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Research Article

An Approach on Screening, Production and Characterization of Laccase from *Fusarium*.

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ABSTRACT
The enzyme Laccase is known to degrade many phenolic aromatic compounds. This enzyme is found in many plant species and is widely distributed in fungi. Laccase play an important role in plant pathogenesis, lignolytic degradation and pigment production. The enzyme can also be used in decolorization and detoxification studies. The current work highlights on isolation of Fusarium species (J 01 to J 10) from different environmental soil samples. Screening of laccase producers and production were also carried out. Partial purification was carried out by salt precipitation (80%). The partially purified enzyme was used for characterization of laccase for effect of pH, temperature and substrate specificity of laccase activity.

KEYWORD
Laccase, dye degradation, partial purification, *Fusarium* and Characterization.

1. INTRODUCTION

Laccase (EC1.10.3.2) is the member of the polyphenol oxidases enzyme family and it is a multicopper-containing enzyme. Laccase reduces molecular oxygen to water and oxidize substrates (diphenols, methoxy-substituted monophenols, aromatic and aliphatic amines) into free radicals [1-3]. The reducing substrate loses an electron and usually forms an unstable free radical which may undergo further chemical changes by laccase-catalyzed oxidation or nonenzymatic reactions including, hydration, disproportionation and polymerization[4-5].

Laccase was first detected in the sap of the Japanese lacquer tree Rhus vernicifera by Yoshida in 1883. Presence of Laccase enzymes have been widely reported in fungi, bacteria, plants and insects. Among Ascomycetous, Deuteromycetes and Basidiomycetous fungi, white rot Basidiomycetous fungi have been widely studied for laccase production and characterization[6].

Laccases have received much attention from researchers in last decades due to their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes. Laccases find wide commercial applications within food industry, pulp and paper industries, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutants and removal of endocrine disruptors[7]. The present investigation was undertaken for isolation, production and characterization of Laccase from *Fusarium* species from various environmental samples.

2. MATERIALS AND METHODS

2.1. Collection of soil samples and Isolation of Fungal strains

Soil samples were collected from different places of Bangalore University Campus. The collected soil samples were used for isolation of fungi by serial dilution method on Czapekdox agar plate. Fifteen fungal strains were isolated. The isolated (plate -1) strains were tentatively identified in the laboratory as described by Rapper and Fennell[8] and were maintained on Czapekdox agar (CZA) slants. Further, the tentatively identified strains were labeled serially as J 01 to J 10.

2.2. Screening of Laccase Producers

The Laccase activity of fungal isolates was screened by using Czapekdox agar medium supplied with bromophenol blue (0.5 to 1.0 ml) (medium pH-6.8). Laccase producing colonies were selected on the basis of clear the color of the medium.

2.3. Biosynthesis of Laccase from Fusarium species

The production of Laccase under submerged fermentation mainly depends on various factors like initial pH, temperature, inoculum size etc. Hence, Czapekdox medium were used for fermentation process. The optimized pH for production is pH 6, optimized temperature is 30^0C and inoculum size (in ml) 1 ml is used for production of laccase.

2.4. Assay of Laccase

Guaiacol as has been reported efficient substrate for the laccase assay. The intense brown color development due to oxidation of guaiacol by laccase can be correlated to its activity often read at 450nm. Guaiacol (2Mm) in sodium acetate buffer (10Mm pH 5.0) was used as substrate. The

reaction mixture contained 3ml acetate buffer, 1ml guaiacol and 1ml enzyme source and enzyme blank contained 1ml of distilled water instead of enzyme source. The mixture was incubated at 30°C for 15 minutes and absorbance was read at 450nm blank using UV spectrophotometer.

2.5. International units (IU)

Enzyme activity was expressed as International Units (IU), where 1 IU defined as amount of enzyme required to oxidize 1 micromole of guaiacol per min. The laccase activity in U/ ml is calculated using the extinction coefficient of guaiacol(12,100 M-1 cm -1) at 450nm.

2.6. Extraction, Purification and characterization of Laccase

The purification was carried out at 4^0C on the crude extract according to the modified method of China etal. (2001). Purification measures adopted by us include:

2.7. Ammonium sulphate fractionation

The crude protein supernatant was precipitated with ammonium sulphate (till 75% saturation) at 4^0C with constant stirring. The proteins were retrieved by centrifuging at 10,000 rpm for 10 min at 4^0C. The pellets of proteins were dissolved with minimum volume of 50 mM Glycine-NaOH buffer (pH 9.0).

2.8. Determination of Optimum pH of Laccase

The pH influences on Laccase activity was studied using different buffers of different pH values ranging from 4 to 9. The Citrate (pH 4.0) acetate (pH 5 & 6.0) and Phosphate (pH 6.0), Tris-Cl buffer (pH 8.0) and Glycine NaOH buffer (pH 9.0) were used. The assay was done with these enzymes and activity was found as mentioned above.

2.9. Determination of Optimum Temperature of Laccase

The reaction rate of partially purified Laccase was measured by various temperatures by following above said assay procedure. The reaction mixtures were incubated at different temperature, 25°C to 45°C and continued with the assay process of the enzyme, as mentioned before.

2.10. Substrate specificity

The partially purified Laccase were tested for substrate specificity by using Starch, Casein and Guaiacol were used in 1% level.

