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Isolation of *Colletotrichum gloeosporioides* gr., a novel endophytic laccase producing fungus from the leaves of a medicinal plant, *Piper betle*

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Abstract

Laccases (E. C. 1.10.3.2, p-diphenol: dioxygen oxidoreductase) are a group of multi-copper containing enzymes that catalyze one-electron oxidation of phenolic compounds with concomitant reduction of oxygen to water. An endophytic fungus *Colletotrichum gloeosporioides* was isolated from *Piper betle*, a medicinal plant and screened for laccase enzyme production.

The amount of enzyme produced by the endophytic fungus was estimated by performing enzyme assay and calculating enzyme activity by using guaiacol as substrate . Effect of different medium compositions (Rose Bengal ,Potato dextrose Broth ,Czapeck Dox, Sabrourd's Media, Basal liquid Media) on the enzyme production was also checked. Further the effect of different parameters like Temperature, pH, Substrate concentration, effect of Magnesium sulphate, Copper sulphate and EDTA on the activity of the crude enzyme was studied. The optimum pH and temperature determined for the enzyme is 6 and 50^{o}C respectively.

Keywords: Characterization, *Colletotrichum gloeosporioides*, , Dye removal, Guaiacol, Laccase ,*Piper betle*, Potato Dextrose

1 INTRODUCTION

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a copper-containing enzyme which is widely distributed within plants, insects and fungi. Laccase catalyzes the removal of electrons from a variety of organic substrates, including phenols, methoxyphenols, aromatic amines, while reducing molecular oxygen to water as a final electron acceptor.[1] In recent years, interest in laccases has been fueled by their potential uses in detoxification of environmental pollutants, prevention of wine decolorization, paper processing, enzymatic conversion of chemical intermediates and production of useful chemicals from lignin [2] Discovery of noval laccases with different substrate specificities and improved stabilities is important

for industrial applications. [3] Endophytic fungi form the promising source for the production of novel products with biological activity .[4]Although the enzymes vary from isolate to another, all the endophytic fungi tested all synthesize in vitro, the enzymes necessary for penetrating and colonizing their plant hosts. However, little information has been reported about laccase production for degrading lignin by endophytic fungi.[5]

2 MATERIALS AND METHODOLOGY

2.1 Isolation of endophytic fungi

The fungus used in this study is one of the twenty endophytic fungi isolated from the leaves of medicinal plants in Nashik city, India. Total 14 endophytic fungi were isolated from (9) medicinal plants. The samples were rinsed gently in running tap water to remove dust and debris.

After proper washing, stem sample were cut into long 0.5-1 cm pieces. Whereas leaves were cut into 3-4 mm x 0.5-1 cm pieces without midrib under aseptic conditions. Surface sterilization was done by 0.1% mercuric chloride for 5 min.

Each set of plant material was treated with tween 20 for 2min. Followed by immersion in 70% ethanol for 30 sec. Later the segments were rinsed three times with sterile distilled water. 100ul of the last and second last wash of distilled water were spread on PDA plates as a sterility control. The explants were blotted on sterile blotting paper. In each petridish, 5-6 segments were placed on potato dextrose agar (PDA). The dishes were sealed with parafilm and incubated at 22°C ± 2°C for 4-5 days in dark room till visible growth of organisms was observed. All the isolated organisms were subcultured and maintained as pure culture on PDA slants at 4°C.[6],[7]

2.2 Screening of isolates for laccase production

Screening was done by two methods by (qualitative) agar plate method and by (quantitative) liquid method

2.2.1 Qualitative method

The fungal isolates were screened for Laccase production by growing them on plates of PDA medium containing 0.04% guaiacol. [8] Petri dishes (15 cm in diameter) each containing 20 ml of medium were used. For inoculum 8mm discs of freshly grown fungal culture were used. A disc was placed on the surface of guaiacol supplemented agar plates. The inoculated plates were incubated at 25 ºC in dark for 7 days. The production of intense brown color under and around the fungal colony was considered as a positive reaction resulting from guaiacol oxidation.

