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AN APPROACH ON MICROBIAL PRODUCTION OF LACCASE FROM *FUSARIUM SP*

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ABSTRACT

Laccases are most stable and powerful biocatalysts with broad applications. It is a copper containing, organic solvents resistance, oxidoreductase enzyme, having potential ability to oxidize the phenolic and non-phenolic compounds. Laccases play an important role in food industry, paper and pulp industry, textile industry, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental phenolic pollutant. Fifteen *Fusarium sp* were isolated from different soil samples from different locations in Bangalore. Isolated fungal strains were screened for Laccase production by plate assay by different substrate using ABTS (2-2'-Azino-bis-[3-ethyl benzthiazoline-6-sulfonic acid]), guaiacol, and tannic acid as a substrates and the culture plates were observed for color change in the media. *Fusarium sp* P 15 were selected as best laccase producer. The *Fusarium sp* P 15 were employed for further production of Laccase studies. The different pH (6), temperature (35^0C) and inoculum size (1.0ml) were optimized and it showed 1.05 IU of Laccase activity.

KEYWORD: Laccase, Submerged fermentation, ABTS, guaiacol, and tannic acid and *Fusarium sp*.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a part of broad group of enzymes called polyphenol oxidases containing copper atoms in the catalytic center and are usually called multicopper oxidases. Laccases contain three types of copper atoms, one of which is responsible for their characteristic blue color. Laccases are thus oxidases that oxidize polyphenols, methoxy-substituted phenols, aromatic diamines, and a range of other compounds (P Baldrin., 2006).

The use of enzymes for the treatment or the removal of environmental and industrial pollutants has attracted increasing attention because of their high efficiency, high selectivity, and environmentally benign reactions.

Laccase are widely distributed in nature ranging from prokaryotes to lower eukaryotes and fungi to plants. In their natural surroundings. Laccases are common enzymes in nature and are found widely in plants and fungi as well as in some bacteria and insects (Minussi, et al., 2007).

The catalysis of laccase occurs with reduction of one molecule of oxygen to water accompanied with one electron oxidation of a wide range of aromatic compounds which includes polyphenols,[10] methoxy-substituted monophenols, and aromatic amines.[11] This oxidation results in generation of oxygen-centered free radical that can be converted to quinone in a second enzyme catalyzed reaction.

The present study highlights isolation of fungal strains for dye degradation and screening of laccase producers by plate assay. *Fusarium Sp* P 15 were emerged as potential strain and employed for the production of laccase by optimizing fermentation parameters.

MATERIALS AND METHODS

1. Collection of Samples

Soil sample was selected for the isolation of Laccase producing fungi. Four different soil samples were collected from different regions from in and around Bangalore city. The soils were selected for isolating Laccase producing fungi and Samples were stored at 4^0C.

2. Media Preparation

The fungi were isolated from the soil sample on Czapek Dox agar (CZA) medium. CZA medium composition is as follows. Glucose, 30g; NaNO3, 2g; K2HPO4, 1g; MgSO4, 0.5g; KCl, 0.5g; FeSO4, 0.010g; Agar, 15g and pH 6.5+_0.2 (1L Distilled water). The media was

sterilized by autoclaving at 121^0C of temperature, 15lbs of pressure for 15 minutes, and then CZA plates were prepared for the inoculation of the soil sample.

3. Screening of Laccase Producers By Plate Assay

The organisms were grown and kept on slants of solid modified Czapek-Dox's medium containing (g/L of distilled water) glucouse-2g; Potassium Hydrogen Phosphate (KH_2PO_4)-1.52g; Potassium Chloride (KCl)-0.52; Magnesium sulphate ($MgSO_4.7H_2O$)-0.52g; Copper nitrate ($CuNO_3.3H_2O$)-trace; Zinc sulphate ($ZnSO_4.7H_2O$)-trace; Iron sulphate ($FeSO_4.7H_2O$)-trace and agar-20g. Modified Czapek Dox's medium was supplemented with different substrate using ABTS (2-2'-Azino-bis-[3-ethyl benzthiazoline-6-sulfonic acid]), guaiacol, and tannic acid as a substrates.

The fungus was inoculated on different Czapek Dox's agar plates containing 3 mM of ABTS (3-4). 4 mM of guaiacol (5) and 4 mM of tannic acid as individually and incubated at room temperature for 7 days. The culture plates were observed for color change in the media (Plate-2 A,B & C).

