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SCREENING AND ASSESSMENT OF LACCASE PRODUCING *TRICHODERMA* SPECIES ISOLATED FROM DIFFERENT ENVIRONMENTAL SAMPLES

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ABSTRACT

The cultivation conditions and high fungal biomass do not support essentially for high laccase yields. In the present study, it was attempted to select *Trichoderma* species for the production of laccase in submerged fermentation. For this purpose, 88 different fungi were isolated, out of which 29 isolates belong to 7 species of *Trichoderma*. Laccases were usually produced in low concentrations by laccase producing fungi, but high concentrations were obtained with the addition of different supplements to media and optimized cultural conditions.29 isolates belonging to 7 species of *Trichoderma* were screened as laccase producers, based on decolorization of guaiacol. *Trichodermaharzianum*, isolated from industrial wastewater was found to be best potential laccase producing fungus while *Trichodermaviridae* showed the least growth. The rest of the *Trichoderma* species showed variable behaviour for laccase activity.

Key words: *Trichoderma*spp, Laccase, Guaiacol, Environment, Pakistan.

INTRODUCTION

Laccases are multicopper enzymes belonging to the blue oxidases group of enzymes (Figure-1) which widely exist in nature and are defined as nomenclature wise oxidoreductases type according to the Enzyme Commission (EC) which oxidize diphenols and allied substances (Kiiskinen *et al.*, 2004). The higher plants and fungi predominantly contains laccases (Mayer and Staples, 2002).

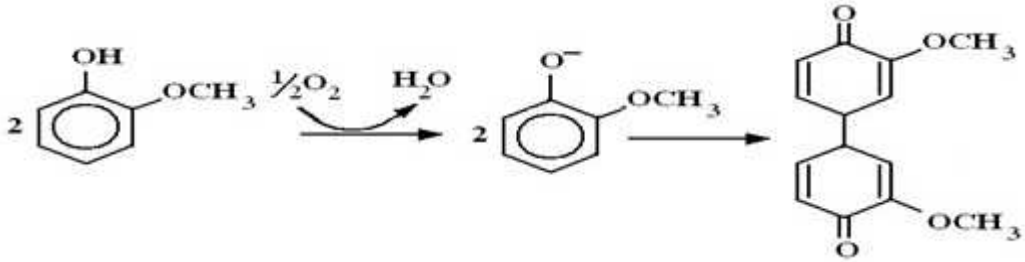

Fig.1. The laccase-catalysed oxidation of guaiacol to its corresponding quinone (taken & modified from Sanchez-Amat and Solano, 1997).

Fungal Laccases have been implicated in degradation of lignin and protection from toxic phenolic monomers of polyphenols. Laccases are helpful for number of industrial applications such as prevention of wine discoloration, paper processing and oxidation of dye, detoxification of environmental pollutants and chemicals production from lignin. Laccases are also useful for the decomposition of azo dyes by oxidative methods (Michael *et al.*, 2005). Due to large scale application of laccase enzyme, the present study was designed to search for highly efficient laccase producing fungi and to reduce the cost of production. Hence, the present work reports the production of laccase by a local isolates of *Trichoderma* spp by submerged fermentation.

MATERIALS AND METHODS

Collection of Samples: Samples for isolation of *Trichoderma* species were collected from different environmental sites of Lahore i.e. agricultural soil, sludge of industrial waste and effluent water of industry. Soil and sludge of industrial waste was collected in clean sterilized plastic bags. The paper and pulp industrial effluent was collected in clean sterilized autoclaved bottles.

Isolation and identification of *Trichoderma*species:The isolation of *Trichoderma* was done on media plates containing malt extract and agar having pH 6. The media was sterilized by autoclaving at 121±1°C for 15 minutes.

It was cooled down and antibacterial was added to avoid bacterial contamination. It was poured in sterilized petriplates in laminar air flow chamber and left for 24 hours at 25±1°C.Serial dilution method was used to isolate *Trichoderma* species from soil. In this method, 1 g of soil was added in 10 ml of sterilized distilled water, it was then allowed to settle for a few minutes and then 1 ml of supernatant was added to another 10 ml of sterilized distilled water. In this way it was diluted 5 times. A 0.2 ml of all dilutions was poured into sterilized solidified petriplates containing malt extract agar and spread over with sterilized glass spreader. These plates were left for one hour and then inverted and incubated at 25±1°C. Some species of *Trichoderma* were isolated from paper and pulp industrial effluent in the similar method. Isolated species were re-cultured. When pure cultures were obtained these were transferred to slants containing malt extract agar with pH 6.Identification of *Trichoderma* species was done on the basis of morphological characterization using 'compendium of soil fungi' and 'The Genus *Trichoderma* in Pakistan'.