2.2.2 Quantitative method

Potato dextrose Broth was used as liquid fermentation media for quantitative estimation of enzyme activities from the selected strains.[9] Two Disc of peripheral growing

fungal isolates were inoculated asceptically in the broth. The tubes were then incubated at room temperature for 3 days. Filter sterilized guaiacol (0.04%) was added in each tube. The tubes were then incubated further at 25 ºC for 7 days. At the end of each growth period, inoculated tubes were collected and centrifugated at 5000 rpm for 5 minutes at 4ºC. [10]The filtrate was tested for laccase enzymes activity as follows and enzyme activity was expressed in U/ml.

2.3 Enzyme Assay

The laccase activity was measured by monitoring the oxidation of 10mM guaiacol buffered with 100mM sodium acetate buffer (pH 5.4) at 470nm for 5 min. The reaction mixture (5ml) contained 1ml of culture filtrate, 3ml buffer and 1ml 10mM Guaiacol. Laccase acivity was calculated as shown in Equation 1. The enzyme activity was expressed in enzyme unit (U) per ml (U ml-1). [11]

Enzyme activity(U/ml)

$$= \frac{\Delta A_{470}\ \text{nm/min} \times 4 \times Vt \times \text{dilution factor}}{€ \times Vs}$$

Where,

Vt=final volume of reaction mixture (ml)=5

Vs=sample volume (ml)=1

€=extinction coefficient of guaiacol=6,740/M/cm

4=derived from unit definition and principle

2.4 Characterization of Maximum producer

Maximum producer was further characterized on the basis of morphological characters from Agharkar Research Institute,Pune, India.It was identified as *Colletotrichum gloeosporioides* gr.

2.5 Screening of different liquid media for laccase production

Different Media like Rose Bengal g/l(peptone ,glucose 10, KH_2PO4 1,$MgSO_4$ 0.5,Rose Bengal 0.05 pH 7.2±0.2) ,Potato dextrose Broth g/l (Dextrose 20, potato infusion 6.5 pH 5.6±0.2),Czapeck Dox g/l (sucrose 4.5,$NaNO_3$ 0.45,K_2HPO_4 0.15,$MgSO_4$ 0.075, KCl 0.075,$FeSO_4$ 0.0015,pH 6.8 ±0.2) , Sabrourd's Media g/l (Peptone10, Glucose 40,pH 5.6), Basal liquid Media g/l(D-Glucose 20, $(NH_4)_2SO_4$ 2, KH_2PO_4 2, $MgSO_4.7H_2O$ 0.5, KCl 0.05, $CaCl_2.2H_2O$ 0.1, $CuSO_4.5H_2O$ 0.01, Malt Extract 4 ,pH 7-7.3) were screened for laccase production.

2.6 Characterization of Enzyme

The effect of temperature on laccase activity was determined by recording the absorbance of

enzyme catalyzed reaction using guaiacol (10mM) as substrate, dissolved in sodium acetate buffer (100 mM pH 5.0), incubated at temperature 4°C, 18°C, 30°C, 37°C, 50°C,70°C,100°C.[12] The reaction mixture was incubated

for 15min. Temperature at which enzyme showed maximum activity, was noted as optimum temperature of enzyme. The influence of pH on laccase activity was studied by recording the absorbance of enzyme catalyzed reaction at optimum temperature, using guaiacol (10mM) as substrate dissolved in buffers of different pH (acetate buffer pH 4, pH 5, phosphate buffer pH 6, pH 7 and tris-glycine buffer pH 8) and incubated at 30ºC for 15min and absorbance were recorded at 470nm. Based on the previous reports effect of compounds like $CuSO_4$ (5mM,10mM,15mM,20mM,25mM,30mM,40mM,50mM), ethanol (1%,2%,3%,4%) and $MgSO_4$(8mM,16mM,24mM,32mM,40mM,48mM,54mM) and EDTA[17] (10mM,20mM,30mM,40mM,50mM,60mM)on enzyme activity was also investigated.

