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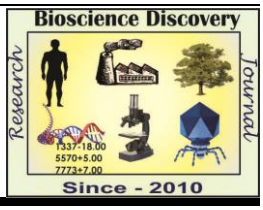

Decolorization of synthetic dyes by microbial crude laccase

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Abstract

In this work, attempts were made to synthesize laccase from local fungal strain. Development of brown colored precipitate and reddish zone after adding tannic acid and guaiacol respectively on PDA plates of *Aspergillus flavus* confirmed the laccase synthesis by fungal strain. Characterization of isolated crude of enzyme from *Aspergillus flavus* showed optimum pH and temperature 5.0-7.0 and 30^0-50^0C respectively. Dye decolorization ability was initially confirmed with zone of decolorization on dye containg agar plates. The enzyme was found to decolorize several dyes. But the highest decolorization value of 76 % was obtained in case of Phenol red.

INTRODUCTION

In recent era so many enzymes are achieving great importance in industrial application for several biotransformation processes. Many of such potential enzymes are widely distributed in nature. Laccases, (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) which are the oldest and most studied enzymatic system are among them (Usha *et al.*, 2014). These are a part of broad group of enzymes called polyphenol oxidases having copper atoms at the catalytic centre and are usually called multicopper oxidases (Brijwani *et al*, 2010).

This enzyme was initially described in 1883 by Yoshida when he extracted it from the exudates of the Japanese lacquer tree, *Rhus vernicifera.* (Kaur and Nigam, 2014). Characteristics of laccase as a metal containing oxidase was reported by Bertrand in 1985 (Giardina *et al.*, 2010). Since then wide presence of these enzymes in various basidiomycetous and ascomycetous fungi has been reported. (Imran *et al.*, 2012).

These enzyme system catalyzes the oxidation of various aromatic compounds (particularly phenol) with the concomitant reduction of oxygen to water (Raheem and shearer (2002). Although the enzyme is present in plants, insects and bacteria, the most important source are fungi and particularly basidiomycetes **(**Urairuj *et al*, 2003**)**. The white-rot fungi are the most efficient microorganisms capable of extensive aerobic lignin degradation (Brijwani *et al*., 2010). And this laccase is considered a key player in lignin degradation and/or the removal of potentially toxic phenols arising during morphogenesis, sporulation or phytopathogenesis. (Valeriano *et al.*, 2009). The role of laccase in lignin and phenolic compound degradations is evaluated in large number of biotechnological applications such as dye degradation, bioremediation of some toxic chemical wastes, wastewater and soil treatments and also biosensor developments (Nandal *et al.*, 201). The mechanism of laccase action can be mediated by its action on nonphenolic substrates by the inclusion of mediators (Ahmad *et al*., 2015). Mediators are reported as the group of low molecular weight organic compounds which can be initially oxidized by laccase resulting highly active cation radicals capable of oxidizing

nonphenolic compounds that laccase alone cannot oxidize (Karp *et al.,* 2015) .

The ability of fungal laccases to catalyze (alone or with the help of mediators) the oxidation of pharmaceuticals and biocides was shown for several substances (Thurston, 1994). Due to this wide range of substrates and the sole requirement of oxygen as the co-substrate, laccases appear to be a promising biocatalyst to enhance the biodegradation of micropollutants in wastewater in a complementary treatment step. (Margot *et al*., 2013). According to recent studies laccases have been efficiently useful in nanobiotechnology due to their ability to catalyze electron transfer reactions without additional cofactor (Shradha *et al*., 2011; Ahmed *et al*., 2014).

In the present study, our objective was to isolate laccase producing efficient fungal strain and to evaluate industrial application of fungal synthesized laccase for decolorization of dyes.

MATERIALS AND METHODS

Fungal strain

Fungal strains (*Aspergillus niger, Aspergillus flavus, Penicilluim chrysogenum, and Penicilluim citrinum*) from the culture collection in the Department of Biotechnology, of Institute of Science, Aurangabad, MS, India were used as the source of laccase enzyme. All strains were microscopically identified and maintained on potato dextrose agar (PDA) at 4^0C and periodically sub-cultured to maintain viability.

Screening of efficient strains:

Synthesis of lacccase by fungal strain was confirmed by using specific substrate of laccase which included tannic acid and Guaiacol. 24 h old cultures of above fungal strains grown on PDA plates were observed for development of brown colored precipitate after adding tannic acid plates and reddish zone in guaiacol containing plates.