Screening of Laccase Producing *Trichoderma* species: Qualitative Analysis: The screening of laccase producing *Trichoderma* species was done on plates composed of media as described by Coll *et al*. (1993). The composition of media used for screening *Trichoderma* species by plate method was(g/l): 3.0 peptone, 10.0 glucose, 1.0 KH_2PO_4, 0.001 $ZnSO_4$, 0.4 K_2HPO_4, 0.0005 $FeSO_4$, 0.05 $MnSO_4$, 0.5 $MgSO_4$, 20.0 agar (pH-6) supplemented with 0.02% guaiacoland then sterilized by autoclaving at 121±1°C and 15 lb/in^2 for 15 minutes. Then it was cooled. Before pouring media into the plates, chloramphenicol was added to avoid any bacterial contamination. Different *Trichoderma* species were inoculated into these plates and the plates were incubated at 25±1°C for 7 days.

Quantitative Analysis: The species of *Trichoderma* that shows positive results for laccase activity during plate assay method by the formation of reddish brown zones in the medium in the presence of guaiacol were selected for quantitative analysis of laccase in liquid culture.For inoculum development, distilled water was sterilized by autoclaving at 121±1°C for 15 minutes. That water was then added in a slant containing pure culture. Fungal mass was scratched gently using a glass rod and spore suspension was poured in sterilized screw capped test tube. That procedure was repeated. All was done under sterilized conditions to avoid any contamination. The number of spores per ml was measured using heamatocytometer. Then 100 ml of media was prepared in each flask of 250 ml capacity by adding all the ingredients as described above except agar. Cotton plug and a piece of aluminum foil were used to cover the mouth of flasks completely. Then these flasks were sterilized by autoclaving at 121±1°C for 15 minutes. As the flasks were cooled, antibacterial was added, and then 1 ml of spore suspension was added in each of the flask. All these steps were performed in laminar air flow chamber under sterilized conditions to avoid any contamination. The flasks were covered properly and placed on a shaker at 30±1°C and 120 rpm for 10 days.Sampling was done after every 24 h under sterilized conditions. A 2 ml of liquid culture was taken. The culture filtrates used in the enzyme determination were obtained by filtering the fermentation medium through a filter paper (Whatman No.1) and subsequently centrifuged at 5000 rpm 5 minutes. Supernatant was transferred to another clean eppendroff for subsequent studies.

Enzyme activity: One unit was defined as the amount of the laccase that oxidized 1 mM of substrate per min. it was calculated as follow,

Unit definition (1U) = 0.001 Abs/min
= 1/0.001 x Sample Absorbance/min

RESULTS AND DISCUSSION

More than 60 fungal strains, belonging to various classes such as Ascomycetes, Basidiomycetes and Deuteromycetes, have been demonstrated to produce laccase (Gianfreda *et al*., 1999). The majority of laccase characterized so far have been derived from efficient lignin degraders such as white-rot fungi (Eggert *et al*., 1996). *Trichoderma* species also active participates in delignification and biodegradation of lignocellulosic compounds in nature. Nevertheless, only a few publications are concernedwith laccase producing *Trichoderma*species (Holker *et al*., 2002). In the present study, 7 species of *Trichoderma* were assessed as potential laccase producers in which,*Trichodermaharzianum*, *Trichodermaviridae*, *Trichodermareesei* produced reddish brown zones.

Screening for Laccase Producing *Trichoderma* species: In order to find *Trichoderma*species, which were potential laccase producers, samples were collected from different environments. Samples were diluted serially and then inoculated on media plates, 88 different fungal isolates were isolated which belongs to several genera, out of which 29 isolates were *Trichoderma*. *Trichoderma* isolates were then re-cultured on malt extract agar until they were purified (Table 1).

Qualitative Analysis (Plate Assay Method): All the identified *Trichoderma* isolates were then checked for laccase production. They were allowed to grow on media plates containing 0.02% guaiacol showing the reddish brown zones on media plates, which was the sign of laccase activity as laccase catalyzes the oxidative polymerization of guaiacol (Fig 2). *Trichodermaharzianum, Trichodermaviridae*and *Trichodermareesei* isolated from

industry effluent water and soil were laccase producers while the rest of the *Trichoderma* species that were *Trichodermakoningii*, *Trichodermagamsii*, *Trichodermahermatum* and *Trichodermaaeroviridae* were very low or negligible laccase producers.