2.7 Application of crude enzyme for dye removal

Crude Enzyme was used for removal of various dye like 1mM Malachite green, 10mM Bromophenol blue, 4.9 mM Commasie blue R-250,10mM Bismark Brown dye. For this 1ml of crude enzyme extract was added to 4ml of 100mM sodium acetate buffer with above dye concentration. Then reaction mixture was incubated at room temperature for 24 hrs .Then absorbance was taken at 613 nm,590nm,546 nm,456nm for Malachite green Bromophenol blue, Commasie blue R-250, Bismark Brown dye respectively after incubation with enzyme and before incubation with enzyme.[13],[14]

Percent removal of dye was calculated as,

$$\% \text{ removal} = \frac{\text{Initial absorbance- final absorbance}\times 100}{\text{Final absorbance}}$$

3 RESULT AND DISCUSSION

3.1 Isolation and Screening of Fungal culture for laccase production

Fourteen different endophytic fungal isolates were subcultured and screened for laccase activity on PD agar with 0.04 % guaiacol. Laccase enzyme react with guaiacol to give reddish brown color product, out of the above 14 isolates 5 isolates were found to be positive. These five fungal isolates were screened by liquid assay. The isolate 14th showed maximum laccase activity which was later characterized as *Colletotricum gleosporiodes* gr

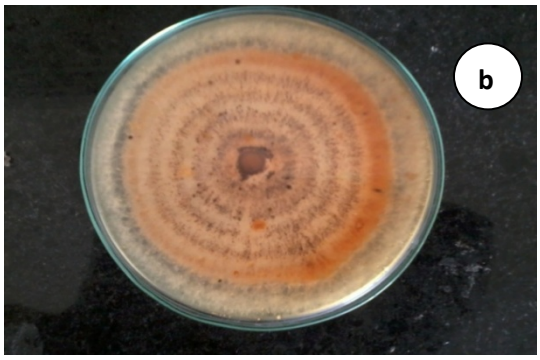

Fig.1. Laccase producing organism "*Colletotrichum gloeosporioides* gr."

a:top view of the plate , b: inverted view of the plate

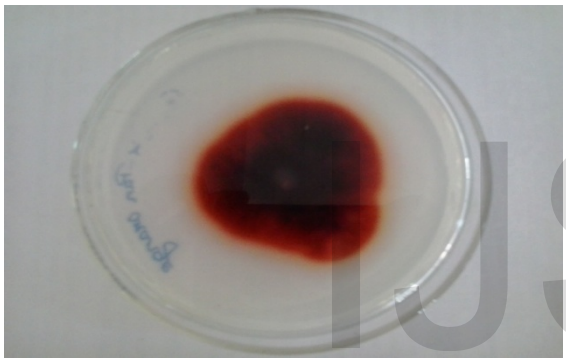

Fig.2. Organism showing positive laccase activity

3.2 Media for laccase production

Different media (Rose Bengal, Potato Dextrose Broth, Czapeck Dox,Sabrourd's Media, Basal liquid Media) were screened for laccase production. Maximum activity was found in Czapeck Dox i.e 38U/ml while Sabrourd's medium showed zero enzymatic activity as it does not showed fungal growth (table 1)

3.3 Characterization of enzyme

3.3.1 Effect of temperature on laccase activity

The effect of temperature values ranging from 4 ºC to 70 ºC was investigated on laccase activity. The optimum temperature for laccase was determined to be 50º C. Enzyme activity declined when the temperature was increased upto 70ºC. According to literature the typical optimum temperature range for laccases is 50ºC to 60ºC. Laccase enzyme extracted from *Trametes versicolor* exhibited high enzyme activity at temperature of 50ºC . Laccase purified from *Stereum ostrea* found to be active and stable at temperature 40ºC. [12]

3.3.2 Effect of pH on laccase activity

The influence of pH on laccase activity was determined at pH values ranging from 4.0 to 8.0. Laccase exhibited the highest activity at pH 6.0. Enzyme activity decreased piercingly as the pH value increased and laccase was almost completely inactivated at pH 8.0. Other investigators found *P. nebrodensis* laccase requires a pH of 5 for it to manifest maximal activity.[15]

3.3.3 Effect of CuSO4, MgSO4 and EDTA on laccase activity

Lower concentration of $CuSo_4$ and $MgSO_4$ induced Laccase activity as compared to control i.e.10.8U/ml. Concentration above 20mM showed sharp decrease in the activity. Laccase activity was almost negligible at concentration above

