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Isolation, Screening and Optimization for Laccase production by *Fusarium* spp. under submerged fermentation

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ABSTRACT

Laccases are oxidases with broad substrate specificity and ability to oxidize various phenolic and non-phenolic compounds. This study was carried out to isolate and characterizes laccase producing fungi from the environmental samples. Soil samples were collected from different locations. Suspensions of the samples (1g in 10 ml sterile physiological saline) were serially diluted, inoculated onto Czapek Dox Agar (CZA) containing antibiotic chloramphenicol and incubated for 7 days at 30 °C. The fungal isolates were characterized macroscopically and microscopically. Screening was done using Czapek-Dox agar was supplemented with 0.1% tannic acid, 0.5% Bromophenol blue, 0.05 ml in 25 ml of Methanol Guaiacol the pure culture of the single colonies from the spread plate method were inoculated on the media and incubated at room temperature for 5-6 days. After 5-6 days the plates were checked for clear transparent zone.

Key words: *Laccase, Fungi, Soil, Media optimization, Submerged fermentation.*

Introduction

Enzyme laccase is discovered in the year 1883, laccase is one of the first most enzyme ever discovered. It has 140 years of history of the research record (Sidhu *et al.,* 2017). Laccase is also called as extracellular enzymes. These extracellular enzymes have achieved great effect in industrial area so that laccase also has wide application in almost all sector (Sivkumar *et al.,* 2010; Viswanath *et al*., 2014). Laccase is an enzyme which can be produced from different species of white rot of fungi. Laccase enzyme are copper containing 1,4-benzenediol oxygen oxidoreductases (EC1.10.3.2). It belongs to a group of benzenediol. Laccase enzyme is also called as one of the enzymatic components required to degrades the major components into lower molecular weights compounds that they can be easily assimilated for fungi nutrition of lignocellulosic biomass (Risdianto *et al.,* 2010; Janusz *et al.,* 2020).

Laccase is a type of copper containing polyphenol oxidases that was discovered in the exudates of the Japanese lacques tree *Rhus verniczfera* and it cross links the monomers, also degrades the polymer and help in ring cleavage of the aromatic compounds (Sivkumar *et al.,* 2010). Fungal groups are considered

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as the major native place of laccase more than 60 fungal strains namely Ascomycetes, Deuteromycetes and Basidiomycetes. They are belonging to the white rot of fungi. These are the huge sources of laccase that we can easily isolated from simple isolation. These fungi are widely distributed in all the soil (Tiwari and Chittora, 2013; Mate and Alcalde, 2017; Hemaraju and Narasegowda, 2018). Laccases produced by the fungi are more advantageous over other laccase sources due to stability, broad substrate specificity and ability to oxidize various phenolic compounds (Risdianto *et al.*, 2010; Vantamuri *et al.*, 2015; 25; Monseef *et al.*, 2016). This study was carried out to screen and identify laccase producing fungi from soil and later laccase production and optimization of the parameters to improve its production under laboratory conditions.

Materials and Methods

Collection of soil samples

Garden Soil samples were collected in Bangalore city, in sterile plastic bag and brought to the laboratory for further processing.

Isolation of the organisms by spread plate method

100 µl of 10^{-4} dilution of soil sample was added on to the petriplate containing Czapek-Doxagar medium and spread all over the plate evenly by the use of sterile glass rod. The petriplate was incubated at room temperature for 5-6 days. Single colonies from the spread plate were selected and grown on fresh Czapek-Dox agar (Patil *et al.*, 2009; Desai *et al.*, 2011).

Screening for laccase producing fungi

Screening was done using Czapek-Dox agar was supplemented with 0.1% tannic acid, 0.5% Bromophenol blue, 0.05 ml in 25 ml of Methanol Guaiacol the pure culture of the single colonies from the spread plate method were inoculated on the media and incubated at room temperature for 5-6 days. After 5-6 days the plates were checked for clear transparent zone (Shraddha *et al.*, 2011; Pundir *et al.*, 2016).

Identification of fungi

The fungal strain was stained with lactophenol cotton blue for studying the morphology of fungi. Here a small amount of mycelium was taken on clean grease free slide, to that a drop of lactophenol cotton blue stain was added and teased gently and clean coverslip was placed on the mount by touching on one side of mountant and lower gently by avoiding air bubbles. The slide was examined under microscope (Hi Media laboratories, 2016; Thakkar *et al.*, 2020).