Laccase production:

The fungal strain isolated as above was used to produce crude laccase using submerged condition. Sterile Olga liquid medium containing 3gm peptone, 10gm glucose, 0.6gm $KH2PO_4$, 0.001gm $ZnSO_4$, 0.4g K_2HPO_4, 0.0005gm $FeSO_4$, 0.05gm $MnSO_4$ and 0.5gm $MgSO_4$ per litre was prepared. Then 2 ml spore suspension (~8 ×106 spores/ml) as an inoculum was added to the medium and incubated in incubator shaker at 200 rpm and 30ºC. 3ml of broth was collected from the fourth day, Fungal mycelium was separated from the broth by filtering it through Whatman No. 1 filter paper. The filtrate collected was used for enzyme assay and all the experiment was carried out in duplicates.

Enzymatic activity assay:

Though laccase is having a wide range of substrates like catechol, lignin, etc, Guaiacol is reported as efficient substrate for laccase assay (Imran *et al*., 2012). Hence Guaiacol (2mM) in sodium acetate buffer (10mM pH 5.0) was used as substrate. The reaction mixture containing 3ml acetate buffer, 1ml guaiacol and 1ml enzyme source was incubated at 30ºC for 15 min. Enzyme blank was also maintained contained as 1ml of distilled water instead of enzyme source, 3 ml acetate buffer and 1 ml guaiacol. After 15 minutes the intense brown color was found to be developed due to oxidation of guaiacol by laccase, which was correlated with laccase activity (Desai *et al*., 2011). The absorbance was taken at 450 nm using UV spectrophotometer (ELICO, SL-218) and laccase activity was expressed as colorimetric units /ml /min (Chavan *et al*, 2013).

Effect of pH and temperature on laccase activity

The influence of pH on laccase activity was studied by measuring the activity at pH 3.0 to 9.0 in sodium acetate buffer, (100mM). The effect of temperature was determined by performing enzyme assays at temperature ranging from 20 to 80◦C (sodium acetate buffer, 100 mM, pH 5.0).

Dye degradation potential:

Five dyes namely Bromophenol Blue, Safranine, Phenol Red, Crystal Violet, Malachite Green (HiMedia, Mumbai) were chosen to test the enzyme's ability to degrade it. Initially Dye degradation potential was checked by plate assay. The culture were plated on the PDA media containing the 20 ppm concentration of each of above dyes.

To calculate dye degrading ability of enzyme, 2ml of the partially purified enzyme extract was added to the volume of 100 ml of the minimal medium containing dyes (20 ppm) and monitored for 3 h and was determined spectrophotometrically (ELICO, SL-218) by monitoring the absorbance at the characteristic wavelength of each dye. The decolorization efficiency (R%) was calculated as follows:

% Dye decolorization = [(Initial absorbance - final absorbance) / (initial absorbance)]x100.

RESULTS AND DISCUSSIONS

During Screening of all fungal strains for laccase synthesis, PDA plate with *Aspergillus flavus* culture showed brown colored precipitate with tannic acid and reddish zone with addition of gguaiacol(fig. 1, fig. 2, fig3) which confirmed us laccase production. This strain was used to produce crude laccase enzyme. After incubation on 4th day, crude enzyme was extracted from broth and its activity was assayed.