40mM. It proves that both compounds act as inducers at lower concentration. In case of EDTA enzyme activity incessantly decreased with increase in concentration as compared to the control mentioned above. EDTA act as Cu chelating agent which inhibits Laccase activity.[16]

Sr no	Media	Enzyme activity U/ml
1	Rose Bengal	2.9
2	Potato Dextrose Broth	29
3	Czapeck Dox	38
4	Sabrourd's Media	0
5	Basal liquid Media	5

Table 1

Screening of different liquid media for laccase production

3.3.4 Application of crude enzyme for dye removal

In order to study the dye removal ability of the *Colletotrichum gloeosporioides* gr. laccase. A dye removal experiment was performed with four different dyes. For Four dyes 1mM Malachite green ,10mM Bromophenol blue, 5 mM Commasie blue R-250 ,10mM Bismark Brown dye % removal was 100%,34.5%,75%.66%,95.31% respectively. Two dyes Malachite green, Bismark Brown showed maximum removal. (table 2) Other investigators found that, Malachite Green was decolourized 75% by C*yathus bulleri* laccase,[15]. Laccases from *T. versicolor* and *A. oryzae* which represented 30.3% and 13.3% decolorization of commassie brilliant blue, respectively. [17]

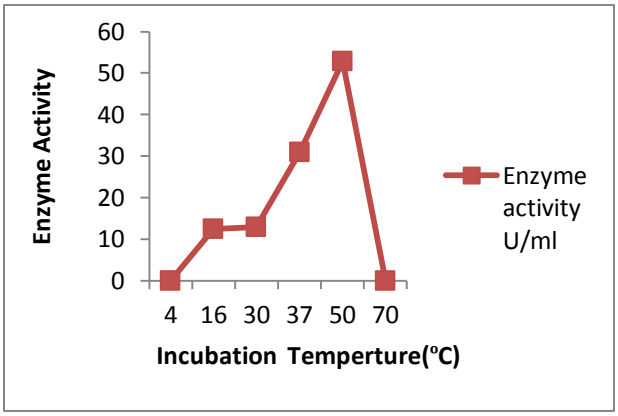

