optical Density)of supernatant was measured using UV Spectrophotometer. The difference in absorbence values of dye solution before and after adsorption yielded the amount of dye removed [7]. Decolorization on solid medium was studied by visual disappearance of color [8]. The fungal strain that showed maximum dye removal was identified and used for further studies.

$$Percentage\ Decolorization = \frac{Initial\ OD - Final\ OD}{Initial\ OD} \times 100$$

2.3 Growth conditions and laccase activity determination

To find out the suitable medium for laccase production, the fungus was cultivated on solid substrates like rice bran,wheat bran, coconut cake and groundnut cake and submerged media like Olga [9] ,Slomczynski[10], Coll[11], B and K[12] and Sabouraud's dextrose broth [13]. Crude enzyme was extracted as filtrate. The protein content was estimated by Lowry's method and standard graph was plotted with

concentration of BSA vs OD [14].

The intense brown color development due to oxidation of guiacol by laccase can be correlated to its activity read at 470 nm [15].

$$Enzyme\ Activity(U/mL) = \frac{[(A/t)\ x\ (V\ x\ DF\ x\ 10]/10000}{V\ (mL)\ x\ Extinction\ Coefficient}$$

2.4 Enzyme purification and Molecular Weight

The crude enzyme was purified first using 40% saturated ammonium sulphate to remove hybrid proteins and subsequently with 80% ammonium sulphate to precipitate laccase. The solution was centrifuged and the pellet was dialyzed against acetate buffer. The desalted sample was loaded on Sephadex G-100 column and eluted fractions were concentrated by lyophilization. The molecular weight was determined by SDS-PAGE [16].

Table 1: Purification table for *Cladosporiumoxysporum*laccase

Purification step	Total volume (mL)	Total enzyme activity (IU)	Total protein content (mg)	Specific activity (IU/mg)	Purification (fold)	Yield (%)
Crude enzyme	30	11.27	0.290	38.86	-	-
$(NH_4)_2SO_4$ Precipitation	10	3.75	0.045	83.33	2.14	33.27
Dialysis	7	3.19	0.033	96.6	1.159	28.30
Gel filtration (Sephadex G-100)	5	2.75	0.025	110	1.138	24.40

2.5 Effect of pH and temperature on laccase activity and enzyme stability

Temperature stability of purified laccase was studied by pre-incubating at different temperatures between 30 and 70°C for 15 min, followed by determination of the residual activity. Temperature at which enzyme showed maximum activity was noted as optimum temperature .The influence of pH on the laccase stability was evaluated by incubating enzyme at 25°C in different pH levels (3–8) for 24 h and determining the changes in absorbance using guiacol as substrate

2.6 Enzyme immobilization

The enzyme was mixed with 4% sodium alginate and the mixture was then extruded into the calcium chloride solution dropwiseand calcium-alginate beads were formed which were allowed to harden [17]. Enzyme immobilised beads were recovered by filtering the solution.

Decolorization experiments were performed by adding the free or immobilized enzyme to each dye solution– Reactive Blue, Reactive Yellow and Reactive Red in a sodium-acetate buffer followed by incubation at room temperature. Decolorization percentage was then determined by monitoring absorbance of the taken samples at intervals of 30 min, using a UV-vis spectrophotometer at the maximum absorbance of each dye.

3. Results and Discussion

Totally seven fungal strains were isolated. These isolates were identified based on their morphology,growth pattern, mycelia structure and spore architecture as the following: *Aspergillusflavus, Aspergillusversicolor, Aspergillus japonicas, Penicilliumchrysogenum, Peniciliumexpansum, Fusariumsolani and Cladosporiumoxysporum* [18].These were screened for their ability to decolorize Reactive Blue, Reactive Yellow and Reactive Red in aqueous solutions. *C.oxysporium* had higher decolorization as compared to the other fungi and hence was selected for further investigative studies.

