Characterization of Fungal Laccase and its Application in Dye Decolorization

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Abstract*: Laccases are blue copper oxidases (E.C. 1.10.3.2 benzenediol: oxygen oxidoreductase) that catalyze the one-electron oxidation of phenolics, aromatic amines, and other electron-rich substrates with the concomitant reduction of O_2 to H_2O. They are currently seen as highly interesting industrial enzymes because of their broad substrate specificity. Characterization of fungal laccase was done using different cultural conditions (incubation time, pH, and temperature) where optimal pH and temperature were 5-6 and 30°C respectively. The fungal isolates were proceeded for its application in dye decolorization using Congo red and RBBR showing decolorization rate of 79.5%, 69.7%, and 59.8%, by using fungal isolate SK3, SK9, and SK17. This study reveals the potential of fungal usage to overcome the persistence of dye pollutants problem, as it is considered an effective, economic and eco-friendly approach for RBBR dye decolorization. These strategies can be suggested to encourage ecologically sustainable development for bioremediation.*

Keywords*: Laccase, Dye decolourisation, Guaiacol, Oxidases*

1. Introduction

Fungi are widespread in nature and they are essential organisms for carbon cycling in nature when inhabit in the soil. In this habitat there are diverse groups, belonging mainly to the Dikarya subkingdom, with a strong dominance of ascomycetous fungi [1]. During last years a wide number of studies have focused on ligninolytic fungi because they have a versatile enzyme system (including laccases, peroxidases and oxidases producing H_2O_2) which participate in the degradation of lignin but also of other recalcitrant aromatic compounds causing environmental problems [2].

Among these groups of extracellular enzymes, laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) have received great attention because they possess huge applicability in different biotechnological and industrial applications [3–5], such as textile dye decolorization, pulp bleaching, detoxification and bioremediation of environmental pollutants and delignification or second generation ethanol production.

Laccases (EC 1.10.3.2; benzenediol: oxygen oxidoreductases) are multicopper enzymes belonging to the group of blue oxidases that catalyses oxidation of a wide variety of organic and inorganic compounds, including diphenols, polyphenols, diamines, and aromatic amines. One electron at a time is removed from the substrate, and molecular oxygen is used as the electron acceptor [6].The substrate loses a single electron and forms a free radical. The unstable radical undergoes

further nonenzymatic reactions including hydration, disproportionation, and polymerization [7].

The substrates of laccases may vary from diphenols and polyphenols to diamines, aromatic amines, benzenethiols, and substituted phenols [7]. They were first discovered by Yoshida in *Rhus vernicifera*, and later, laccases were widely found in nature. Laccases belong to the superfamily of multi-copper oxidases, and have been found in bacteria, fungi, plants and insects. Due to their highly diverse origin, the properties and functions of various laccases differ significantly, and execute different physiological functions in different species [8-12].

White rot fungi are filamentous fungi that can cause the decay of wood. Most of them are basidiomycetes which can degrade lignin, causing a white discoloration of the wood and playing an important role in the material circulation in ecosystems. White rot fungi can synthesize and secrete a variety of oxidases including laccase. [8-12].They have a strong ability to degrade various natural and synthetic chemicals with different structures, such as benzene, aniline, cyanide, polycyclic aromatic hydrocarbons and azo dyes [13-15]. Thus the practical uses of laccases in industry and biotechnology have attracted much research attention.

Laccases are generally glycoproteins containing four copper atoms, and the identified fungal laccases show a similar structure [16,17]. The active site contains four copper atoms of three different types, which play an important role in the catalytic reaction of laccase. One type-1 (T1) copper with a maximum absorption at 600 nm makes the laccase blue. T1 copper, associated with the oxidation of substrate, accepts electrons from the substrate. One type-2 (T2) and two type-3 (T3) copper atoms arrange in a cluster. The electron from T1 is transferred to T2/3 where molecular oxygen is reduced to generate water. During the reaction, O_2 in the air is consumed, and the only byproduct is water. Therefore, laccase is considered a 'green' enzyme. [18].

Potential application of laccases includes textile dye bleaching, pulp bleaching, effluent detoxification, biosensors, and bioremediations [1, 10]. However, a serious problem often encountered with industrial exploitation of fungal laccases is the low production level by the native hosts. This problem may be overcome by heterologous production in fungal hosts capable of producing high amounts of extracellular enzymes generally *Trichoderma reesei* or *Aspergillus* sp. [19-28].
The aim of the present paper is to screen and isolate laccase-producing fungi from decayed wood samples using plate assay method [29] and identifying a new source of extracellular laccase. The extracellular fungal laccase in the culture medium was subjected for characterization using culture conditions and its application for human welfare.

2. Material and Methods

2.1.Sample collection Decayed wood, bark and tree samples for isolation of fungal laccases producers were collected from various sites of Ahmedabad and Dahod, Gujarat. The samples were collected in sterile plastic bags swapped using alcohol and were sealed and brought to the lab aseptically for further processed inside laminar air flow.