Fig. 1, Brown colored precipitation in tannic acid containing PDA

Fig. 2, Reddish color on Guaiacol containing PDA plate- Day 2

Fig. 3, Reddish color on Guaiacol containing PDA plate - Day 5

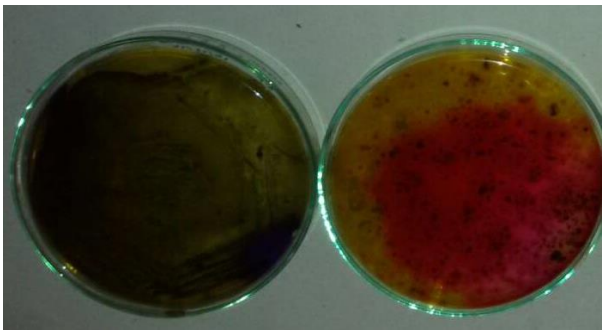

Fig. 4 Decolorization of 20ppm bromophenol blue and phenol red

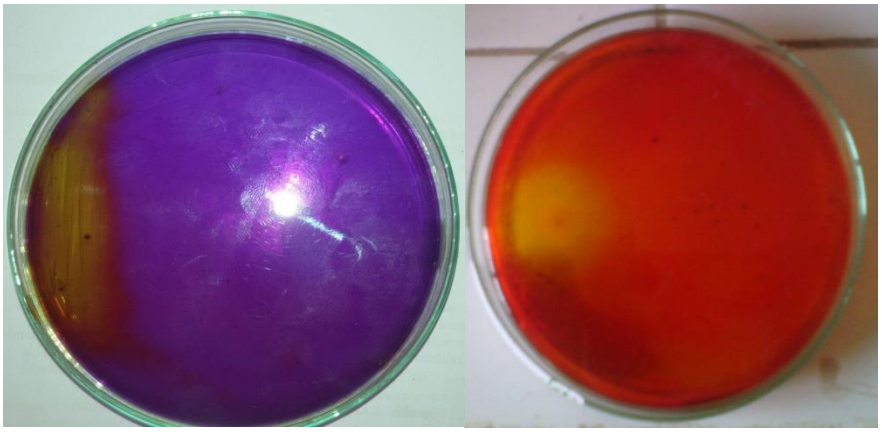

Fig. 5, Decolorization of 20ppm crystal violet and safranine

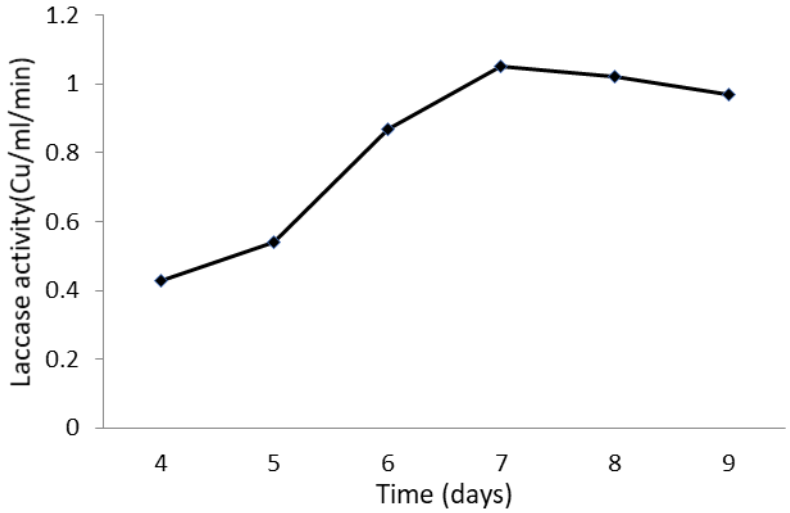

Fig 6. Kinetics of laccase activity estimated at different time interval.