Quantitative assay of laccase activity

Karla *et al.* (2013) was reported as enzyme assay. Guaiacol is notified as an effective substrate for the assay of laccase. The development of brown colour because of oxidation of Guaiacol by an enzyme and can be correlated to its activity and absorbance can be checked at 450 nm (Venkamuri *et al.*, 2015).

The reaction mixture contained 3 ml acetatebuffer, 0.5 ml enzyme, 0.5 ml guaiacol and blank contained 0.5 ml of distilled water is replaced with enzyme. The reaction mixture was incubated at 30 ^{0}C for 15 minutes and absorbance was checked.

Optimization of fermentation parameter for biosynthesis of laccase

Optimization of pH for laccase

50 ml of test tube contained 25 ml of fermentation media were prepared and initial pH of the media were adjusted. The adjusted initial pH of fermentation media was ranging from 3-10 with difference of 1.0 using 0.1N HCL and 0.1N NaOH, consequently test tube were plugged with sterilized cotton and sterilized by autoclave at 15lbs, 121 ^{0}C for 15 minutes. The test tube were aseptically inoculated with freshly prepared spore suspension and incubated. For every 24-hour enzyme assay is performed till 6th day (Venkatamuri *et al.*, 2015; Chenthamarakshan *et al.*, 2017).

Optimization of temperature for laccase

50 ml of test tube contained 25 ml of fermentation media were prepared for four different temperature the selected pH was adjusted to all test tube and prepared test tubes were cotton plugged and sterilized by autoclave at 15 lbs, 121 ^{o}C for 15 minutes. The test tube was aseptically inoculated with freshly prepared spore suspension and incubated at different temperatures like 25 ^{o}C, 30^{o}C, 35^{o}C and 40^{o}C. For every 24-hour enzyme assay is performed till 5th day(Venkatamuri *et al.*, 2015).

Optimization of inoculums size for laccase

The fungal spore suspension was prepared from a 6

days oldculture grown on Czepek-Dox agar slants. 10 ml distilled water of 20% tween -80 solution was added and scraped with loop to suspend spores. 50 ml of test tube contained 25ml of fermentation media were prepared for five different test tube. The selected pH and temperature are maintained and the prepared inoculum at different levels like 0.25 ml, 0.50 ml, 0.75 ml, 1.0 ml, 1.25 ml and then inoculated and for every 24 hours the enzyme assay was checked (Venkatamuri *et al.*, 2015).

Results and Discussion

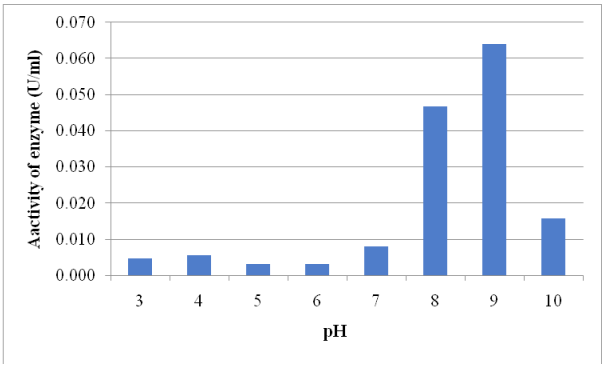
Soil is excellent source of filamentous fungi exhibiting several physiological functions. Environmentally stress soil where limitation of nutrients provides stress-tolerant fungal strains. Fifteen (Jaber *et al.*, 2017). Isolates isolated from soil samples collected at varying environmental stress conditions. In Bangalore the required samples of soil with different soil texture are collected, Karnataka for the isolation of fungi able to produce Laccase. Out of three soil samples 15 fungal strains were obtained each fungal strain was cultured on freshly prepared czapek dox agar medium.

All the 15 fungal strains isolated were screened to check laccase activity which can produce by culturing the fungi in czapekdox agar media supplemented with 0.1% Tannic acid0.5%Bromophenol blue, 0.05 ml in 25 ml of Methanol Guaiacol. The strains showing the clear transparent zone were selected. Out of 15 fungal strains 4 fungal strains were positive for laccase activity. Only with Tannic acid as substrate show a clear zone and positive compare to other substrate so that fungal strain which showed clearer zone was selected for further work (Sonali *et al.*, 2017). The selected fungal strain was stained using lactophenol cotton blue to study the morphology of fungi, by staining the positive fungal strain was found to be a species of *Fusarium* (Anthony, 1973; Srinivasan *et al.*, 1995).