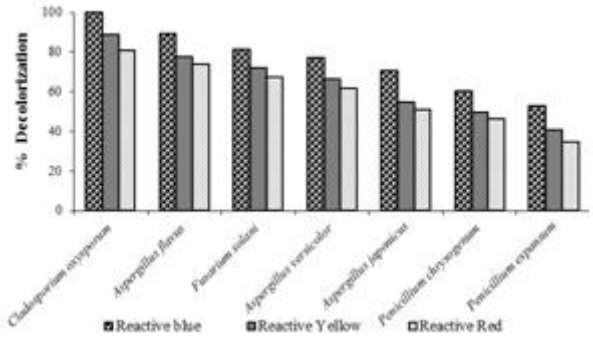


Figure 1: Ranking of fungal isolates based on their adsorption ability towards Reactive dyes

Guiacol was used as an indicator to test the ability of microorganisms to produce laccase enzyme. Laccase produces brown color in culture medium due to oxidative polymerization with guiacol [19, 20]. In the present study, *C.oxysporum* grown on Kirk's nutrient salt medium containing gluiacol showed formation of reddish brown color zone around the colonies on agar plate. Similarly in Kirk's nutrient broth when gluiacol was added, reddish brown color in the medium was observed withing 24 hrs.

Among the various substrates used for solid state fermentation (SSF), rice bran (21.666 U/g) yielded the highest laccase production followed by wheat bran (5.701) ,coconut cake (1.854) and groundnut cake (0.973) [21,22]. *C. oxysporum* utilize xylan, cellulose and mannan in rice bran as carbon sources in initial growth phase. As these sources deplete, laccase synthesis is induced by phenolic compounds in rice bran, leading toincrease inlaccaseproduction. Maximum enzyme production was obtained on 14th day of fermentation. Submerged fermentation was carried out in five synthetic media: Olga, Slomczynski, Coll, B and K, and Sabaurd's dextrose broth. Among the five, Sabourd's dextrose broth yielded maximum laccaseactivity as it has high concentrations of peptone and glucose compared to other media [23].

The enzyme was partially purified by sequential steps of sulphate precipitation, dialysis, and Sephadex G-100 column chromatography and the recovery and purity of enzyme was analysed. The results revealed that gel filteration increased the enzyme activity from 21.67 to 110 with the purification

fold of 1.9. The molecular mass of the laccase purified in this study was 46KDa which was in accordance with the previous reports [24].

The effect of pH on laccase activity was studied in the range of 3 to 8. The partially purified enzyme of C. oxysporum exhibited the highest activity at pH 5.0. A sharp decrease in laccase activity was observed as the pH was increased towards alkaline range. The optimum temperature for the laccase of *C.oxysporum* was found to be 40 degrees [25].

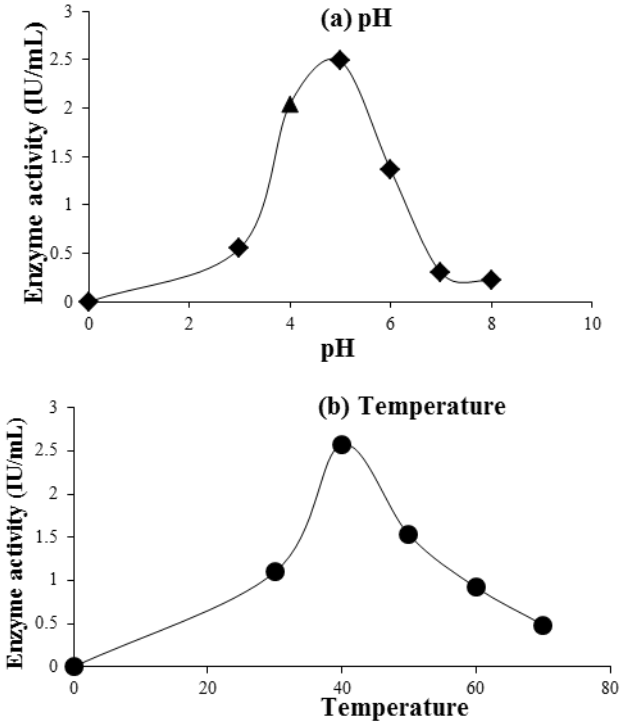
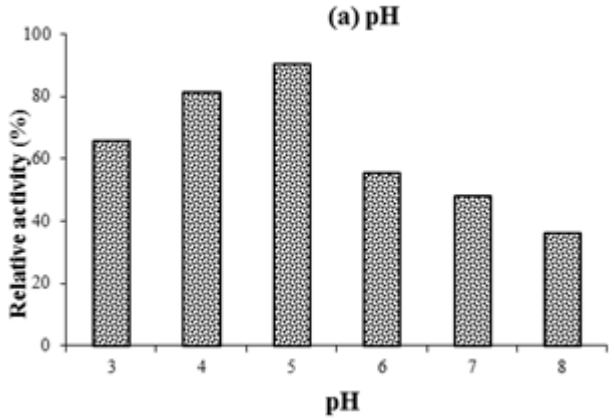


Figure 3: Effect of pH and temperature on laccase activity

Enzyme activity declined as temperature was increased from 40 to 70. pH tolerance and thermal stability are essential for industrial application of enzyme. Laccase enzyme activity was stable at pH 5.0 retaining 90.34% activity, however raising the pH above 5.0 or decreasing below 4.0 rapidly decreased laccase activity. Not much loss in laccase activity was detected till 50°. At 60° 33.17 % of the residual laccase activity remained after 1 hour, indicating it to be a thermostable enzyme.



