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Synthetic Dye Decolourization Activity of Laccase Enzyme: A Novel Approach to Brawl Against Environment Pollution

KEYWORDS: dye decolourization, Laccase, Environment Pollution

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ABSTRACT *A research was carried out on production of laccases by fungi isolated from soil. Decolorization of two synthetic dyes (Reactive Black 5 and Reactive Blue 171) using three sources of fungal laccase with the origin of Rhizopus oryzae, Aspergillus flavus and Fusarium sporotrichioides was investigated. Among them, the enzyme from Rhizopus oyzae was found to the most efficient which decolorized 70% RB5 and 64% RB171 followed by Fusarium sporotrichioides and Aspergillus flavus. All conditions used were important for dye decolorizing activity of this crude laccase enzyme. The optimum pH of decolorization was tested at 30 °C and it was around 3.0. This activity was highly reduced at pH 4.5–5.0. This crude laccase could also decolorize the mixed dyes (Reactive Black 5 and Reactive Blue 171) and synthetic wastewaters. Rapid and high textile dye decolourization through the selection of appropriate conditions could facilitate the development of more economical and environmentally friendly processes.*

1. Introduction:

Wastewaters from textile or dyeing industries contain various textile dyes. These dyes give these wastewaters their characteristic deep color. Because of their deep color, their disposal into waters may affect the ecosystem by reducing the photosynthetic activity and, therefore, affecting dissolved oxygen concentration (Robinson et al., 2002).

Thus, these wastewaters need to be treated before being discharged into aquatic ecosystems. There are great attempts to develop new and effective environmentally friendly biological alternatives for decolorizing these dye-containing waste waters (Yesilada, 1995; Murugesan et al., 2007b; Wang et al., 2009; Yesilada et al., 2010; Bonugli-Santos et al., 2012; Gul and Donmez, 2012, 2013).

The solution could be specific microorganisms or enzymes (Yeşilada and Ozcan, 1998; Yesilada et al., 2010; Zeng et al., 2011; Hadibarata et al., 2012). The main advantages of using enzymes for decolorization of dyes may be their rapid decolorization activity and their ability to retain their activity even under unfavourable conditions (Murugesan et al., 2007b; Zeng et al., 2011).

The copper containing oxidase, laccase (benzenediol oxygen oxidoreductase, EC 1.10.3.2), which is mainly produced by white-rot basidomycetes and other fungal (Baldrian P., 2006) and bacterial strains (Telke AA. et al., 2009)and also some plants have been used in various biotechnological and environmental processes. Lack of substrate specificity introduced laccase as an enzyme able to oxidize wide range of chemical compounds.

This research work is therefore aimed at isolating and producing laccase enzyme from soil fungi and checks their decolorizing ability against various dyes.

2. Materials and Methods:

2.1. Sample Collection:

A total of five (5) samples of saw dust were collected from different areas. The samples were collected in clean polyethene bags and were immediately transported to Laboratory for further analysis.

2.2. Serial Dilution:

One gram of each sample was diluted three (3) times in sterile physiological solution. Serial dilution was carried out in accordance with the procedure of Cheesbrough, (2000).

2.3. Isolation of Fungi:

The Suspension obtained after vigorous mixing of the soil samples were left to stand for 30 mins. 0.5ml of different aqueous dilutions of soil suspensions were inoculated on Sabouraud Dextrose Agar plates. To which Streptomycin was had been incorporated to reduce bacterial contamination. The inoculated plates were incubated upright at 25°C for 5 days. All observed colonies were subculture to obtain and pure cultures were obtained.

2.4. Subculture:

The Colonies from the plates were observed for morphological difference. Each colony that differs morphologically from another was picked with a sterilized inoculating needle and inoculated on a freshly prepared Sabouraud Dextrose agar plates and incubated at 25°C for 3-5 days in order to obtain pure colonies of the isolates.