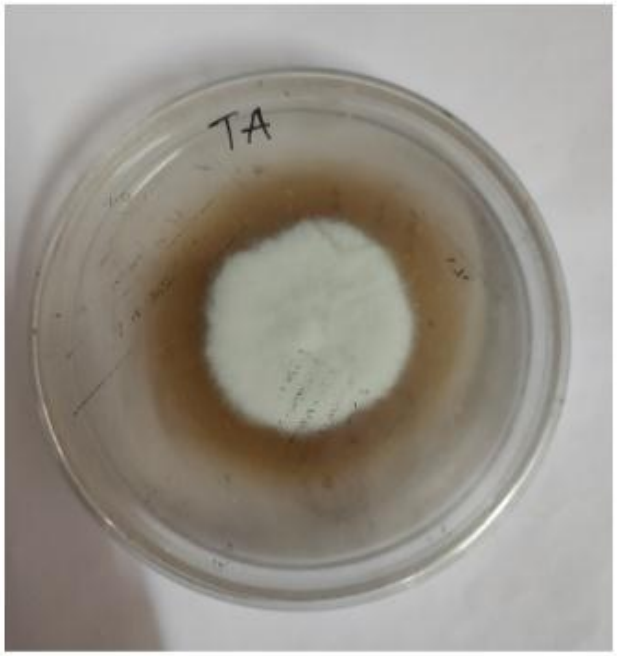

Plate 2 A: Plate assay-Tannic acid used as a substrate.

Plate 2 B: Plate assay-Bromophenol blue used as a substrate.

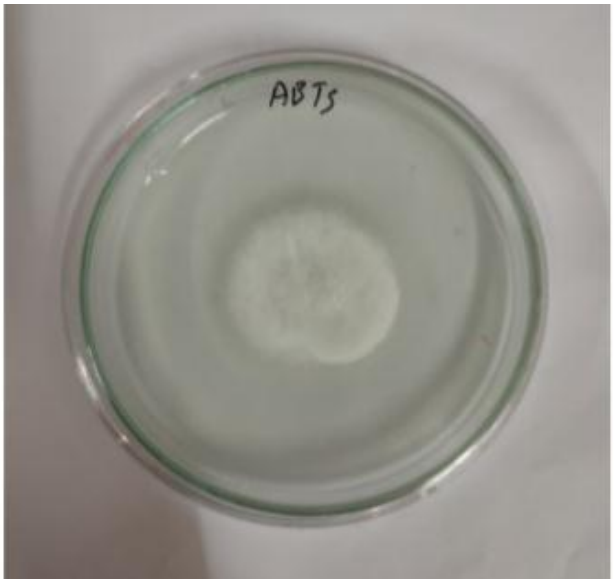

Plate 2 C: Plate assay-ABTS used as a substrate.

Fermentation Medium Composition

The selected *Fusarium* sp P 15 were cultured on production medium. The production medium consists (1L of distilled water) 30.0g of Sucrose; 2.0g of Sodium nitrate-; 1.0g of K_2HPO_4, 0.5g of $MgSO_4$. $7H_2O$; 0.5g of KCl and 0.01g of $FeSO_4$ with pH 6.8 for 96 -120h.The selected *Fusarium* sp P 15 were cultured on production medium. The production is carried out in Czapek Dox broth for better yield and pH 6.0, temperature 35^0C and 1.0 ml inoculum were used.

Quantitative Assay of Laccase Activity

Kalra et al. (2013) were reported for laccase assay Guaiaicol 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 guaiaicol 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.

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 guiaicol (12,100 M-1 cm -1) at 450nm.

Optimization of Fermentation Kinetics for Biosynthesis of Laccase

Effect of Initial Ph on Laccase

250 mL Erlenmeyer flasks contained 100 mL of fermentation media were prepared and initial pH of the media were adjusted. The adjusted initial pH of fermentation media were ranging from 3-7 with increments of 1.0. Consequently prepared flasks were cotton plugged and sterilized by autoclave at 15 lbs, 121^0C for 15 min. The flasks were aseptically inoculated with freshly prepared spore suspension and incubated.

Effect of Temperature on Laccase

100mL of the fermentation media were collected separately in 250 ml Erlenmeyer flasks and prepared for submerged fermentation. Thus prepared flasks were incubated at different temperatures like 25^0C, 30^0C, 35^0C and 40^0C.

Effect of Inoculum Size on Laccase

The inoculum was prepared by 168h freshly prepared culture of *Fusarium* sp P 15 at different levels i.e., 0.25, 0.50, 0.75, 1.0 and 1.25 mL and then inoculated and fermentation studies were carried out. As it was mentioned before, the media was extracted during every condition of pH or incubation temperature or inoculum size to prepare crude enzyme for each and was used to assay the Laccase activity.