3. RESULTS AND DISCUSSION

3.1. Collection of soil samples and isolation of fungi

Collection different soil samples from different regions from Bangalore such as Bangalore university campus, Near Garden soil, near Sewage Chanel and Soil Near coconut tree etc. the collected soil samples were used for isolation of fungi by serial dilution method on Czapekdox agar plate. Ten fungal strains were isolated. The isolated (plate - 1) strains were tentatively identified in the laboratory as described by Rapper and Fennell [8] and were maintained on CzapekDox's agar (CZA) slants. Further, the tentatively identified strains were labeled serially as J 1 to J 10.

3.2. Screening and Production of Laccase

The CZ broths were supplemented with bromophenol blue and inoculated with *Fusarium species* and then incubated 48-72 hrs. The control flasks were also used visual disappearance of color from the media of the flask, when compared to their respective controls and observed the disappearance of the color from the flask. Total six isolates were showed positive results those are *Fusarium* species J1,J3,J5,J6,J8 and J9.out of these were *Fusarium* J9 were showed better yield in quantitatively.

Further studies were carried out for fermentation work through submerged state with optimized pH 6, temperature 30 ^{0}C and 1 ml of inoculum size (1ml contains 1X10^7 spores/ml) the maximum enzyme production was found 8.5 IU at 96 hour of fermentation period. Laccase producing fungus, *Pleurotus ostreatus* IMI 395545 was isolated from Kolli hills, Namakkal Dt., Tamil Nadu, India. This fungal strain was further used in decolorization of dyes like Poly R-478 and RBBR[9].

Adiveppa B.V and B.B Kaliwal[10] were also reported on production of laccase from *Pleurotus* sp. and *Marasmius* sp were showed for the biosynthesis of laccase through submerged fermentation and our results are coincides with Adiveppa B.V and B.B Kaliwal[10].

3.3. Partial purification and characterization of Laccase

The crude enzyme was precipitated with ammonium sulfate solution (till 80% saturation) at 4^{0}C with constant stirring. The centrifuged pellets of precipitated proteins were dissolved in 50 mM glycine-NaOH buffer (pH 9.0) and used for characterization of Laccase enzyme.

3.4. Effect of pH on Laccase activity

The results of characterizing Laccase activity of optimum pH were shown in Table-1. It declares that Laccase was active over broad range of pH (4.0 to 11.0). The highest Laccase activity was found to be at pH 9.0. So the enzyme is considered as alkaline Laccase by using *Fusarium* sp J9.

The optimum pH for the enzyme was identified as 3.0, which was consistent with the optimum pH for the laccase isozyme from *C. subvermispora* and enzyme from C. *hirsutus*[11-12]. Other studies have also reported very low optimal pH (between 3.0 and 5.7) for fungal laccases, except for the laccase from *Rhizoctonia praticola*, which exhibited a neutral optimal pH with various substrates[13] and also our results were coincides with Bollag, J. M. and A. Leonowicz[13].

Table 1. Effect of pH on Laccase activity.

pH	Laccase Enzyme activity (IU)
4	0.018
5	0.023
6	0.015
7	0.061
8	0.030
9	0.028
10	0.311

3.5. Effect of Temperature on Laccase activity

The results of determination of Laccase activity of optimum temperature were shown in Table-2. It declares that Laccase was increasingly active from 25^0C. The highest Laccase activity was recorded at 25^0C. This is one of the important characteristics for Laccase for *Fusarium* species J 9.

The optimal temperatures of laccases from different sources of fungi vary widely[14], ranging from 20^0C to 80^0C. Strains of the same species from different regions may also show significant differences in their biological characteristics due to their adaptation to the environment. The laccase produced from Trametes sp. MA-X01 was a thermophilic enzyme, the laccase activity increased as the reaction temperature increased, up to a maximum activity at 60^0C. After that, the activity decreased rapidly[15].

Table 2. Effect of optimum temperature on Laccase activity.

Temperature ^{o}C	**Laccase Enzyme activity (IU)**
25	0.311
30	0.246
35	0.251
40	0.223
45	0.14

3.6. Substrate Specificity of Laccase

The substrate specificity of the enzyme is presented in Table 3. The results revealed that the enzyme was 100%, active towards Guaiacol, starch and casein respectively. The data indicated that the enzyme extracted from *Fusarium* J 9 is very much specific to its natural substrate Guaiacol. The substrate specificity of *Fusarium* J 9for Laccase was investigated.

Ben Younes et al., [16] were reported on substrate specificity of Guaiacol by using white rot fungus *Perenniporia tephropora* and the substrates are used at a concentration of 5 mmol l^{-1}.

Table 3. Substrate specificity on Laccase activity.

Substrate	**Laccase Enzyme Activity (IU)**
Guaiacol	0.128

Starch	0.083
Casein	0.099

The enzyme Laccase is known to degrade many phenolic aromatic compounds. This enzyme is found in many plant species and is widely distributed in fungi. Laccase play an important role in plant pathogenesis, lignolytic degradation and pigment production. The enzyme can also be used in decolorization and detoxification studies. The current work highlights on isolation of Fusarium species (J 01 to J 10) from different environmental soil samples. Screening of laccase producers and production were also carried out. Partial purification was carried out by salt precipitation (80%). The partially purified enzyme was used for characterization of laccase for effect of pH, temperature and substrate specificity of laccase activity.

4. CONCLUSION

The laccase enzyme producing microbes to isolate, screen, produce and characterization is utmost important. This enzyme is used in dye degradation studies. Hence the isolated *Fusarium sp*. Showing all the potentiality to do the degradation of dye and we conclude this organism is utmost important.

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