Fig.3.Graph displaying effect of temperature on enzyme activity

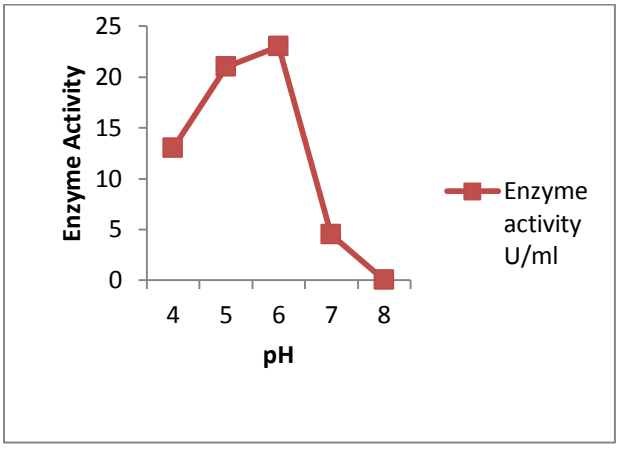

Fig.4.Graph displaying effect of pH on enzyme activity

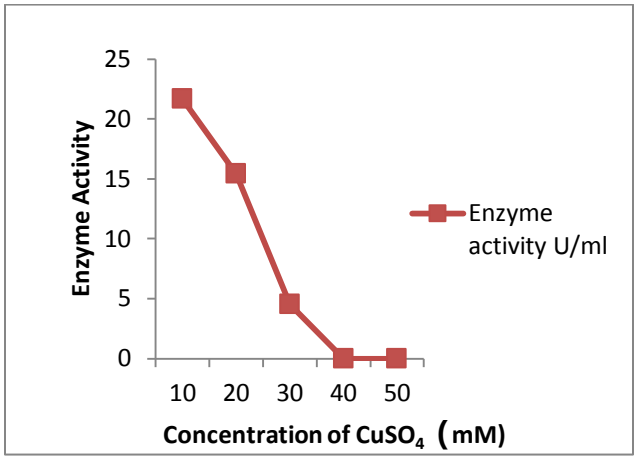

Fig.5.Graph displaying effect of CuSo4 on enzyme activity

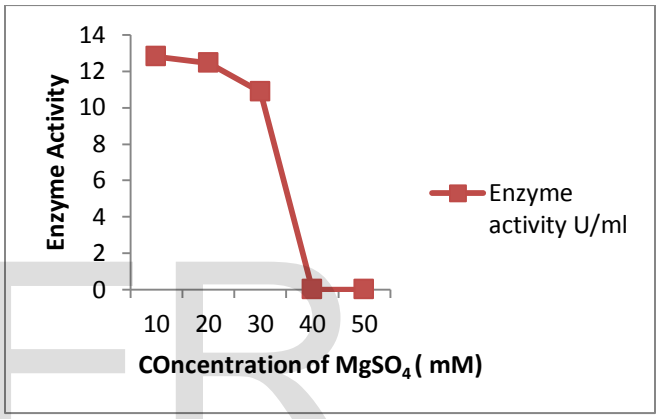

Fig.6.Graph displaying effect of MgSo4 on enzyme activity

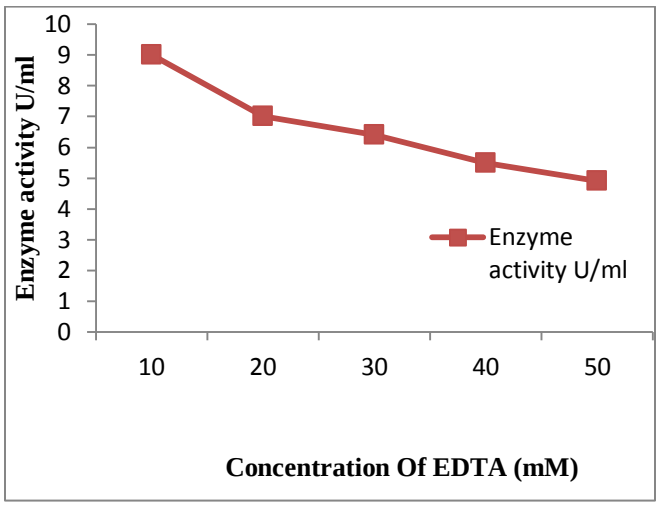

Fig.7.Graph displaying effect of EDTA on enzyme activity

Sr.no	Dye	% Removal
1	Malachite green	100
2	Bromophenol Blue	34.35
3	Commaise Blue R250	75.66
4	Bismark Brown	95.31

Table 2

% Dye Removal by Crude Enzyme

4 CONCLUSION

Present isolate *Colletotrichum gloeosporioide gr.* from the leaves of a medicinal plant, *Piper betle* is found to be a novel endophytic laccase-producing fungus. Enzyme characterization experiments confirmed that optimum pH for laccase is pH 6 and optimum temperature is 50^{o}C. Enzyme activity is induced by the presence of $CuSo_4$ and $MgSO_4$ at lower concentration upto 20mM. Crude enzyme can be successfully used for removal of Malachite green, Bismark Brown dyes. Future research is focused on immobilization and application of laccase in biodegradation of recalcitrant environmental pollutants.