2.2.Screening of Laccase producing Fungi The samples were homogenized and suspensions were made with dilution up to 10^{-5} in sterile distilled water. The prepared suspensions were used for inoculating onto the plates. Plate assay method was used for primary screening of fungal laccases using Potato Dextrose Agar medium consisting 4 mM tannic acid as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of dark brown coloured halo around the fungal colonies. The isolates showing positive results were used for further

studies. Another plate assay method was used for primary screening of fungal laccases using Potato Dextrose Agar medium consisting 3 mM ABTS (2-2'-Azino-bis-[3- ethyl benzthiazoline-6-sulfonic acid]) as substrate and incubated at room temperature for 3 days. The production of laccase enzyme was indicated by the formation of green coloured halo around the fungal colonies.

Bavendam test: Confirmation of laccase production was done by Plate assay technique using 10 mM guaiacol as a substrate. The colour change from colour less to reddish brown colour was to be observed in fungal culture plate for presence of laccase (Soponsathien, 1998).

2.3. Characterization of Laccase activity

Effect of pH on laccase activity The reactive mixture was 3 ml acetate buffer (10 mM) of different pH 3.6 to 5.6, 1 ml guaiacol (2 mM) and 1 ml culture medium of different age (6,10and 15 day). The contents were mixed well and incubated at 50◦ C for 5 min [8].The brown color formed was spectrophotometrically read at 460nm using Beckman DU-530spectrophotometer. 2.4.2.

Effect of temperature on laccase activity The reactive mixture was 3 ml acetate buffer (10 mM, pH 4.8), 1 ml guaiacol (2 mM) and 1 ml culture medium. The contents were mixed well and incubated at different temperature 30 to 90˚C for 5 min [2]. The brown color formed was spectrophotometrically read at 460nm using Beckman DU-530 spectrophotometer.

2.4. Dye decolorization studies

The Potato dextrose agar medium (100 ml) was prepared; 50 ppm dye was added to the medium and sterilized. Mycelial discs 6mm from 5-day-old fungal culture were transferred to the medium and incubated at room temperature (30±2°C). The culture was allowed to grow for 5 days.

A sterile control, without mycelial discs was maintained and duplicates of cultures were also maintained under same conditions. Culture filtrates were harvested at every 24 h interval and monitored for decolorization for 3 days at temperature (30±2°C) and pH 6. The culture filtrate was centrifuged at 5000 rpm for 10 minutes and the supernatant was taken for the measurement of decolorization in the spectrophotometer.

Phenol red and RBBR were used for dye decolorization assay, 100 mg/L dye were used for the experiment. Dye content was monitored photometrically at 540 nm and 665 nm for Phenol red and RBBR respectively using spectrophotometer, which is the maximum visible absorbance of these dyes. A control was kept un inoculated.

3. RESULTS AND DISCUSSION

3.1.Isolation and Screening of Laccase producing Fungi Primary screening was done using (ABTS 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid) and tannic acid, where secondary screening was done using Bavendam test with guaiacol as substrate.
Reduction zone observed for ABTS was green coloured, brown colour for Tannic acid and red colour for guaiacol.

Quantitative estimation of laccase was done using guaiacol as substrate at pH 6 and temperature 30°C for 6 fungal isolates which showed highest zone of reduction. (SK3.SK5, SK6, SK9,SK12,SK17)

Enzyme assay was carried out with 3 fungal isolates SM 5, SM 9 and SM17 which were showing prominent enzyme activity, (0.8, 0.3,0.1 U/ml) were used for further proceeding.

3.2. Characterization of Fungal Laccase The effect of incubation time, temperature and optimal pH was evaluated for laccase activity using fungal isolate SK5,SK9,SK17. As per result, it is observed that the maximum yield of pH, temperature and incubation time is 10 minutes, with pH 6 and temperature of 30 °C.

Table 1 Effect of pH on fungal laccase activity (U/ml)

	pH 5	***pH 6***	***pH 7***	***pH 8***
SK17	*0.01*	*0.05*	*0.05*	*0.01*
SK5	*0.02*	*0.06*	*0.04*	*0.02*
SK9	*0.03*	*0.08*	*0.05*	*0.01*

Table 2 Effect of temperature on fungal laccase activity(U/ml)

	20°C	**30°C**	**40°C**	**50°C**
SK17	0.02	0.06	0.05	0.02
SK5	0.01	0.04	0.03	0.015
SK9	0.03	0.06	0.04	0.014

Table 3 Effect of incubation on fungal laccase activity (U/ml)

	5 min	**10 min**	**15 min**	**20 min**
SK17	0.02	0.05	0.05	0.04
SK5	0.01	0.03	0.03	0.02
SK9	0.03	0.04	0.04	0.03

3.3. Dye decolorization studies

Decolorization efficiency was been measured after 72 h of incubation. Selected fungal isolates showed dye decolorization efficiency of 69.79% of RBBR and 62.59% of Congo red respectively in duration of 8 days.

4. Conclusion

Based on the screening results using various substrates of ABTS, guaiacol and tannic acid, laccase positive test were confirmed and production was carried out. Enzyme characterization showed that enzyme work at 30ºC as optimum temperature, pH 6 and incubation time of 10 minute. Laccase exists in decolorization mechanism could be concluded using dye decolorization mechanism results showed that laccase enzyme was responsible for 69.79% of RBBR and 62.59% of Congo red respectively in time interval of 72 h. The use of the laccase may conceivably be extended to other anthraquinone-type textile dyes, indeed suggesting a potential application field for the removal of dyes from industrial effluents.

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