Optimization of culture conditions for the laccase production

Influence of pH on laccase production

The effect of pH for production of enzyme was studied by varying pH of the culture medium from 3 to 10. Optimum pH for the effective production of enzyme was found to be at pH 9 with the enzyme activity 0.144 U/ml. Hence the whole fermentation process was maintained at pH 9 for the better results. Results are as represented in Table 2 and Graph 1.



Graph 1. Optimization of pH

Influence of temperature on laccase production

By maintaining pH of the culture medium was 9, optimum temperature was studied by incubating the culture medium at different temperature conditions and finally the optimum temperature supportive for the growth of organism and production of enzyme was found out. By the present study optimum temperature suitable for the growth of organism and production of Laccase enzyme at high rate

Table 2. Optimization of pH

pH	Activity of enzyme U/ml					
	24h	48h	72h	96h	120h	144h
3	0.017	0.016	0.022	0.014	0.005	0.022
4	0.013	0.014	0.018	0.016	0.006	0.140
5	0.044	0.018	0.015	0.015	0.003	0.142
6	0.031	0.015	0.020	0.012	0.003	0.142
7	0.012	0.016	0.017	0.012	0.008	0.138
8	0.013	0.015	0.016	0.041	0.047	0.121
9	0.013	0.013	0.030	0.050	0.064	0.144
10	0.095	0.158	0.010	0.016	0.016	0.038

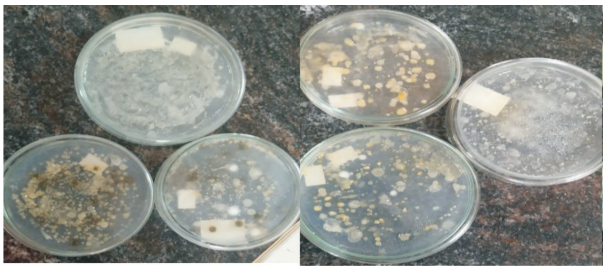


Fig. 2. The isolation plates containing fungal colonies after incubation.