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REFERENCES

1. Zhongyang Ding,Lin Peng, Youzhi Chen ang Zhang, ZhenghuaGu, Guiyang Shi and Kechang Zhang, "Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation," *African Journal of Microbiology Research*, vol.6 ,no.6, pp.1147-1157, 2012
2. Rosana C.Minussi, Marcio A.Miranda, jose A.Silva,Carmen V. Ferreira, Hiroshi Aoyama, Sergio Marangoni, Domenico Rotilio, Glaucia M.Pastore and Nelson Duran, "Purification, characterization and application of laccase from Trametes versicolor for colour and phenolic removal of olive mill wastewater in presence of 1-hydroxybenzotriazole,"*African Journal of Biotechnology*, vol. 6 no.10,pp. 1248-124,2007
3. L.-L. Kiiskinen, M.Ratto and K.Kruus, "Screening for noval laccase-producing microbes," *Journal of Applied Microbiology*, vol.97, pp.640-646, 2004
4. N. Bhagya, Sana Sheik, M. Samhitha Sharma, K. R. Chandrashekar, "Isolation of Endophytic *Colletotrichum gloeosporioides* Penz. from *Salacia chinensis* and its Antifungal Sensitivity" *Journal of Phytology* , vol 3,no 6 ,pp.20-22,2011
5. El-Zayat S.A., "Preliminary studies on laccase production by *Chaetomium globosum* an Endophytic fungus in *Glinus lotoitks," American- Eurasian J. Agruc.& Environ. Sci.*, vol3 ,no.1,pp.86-90 ,2008
6. R. Bharathidasan and A. Panneerselvam, "Isolation and identification of endophytic fungi from *Avicennia marina* in Ramanathapuram District, Karankadu, Tamilnadu, India," European Journal of Experimental Biology,vol 1 ,no.3,pp.31-36,2011
7. T.S. Suryanarayanan, G. Senthilarasu and V. Muruganandam, "Endophytic fungi from Cuscuta

reflexa and its host plants," Fungal Diversity,pp.117-123

8. K.Kalra , R.Chauhan,M.Shavej , S.Sachdeva S, "Isolation of lacase producing Trichoderma spp. And Effectof pH and Temperature on its activity,"*International Journal of ChemTech Research*, vol.5 ,no 5,pp.2229-2235, July/Sept.2013

9. R. Periasamy, "Isolation, Screening And Identification Of Laccase Producing Fungi From Kolli Hills," Shodganga ,2013

10. Monnat Theerachat, Sandrine Morel, David Guieysse, Magali Remaud-Simeon and Warawut Chulalaksananukul, "Comparison of synthetic dye decolorization by whole cells and a laccase enriched extract from *Trametes versicolor* DSM11269", *African Journal of Biotechnology*, Vol. 11 no.8, pp. 1964-1969, 26 January, 2012

11. A.Jhadav , K.K.Vamsi ,Y. Khairnar, A.Boraste , N.Gupta , S.Trivedi , P.Patil ,G. Gupta, M.Gupta ,A.K. Mujapara , B.Joshi ,D. Mishra , "Optimization of production and partial purification of laccase by *Phanerochaete chrysosporium* using submerged fermentation," *International Journal of Microbiology Research*, Vol.1,no2, pp.9-12,2009

12. S.S. Desai, B.G. Tennali, N. Channur, A.C. Anup, G. Deshpande, B.P. Murtuza, "Isolation of laccase producing fungi and partial characterization of laccase," *Biotechnol.Bioinf.Bioeng.*, vol.1 ,no.4,pp.543-549,2011

13. V.V.Kumar, S.D. Kirupha, P. Periyaraman and S.Subramanian, "Screening and induction of laccase activity in fungal species and its application in dye decolorization, " *African Journal of Microbiology Research* ,vol.5, no.11, pp.1261-1267, 2011

14. S.S.Mishra , V.S.Bisaria , "Decolorization and detoxification of textile dyes and black liquor by laccase of *Cyathus bulleri*," *Journal of scientific and industrial research*,vol.66,pp684-688,2007

15. Guo-Ting Tian, Guo-Qing Zhang, He-Xiang Wang and Tzi Bun Ng, "Purification and characterization of a novel laccase from the mushroom *Pleurotus nebrodensis ABP* ,"vol.59 ,no.3,pp.407-412,2012-PH

16. Benny Chefetz, Yona Chen , and Yitzhak Hadar, "Purification and Characterization of Laccase from Chaetomium thermophilium and Its Role in Humification," *Appl Environ Microbiol*. Vol.64 ,no.9,pp.3175–3179,1998-EDTA

17. Hamid Forootanfar, Atefeh Moezzi, Marzieh Aghaie-Khozani, Yasaman Mahmoudjanlou, Alieh Ameri, Farhad Niknejad, and Mohammad Ali Faramarzi, "Synthetic dye decolorization by three sources of

fungal laccase," *Iranian J Environ Health Sci Eng*.

Vol.9,no.1, 2012

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