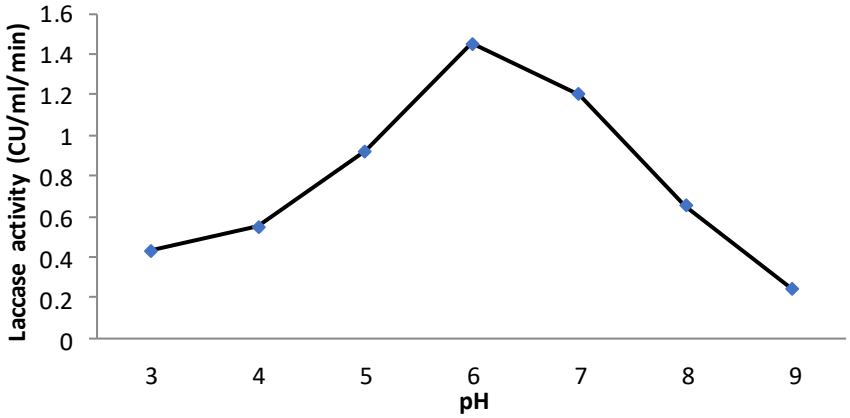

Fig 7. Kinetics of laccase activity estimated at different pH

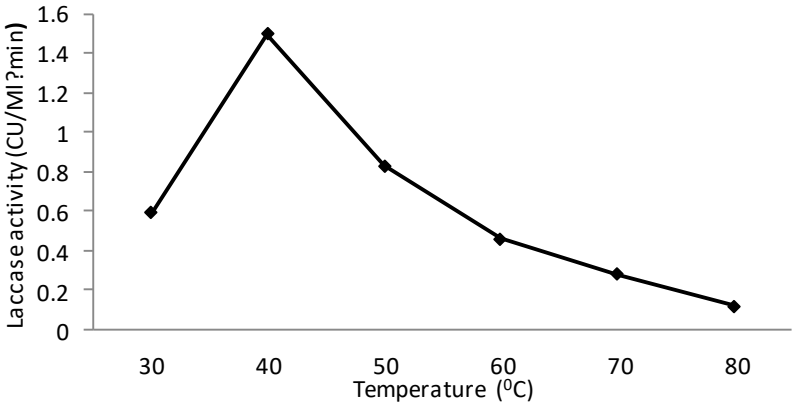

Fig 8. Kinetics of laccase activity estimated at different temperature

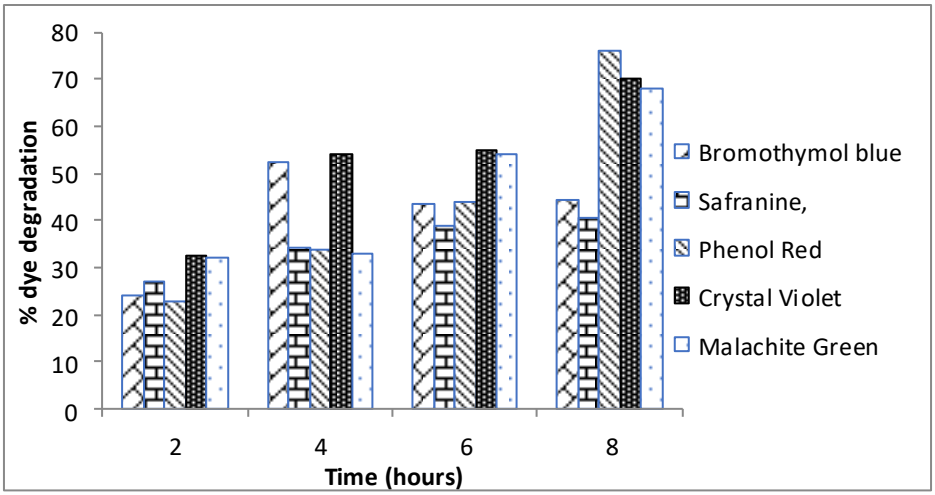

Fig.9: Decolorization of various dyes (20ppm) by crude enzyme

Enzyme Characterization:

Laccase activity was determined using a colorimetric assay by measuring the oxidation Guaiacol (2mM) in in sodium acetate buffer (100mM pH 5.0). The crude extract of the laccase produced by *Aspergillus flavus* showed the increasing activity upto 48 hours (1.05 CU/ml/min). After 48 hours lacase activity was found to be declined (fig.6). Maximum laccase activity was reported at pH at 5.0-7.0 and at 30^0-50^0C temperature. Tannic acid and Copper Sulphate induced the enzyme activity by oxidizing Guaiacol in lesser time than the inducer free enzyme.

Dye decolorization ability:

The fungus initiated decolorization of the 20 ppm dye concentration in the third day after inoculation on agar plates containg these dyes. Initially up to 48 hours mycellial growth on agar plates was observed, but after 48 hours fungal mycellias may start to synthesize laccase, due to which decolorization zone on plates were observed (fig.4 and fig.5). Quantitative measurement of all dyes decolorization. Decolorization of all five of the used dyes exceeded 50% within 6 h and was confirmed by the decrease in absorbance in the characteristic wavelength of every dye. Maximum 44.5 % decolorization of bromothymol blue by crude laccase was found within 8 h. Safranine, crystal violet and malachite green was decolorized up to 40.8%,70 % and 68 % respectively. The highest decolorization value of 76 % decolorization was obtained in case of Phenol red (fig.6). The most efficient lignolytic fungi are the basidiomycetes (Ahmed et al, 2015)which could be either white or brown-rot fungi. Almost all species of white-rot fungi were reported to produce laccase to varying degree (Hatakka 1994).

But after screening of above four fungal strains, *Aspergillus flavus* proved as an efficient laccase producer. Thus The present study proposes that the non white rot fungi isalso capable of producing Laccase enzyme which is, used in many industries for bioleaching and dye degrading industries. This enzyme works well at room temperature and can tolerate the bit high temperature and a stable pH range which shows its application in waste water or industrial effluent treatment. This will help reducing the environmental water pollution by safer way.

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