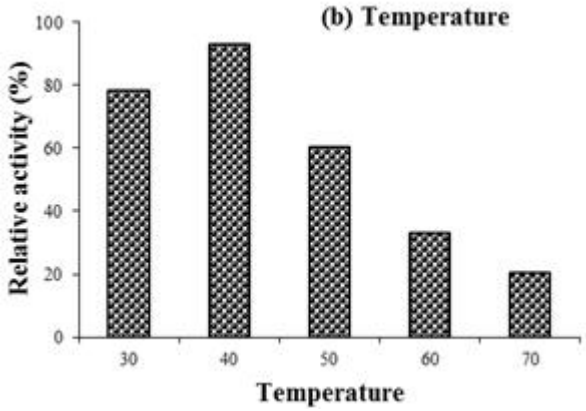


Figure 4: pH and temperature stability of laccase

The dye decolorization depends on the structure of dye and substrate specificity of lacasse enzymes. In the present study, the free laccase of *C.oxysporum* yielded 41.9, 34.18 and 27.87 % decolorization in Reactive Blue, Reactive Yellow and Reactive Red at 10mg/L at 210 min. For application in industrial process, a polymeric-alginate was used to entrap laccase to study the relative efficiency in dye removal [26]. The optimum conditions were found to be 10 mg/L dye concentration, 210 min incubation and ten immobilized beads. At these conditions, the immobilized enzyme yielded 61.20, 50.51 and 39.34 % decolorization for Reactive Blue, Reactive Yellow and Reactive Red respectively. After 210 min the alginate composition affected the reactive dye decolorization.

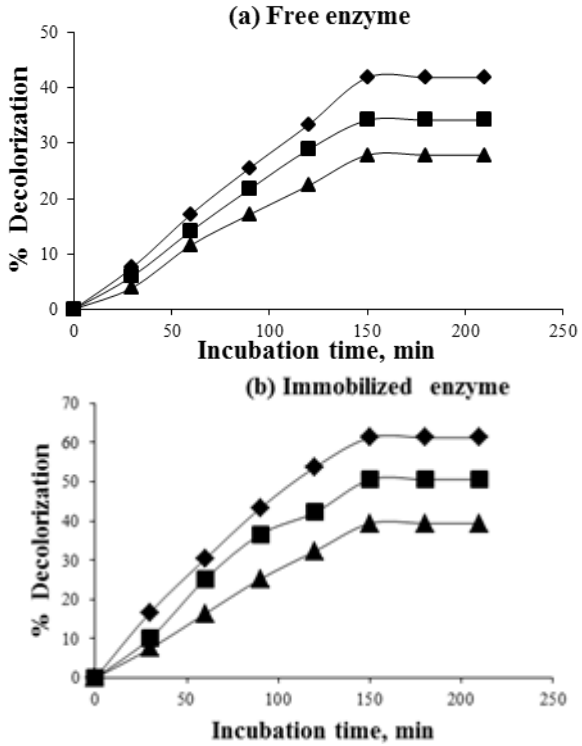


Figure 5: Comparison of percentage decolorization of free enzyme and immobilized enzyme

When comparing the results of free enzymes with that of immobilized enzymes especially in the fields of bioremediation, immobilized enzymes can be easily recovered for reuse and are more stable towards harsh conditions like high temperature. This turns the enzyme

based process into more economically viable approach [27, 28].

4. Conclusion

As the fungus *C.oxysporum* is easily cultivable and biodegradable, thereby avoiding the problem of sludge. The dye adsorbed on the surface can be easily desorbed and reused as low grade dye for coloring purposes, while the biomass could be reused for several cycles for effluent treatment. Hence the process developed in this study is recommended for decolorization of dye industry effluents prior to secondary and tertiary treatments so that the cost of these advanced techniques will be very much reduced

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