Table 1. Isolation of *Trichoderma* species from different environmental samples

Sampling Sites	No. of Colonies	Total No. of*Trichoderma*isolates	*Trichoderma*species	No. of*Trichoderma*isolates
Effluent Water of industry	29	9	*T. harzianum*	4
			T.reesei	2
			T.hermatum	3
Sludge of industrial waste	37	7	*T.koningii*	5
			T.viridae	2
			T. harzianum	3
Agricultural soil	22	13	*T.gamsii*	4
			T.reesei	5
			T.aeroviridae	1
Total	88	29	9	29

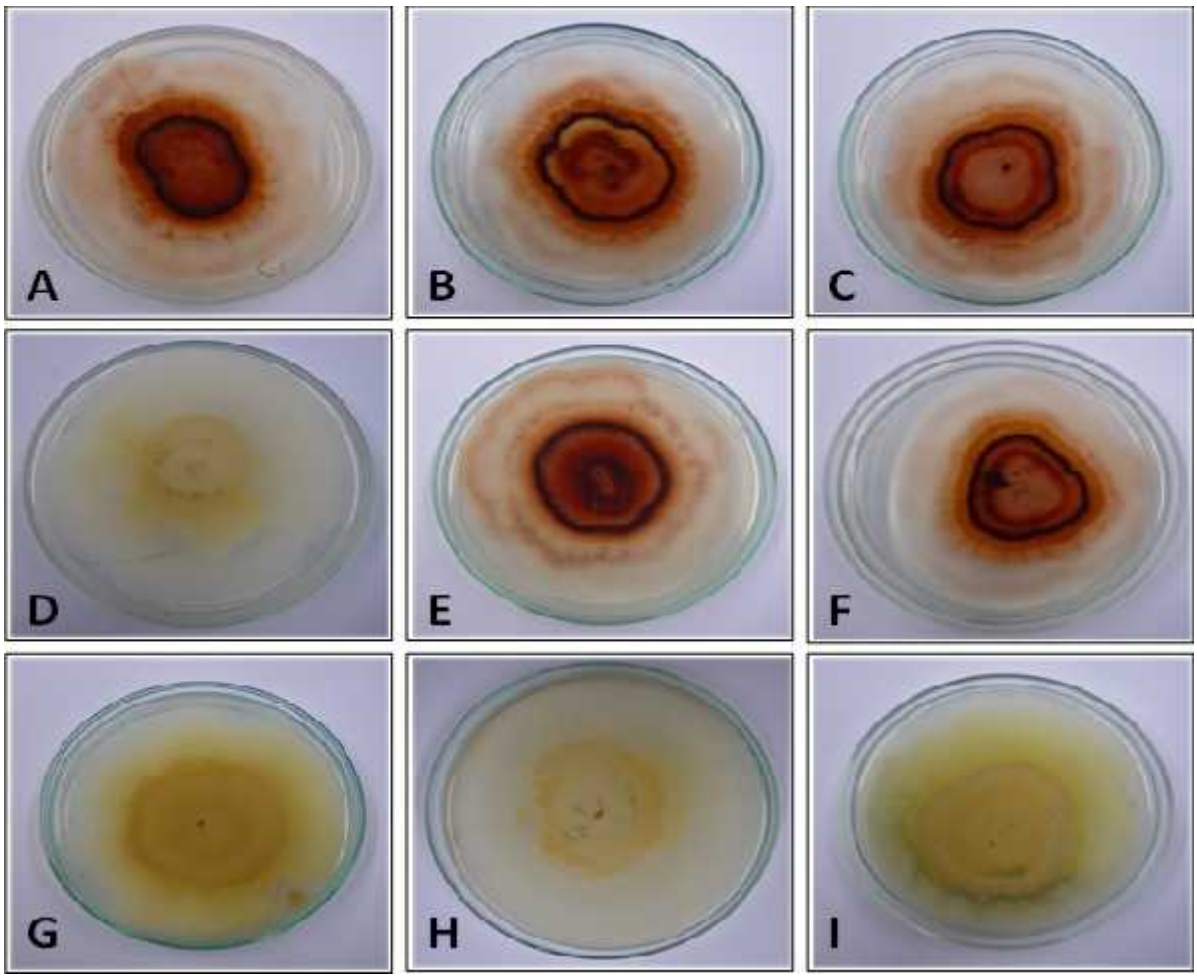

Fig 2. Screening of *Trichoderma* species by plate assay method. A: *Trichodermaharzianum* (industry effluent water), B: *Trichodermaharzianum* (soil), C: *Trichodermaviridae*, D: *Trichodermakoningii*, E: *Trichodermareesei* (industrial effluent sludge), F: *Trichodermareesei* (soil), G: *Trichodermagamsii*, H: *Trichodermahermatum*, I: *Trichodermaaeroviridae*.