2.5. Identification of Fungi:

The growth rate, colour, texture, colonial morphology and diffusible pigmentation of each sample were examined macroscopically. Tease mount using lactophenol cotton blue was adopted and microscopic features such as spore and hyphae morphology were observed and compared with the standard colored atlas as described by of Cheesbrough, (2000).

2.6. Production of Laccase:

A total of four (4) fungal strains were inoculated onto the plates containing plain agar with the following composition (g/L): peptone 3g, K2PO4 0.4g, MgSO4 0.5g, ZnSO4 0.001g, FeSO4 0.005g, MnSO4 0.5g and Carboxyl methyl cellulose (CMC) 0.5g and 1g respectively. The plates were incubated at 30°C for 5 days. The culture form these plates were cultured in 100 mL of Sabouraud dextrose broth (SDB) for 5 days at 30 °C and 150 rpm to produce preinoculum. After incubation, the pre-inoculum was homogenized and 7mL of this homogenized preinoculum was used to inoculate 600mL of fresh SDB. This culture was incubated for 5 days. The pellets obtained from this fermentation were then used to produce laccase enzyme during repeated-batch studies. In this process, 50mL of stock basal medium (SBM) in 250mL flasks was inoculated with pellets and this culture was incubated for 24 h. The culture medium was then completely removed and replaced with 50mL of fresh SBM without removing the pellets, which was then incubated as stated above for the next

Volume : 4 | Issue : 6 | June 2014 | ISSN - 2249-555X

cycle (Birhanli and Yesilada, 2010). This mode was repeated 5 times and then the culture was filtrated on fifth day and the obtained culture filtrate was used as crude laccase enzyme for decolorization experiments. The composition of SBM used was (g/L): KH2PO4 0.2; CaCl2.2H2O 0.1; MgSO4.7H2O 0.05; NH4H2PO4 0.5; FeSO4.7H2O 0.035; glucose 2, yeast extract 1, and CuSO4.5H2O 0.125.

2.7. Enzymatic dye decolorization:
The crude laccase enzyme from repeated-batch culture of three fungi was used as a biological system for dye decolorization. The diazo dyes Reactive Black 5 (RB 5) and Reactive Blue 171 (RB 171) were used in the current study and were prepared as stock solutions (final concentration of 200 mg/L) by dissolving them in citrate phosphate buffer solutions with different pH values. These stock solutions were used according to the experimental design. The crude enzyme sample was added into citrate phosphate buffer containing dye and incubated for 30, 60, and 300s. Decolorization was determined by monitoring the absorbance changes at the maximum absorbance wavelength of the dyes used and was expressed in terms of percentage. Unless otherwise stated, the temperature and pH were 30 °C and 4.5, respectively.

3. Results
A total number of four (4) species of fungi were isolated. Table 1 indicates the species of fungi isolated from the soil sampled. Table 2 represents the results of fungal isolates producing laccase. The species include *Aspergillus niger, Aspergillus flavus, Fusarium sporotrichioides* and *Rhizopus oryzae.*. All the fungal species were further used for Laccase production. The repeated-batch method, which is a completely different fermentation method than the submerged and solid-state fermentation methods, was used to produce laccase enzyme. The culture filtrate obtained after 5 cycles of incubation was used as crude laccase enzyme to decolorize textile dyes. Only laccase enzyme was detected with this culture. Out of the four, 3 were laccase positive and 1 laccase negative. The laccase positive fungi were *Aspergillus flavus, Fusarium sporotrichioides* and *Rhizopus oryzae.*