RESULTS AND DISCUSSION

Soil is excellent source of filamentous fungi exhibiting several physiological functions. Environmentally stress soil where limitation of nutrients provides stress-tolerant fungal strains. Fifteen (15) isolates isolated from soil samples collected at varying environmental stress conditions.

Out of fifteen fungal strains were screened for laccase production. The fungal strain *Fusarium sp* P 15 was selected as potential strain for laccase producer from plate assay and used for further studies (Plate 2 A,B &C). Srinivasan *et al*.(2005), were also reported screening Laccase producers by plate assay. Our results are good agreement with Srinivasan *et al*. (2005).

The results obtained in the present study on the effect of initial pH in submerged fermentation of Laccase production by *Fusarium* P 15 is represented in Table-1. It reveals that the Laccase production with the increasing of pH of the medium from pH 3.0, up to pH 6.0 and then further increase in initial pH caused the declining of Laccase yield. These increasing peaks were observed up to 120 hours of fermentation period and thereafter the decreased yield as fermentation period increased. The maximum Laccase activity 0.34 IU was obtained at pH 6.0 for 120 hours of fermentation period. The least Laccase activity was obtained at pH 3.0 with *Fusarium* P 15 strain and it showed 0.17 IU at 120 hours of fermentation period

Amanpreet *et al.,* (2017) have reported pH 7 were optimum for the maximum production of Laccase under SmF process respectively. The optimum pHof laccase production, as reported in many fungi, falls between 5.0 and 6.0 [6,7 &8]. Maximum titres of laccase and biomass were observed in the medium adjusted to pH 6.0 by *Fomes sclerodermeus*, white-rot basidiomycetes. Our results were close agreements with Amanpreet *et al.,* (2017).

The results obtained in the present study on the effect of temperature in submerged fermentation of Laccase production by *Fusarium* sp P 15 is represented in Table 2. It reveals that the Laccase production was increased along with the increase of temperature of the medium from 25^0C, up to temperature 35^0C with optimized constant pH of 6.0. These increasing peaks were observed up to 120 hours of fermentation period and thereafter the decreased yield as temperature levels and fermentation period increased. The maximum Laccase activity 0.44 IU was obtained at temperature 35^0C for 120 hours of fermentation period. The least Laccase activity was obtained at temperature 25^0C with *Fusarium* sp P 15 strain and it showed 0.29 IU at 120 hours of fermentation period.

Amanpreet *et al.,* (2017) have reported temperature were optimum for the maximum production of Laccase under SmF process respectively. It has been found that the optimal temperature for fruiting body formation and laccase production is 25^0C in the presence of light but 30^0C for laccase production when the cultures are incubated in the dark (Thurston,1994). Our results were close agreements with Amanpreet *et al.,* (2017).

The importance of inoculum size on microbial fermentation process is widely accepted. The results obtained in the present study on the effect of inoculum size in submerged fermentation of Laccase production by *Fusarium* sp P 15 is represented in Table-3. Out of five inoculum size tested (0.25, 0.50, 0.75, 1.0 1.25 and 1.5 mL), 1.25 mL inoculum was found to be the most suitable for high production of Laccase by *Fusarium* sp P 15 in submerged fermentation at 120 hours of fermentation and it showed 1.05 IU and lowest Laccase activity 0.22 IU were found at 0.25 ml inoculum size. At 120 hours of fermentation period.

Amanpreet *et al.,* 2017 (5) have reported varied inoculum size (1 to 5 mycelial disc) were used for the maximum production of Laccase under submerged fermentation process respectively. A lower level of inoculum may not be sufficient to initiate the growth, whereas a higher level may cause competitive inhibition (pate et al., (2009)). Our results are good agreement with Amanpreet *et al.,* (2017).

Table 1: Effect of different pH on Laccase production.

pH	Laccase Enzyme activity (IU)
3	0.17
4	0.22
5	0.30
6	0.34
7	0.26
8	0.11

Table 2: Effect of temperature on Laccase production.

Temperature in ^{0}C	Laccase Enzyme activity (IU)
25	0.29
30	0.38
35	0.44
40	0.28
45	0.22

Table 3: Effect of Inoculum size on Laccase production.

Inoculum Size (in ml)	Laccase Enzyme activity (IU)
0.25	0.22
0.50	0.39
0.75	0.63
1.00	0.75
1.25	1.05
1.5	0.78

CONCLUSSION

Dye degrading laccase enzyme producing fungi were isolated and potential strain were screened by plate assay by using different substrates. Optimization of fermentation kinetics were employed for the production by using *Fusarium sp* P 15 and it is utmost important in dye degradation.

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