Quantitative Analysis (Enzyme Assay Method): Out of 9 selected *Trichoderma*isolates, 5 showed as laccase producers. These species were further checked. Quantitative analysis was carried out to select one of the best laccase producers among the rest five. Fig 3 shows *Trichodermaharzianum*, isolated from industrial effluent water has the highest laccase activity while *Trichodermaviridae*showed the least growth. The rest of the *Trichoderma* species showed variable behaviour for laccase activity.

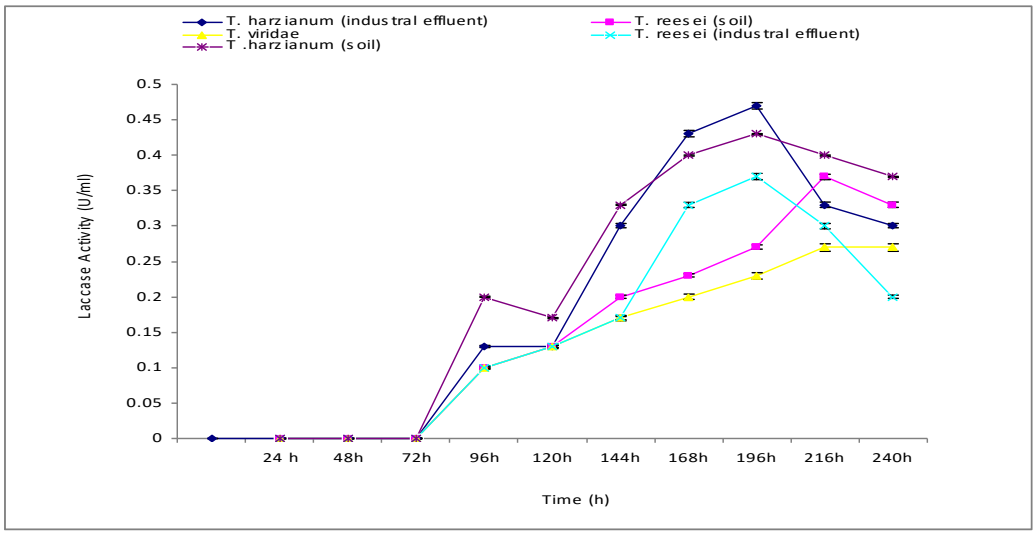

Fig.3. Screening of Laccase producing *Trichoderma*species by Enzyme assay method.

In order to find laccase producing fungi from the fungiisolated from various environmental samples, a simplescreening method was followed using solid media containing indicator compound guaiacol. A total of 12species were screened and among these, 6 were laccasepositive (Table 1). From these laccase positive fungi *Trichoderma* spp were selected for further studies as the oxidative polymerization of guaiacol to form reddish brown zones in the medium was high inthese two organisms compared to other fungi (Fig 2).

During present investigation, the inducers enhance laccase production except ethanol. Trypan blue is a dye that increased laccase yield up to 12.6 U/ml by *Trichodermaharzianum*, however, 1-butanol showed the maximum fungal growth. According to Souza *et al*. (2006), Trypan blue, methylene blue and crystal violet did not induce laccase activity in marine fungus.

Sadhasivam*et al*. (2008) reported during partial purified studies that in culture filtrate, total protein contents were 752.0 mg, and enzyme activity was 653.0 U while in partial purified form total protein contents were 128.8 mg and enzyme activity was 168.6 U. However, in present assessment, crude enzyme yield was 11.95 U/ml and total protein contents were 3.54 mg/ml while in partial purified form, laccase activity was 16.68 U/ml and total protein was estimated as 2.96 mg/ml. Influence of pH on the stability of Laccase was studied in this work over a pH range of 2.0–11.0. Laccase was stable at pH 4.5, but as pH become alkaline or acidic, the stability of the laccase also decreased.

It was found in the present study that 20°C, 30°C and 40°C has minor effect on relative activity of laccase, however relative activity of enzyme decreased as the time of incubation increased. At temperatures 50°C, 60°C and 70°C, enzyme become inactive as the time of storage increased.

Conclusions: Fungal Laccases have been implicated in degradation of lignin and protection from toxic phenolic monomers of polyphenols. Laccases are helpful for number of industrial applications. Laccases were usually produced in low concentrations by laccase producing fungi, but high concentrations were obtained with the addition of different supplements to media and optimized cultural conditions. Out of 29 isolates belong to 7 species of *Trichoderma* were screened as laccase producers, based on decolorization of guaiacol. *Trichodermaharzianum*, isolated from industrial wastewater was found to be best potential laccase producing fungus while *Trichodermaviridae*showed the least growth.

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