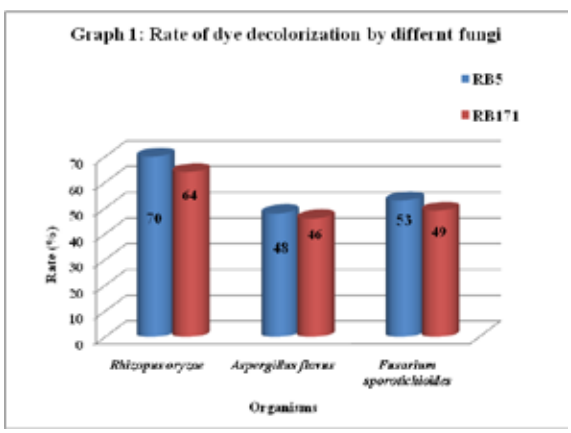
The decolorization activity of crude laccase obtained from three different fungi against these 2 dyes was different. Incubation time was detected as an important factor for decolorization. Graph 1 represents the Comparative decolourization rates of all the three fungi. *Rhizopus oryzae* showed highest rate of decolourization for both the dyes followed by *Fusarium sporotrichioides* and *Aspergillus flavus.*

4. Discussion
Laccase producing microorganisms especially white rot fungi were extensively applied for dyes decolorization experiments. Decolorization ability of five indigenous white rot fungi on vat dyes during 10 days was studied by Asgher et al. 2008 and it was determined that Coriolus versicolor IBL-04 showed excellent decolorization potential on all tested dyes. Decolorization potential of laccases even on a same dye shows variation and depends on the biological sources of producing microorganism.

In this study, enzymatic decolorization of reactive dyes was tested. Because laccase is a primary enzyme with the ability of textile dye decolorization, culture filtrate containing a high amount of laccase enzyme was obtained from three different fungi. Various factors such as culture conditions, fermentation mode, type and amount of nutrient, and inducers may affect the laccase production by influencing the expression of laccase isozymes (Giardina et al., 2010; Parenti et al., 2013). Therefore, laccase production ability of fungal strains was investigated under repeated-batch method and culture filtrate with 57 U/mL, 45U/ml and 51 U/mL laccase was obtained from *Rhizopus oryzae* , *Fusarium sporotrichioides* and *Aspergillus flavus* respectively under these conditions. These cultures, containing high amounts of laccase, were used as crude laccase enzyme to decolorize reactive textile dyes. In this study, textile dyes RB 5 and RB 171 were decolorized to different extents by crude laccase.

The optimum pH of decolorization was around 3.0 at 30 °C and dye decolorization activity was highly reduced at pH 4.5–5.0. The pH and time of incubation were detected as the important parameters for obtaining high decolorization values (Kokol et al., 2007). Michniewicz et al. (2008) reported an optimum pH value of around 3.5 for decolorization of azo and anthraquinone dyes by crude laccase from *C. unicolor.*

In conclusion we can say that, it was possible to enhance the decolorization activity of this crude laccase from repeated batch culture of *Rhizopus oryzae* and other two fungi by optimizing the conditions without a mediator. Because the high price and toxicity of some of the mediators may affect application, decolorization potential without any mediator may be advantageous for application of this crude laccase to biological dye treatment systems. This crude laccase obtained from Rhizopus oryzae source showed high decolorization potential in a short time. It could also decolorize the mixed dyes (RB 5 and RB 171) and synthetic waste waters. This could be an alternative environmentally friendly solution to solve the textile dye pollution problem.



Sr. No.	Colony Description	Morphology	Identified Organism
1	It is white and colony with aerial growth	It has pigmented rhizoids and sporangiophore and numerous stolons	Rhizopus oryzae
2	It is green in colour and powdery	They have aerial hyphae bearing conidiophores	Aspergillus flavus
3	It is black in colour and powdery, the reverse is yellow	The conidiophores terminate is vessels and the conidia are in chains	Aspergillus niger
4	Colony whitish and Aerial mycelium floccose or cottony	Conidiophores branched, formed in aerial mycelium bearing cylindrical phialides which often proliferate sympodially	Fusarium sporotrichioides

Table 1. Identification of Fungal Isolates from Soil

Volume : 4 | Issue : 6 | June 2014 | ISSN - 2249-555X

Fungal Isolates	Production of laccase
Rhizopus oryzae	+
Aspergillus niger	-
Aspergillus flavus	+
Fusarium sporotichioides	+

Table 2. Fungal isolates producing laccase

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