Fig. 3. Highest zone formed in the Tannic acid plate – 9mm zone size

Fig. 4. Microscopic view of laccase producing fungi

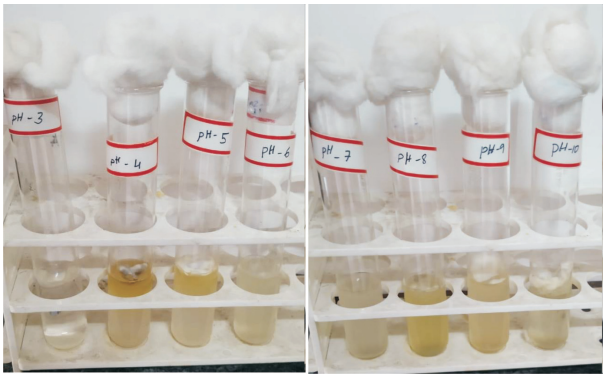


Fig. 5. Optimization of pH

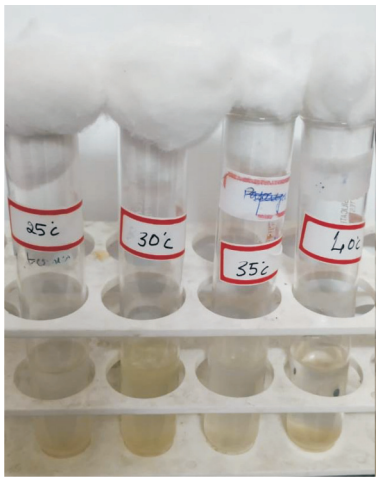


Fig. 6. Optimization of temperature

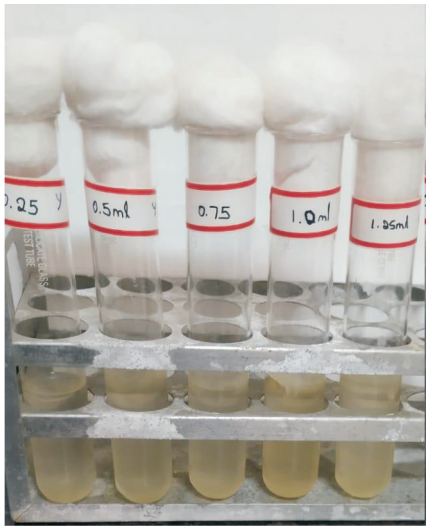


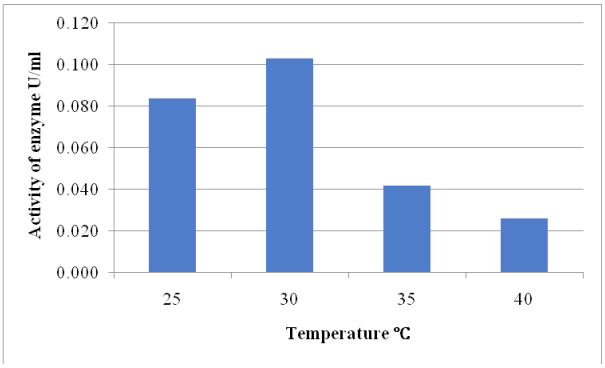
Fig. 7. Optimization of inoculum

using *Fusarium sps* was found to be 30 °C with the enzyme activity 0.103U/ml, obtained results are represented in Table 3 and Graph 2. Results obtained are similar to the results obtained by Risdianto *et al.*, (2010) who got maximum laccase production by under solid state fermentation in between 25R C and 31 °C by *Marasmium* sp. Nor-

Table 3. Optimization of Temperature

Temperature °C	Activity of enzyme U/ml				
	24h	48h	72h	96h	120h
25	0.010	0.009	0.020	0.024	0.084
30	0.009	0.010	0.025	0.036	0.103
35	0.007	0.009	0.027	0.039	0.042
40	0.009	0.007	0.017	0.028	0.026

mally, the fermentation processes (SSF as well as SmF) develop with mesophilic microbial strains (Risdianto *et al.,* 2010; Dhakar and Pandey, 2013). As *Scytalidium lignicola* also belongs to mesophilic organisms, the growth temperature is in the range of 20-40 °C.



Graph 2. Optimization of Temperature

Influence of inoculum size on laccase production

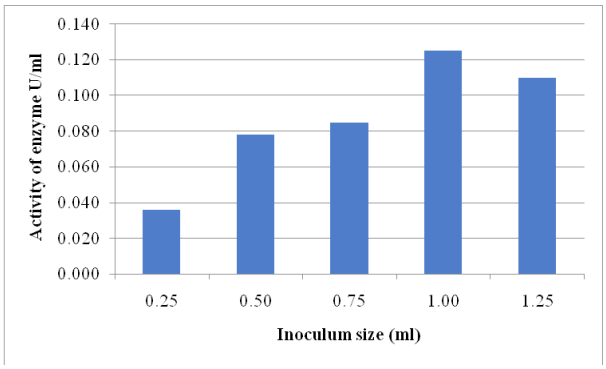
Inoculum size plays a significant role in any fermentation. A lower level of inoculum may not be sufficient to initiate the growth, whereas a higher level may cause competitive inhibition (Patel *et al.,* 2009). Therefore, determining exact inoculum size is critical step (Patel *et al.,* 2009). By maintaining the previously studied optimum culture conditions pH and temperature the optimum inoculum size required for the fermentation process was studied. By the study inoculum size optimum for the production of laccase was found to be 1.0ml showing enzyme activity of 0.125U/ml, (Table 4. Graph 3) which supports the active growth of the fungi to produce the enzyme.

Production of laccase by submerged fermentation process

For the production of enzyme 1000 ml Erlemeyer flask contained with 400ml of fermentation media were prepared the selected pH is adjusted for the media flask were cotton pluged and sterilized by autoclave at 15lbs, 121 °C for 15 minutes. The flask is aseptically inoculated with freshly prepared spore suspension of selected inoculums size of 16 ml and incubated at selected temperature for 5 days. After 5days the media is taken out and filtration of the fermentation taken place aseptically later the sample was carried out for centrifugation process with rpm of 5000 and with time lap of 20 minutes. The pellet was discarded and the supernatant (crude enzyme) was collected for the purification process (Karp *et al.,* 2012; Renge *et al.,* 2012; Dhull *et al.,* 2020).

Table 4. Optimization of Inoculumn size

Inoculumn size (ml)	Activity of enzyme U/ml				
	24h	48h	72h	96h	120h
0.25	0.023	0.051	0.056	0.036	0.042
0.50	0.026	0.064	0.070	0.078	0.057
0.75	0.030	0.063	0.084	0.085	0.075
1.00	0.028	0.115	0.124	0.125	0.099
1.25	0.027	0.081	0.089	0.110	0.104



Graph 3. Optimization of Inoculum size

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