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Isolation Of Laccase Producing Trichoderma Spp. And Effect Of PH And Temperature On Its Activity

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Abstract: Laccase is a copper-containing polyphenol oxidase that acts on a wide range of substrates. This enzyme is found in many plant species and is widely distributed in fungi including wood-rotting fungi where it is often associated with lignin peroxidase, manganese dependent peroxidase, or both. In the present study, water samples were collected and inoculated for the isolation of fungal species present in water samples. The cultures were purified by repeated transfer to agar plates and grown at 25°C for different periods of time. These cultures were then screened for laccase production. All isolated organisms were cultured on media supplemented with 0.04% guaiacol. Solid State Fermentation process shows higher enzyme activity (11.7 U/ml) than Submerged Fermentation (8.67 U/ml). The optimum pH and temperature for the phenolic substrate guaiacol were 4.9 and 55˚C, respectively. The enzyme remained stable within an acidic pH range from 3.0 to 6.5 and the thermal stability of the enzyme was also determined and the enzyme remained stable up to 40˚C.
Key words: Trichoderma, laccase, guaiacol, fungi, submerged fermentation, solid state fermentation.

INTRODUCTION AND EXPERIMENTALS

Large quantities of Lignocellulosic wastes (LCW) are released from various industries such as food, agricultural, forestry, paper-pulp, and timber. These wastes cause serious environmental pollution, but they can be reused constructively rather than burned due to their rich sugar contents. The chemical properties of these lignocellulosic wastes make them a crucial and cost-effective fermentation medium for biotechnological applications. However, while the hemicellulose and cellulose components of lignocellulosic materials are used by numerous microorganisms, the lignin, which its component is the most resistant material to microbial degradation, is converted efficiently by only a limited number of organisms such as white rot fungi. White rot fungi are the best laccase-producing organisms and they can be easily grown on lignocellulosic wastes.

In recent years, enzymes have gained great importance in industries; laccases are among one of them which are widely present in nature. Laccases are the oldest and the most studied enzymatic systems[1]. Laccase was first discovered in the sap of the Japanese lacquer tree, *Rhus vernicifera* and its characteristic as a metal containing oxidase was discovered by Bertrand in 1985[2].

Laccases (E.C.1.10.3.2, benzenediol: oxygen oxidoreductase) are copper containing oxidoreductase able to catalyse the oxidation of various aromatic compounds (particularly phenol) with concomitant reduction of oxygen to water[3]. Basically, general oxidation takes place by three types:

a) By the addition of O_2 to the substrate.
b) By the removal of H from the substrate.
c) By the removal of e^- from the substrate.

In the case of laccase it oxidizes the substrate by the removal of e^- and because it belongs to the oxidase enzyme family, it requires O_2 as a second substrate for enzymatic action. The copper is bound in several sites: type 1, type 2 and/or type 3. The way of assembly of types 2 and 3 is called a trinuclear cluster. Laccase contains 15-30% carbohydrate and have molecular mass of 60-90 KDa.

SOURCES OF LACCASE

Fungi: Fungi from the deuteromycetes, ascomycetes[4] as well as basidiomycetes are the good producers of laccase[5]. White-rot fungi produces efficient amount of laccase among basidiomycetes[6]. *Trametes versicolor, Chaetomium thermophilum* and *Pleurotus eryngii* are well known producers of laccase and it has been reported that *Trichoderma* species has the ability to produce polyphenol oxidases[7].

Plants: The plants in which the laccase enzyme has been detected includes lacquer, mango, mung bean, peach, pine[8]. Recently laccase has been expressed in the embryo of maize seeds[9]. Laccases are produced by both plants and fungi but when they are produced by former then it takes part in radical based polymerization of lignin[8 and 10]) whereas fungal laccase contributes to lignin biodegradation because of which it has many industrial applications[8].

Bacteria: First of all laccase was found in plant root associated bacterium *Azospirillum lipoferm*[11 and 12]. Nowadays laccase is found in many bacteria including *Bacillus subtilis, Bordetella compestris, Escherichia coli, Pseudomonas syringae* [8 and 13].

Insects: Different insects have also been reported for the production of laccase. Some of them are Bombyx, Calliphora, Diploptera[8].

MECHANISM OF LACCASE

Basically laccase reduce one oxygen molecule to water accompanied with the oxidation of one electron with wide range of aromatic compounds which include polyphenols[14] and aromatic amines (Figure-1) [15and 16] .The four copper ions are classified into three categories- type 1 (T1), type 2 (T2) and type 3 (T3). These 3 types can be distinguished by using visible electronic paramagnetic resonance (EPR) spectroscopy. Type 1 copper gives blue colour to protein at an absorbance of 610 nm which is EPR detectable. Type 2 copper doesn't gives colour which is EPR detectable. Type 3 copper gives weak absorbance in near UV region but not detected by EPR signal[3].

When laccase oxidizes the substrate, free radicals are generated. The lignin degradation proceeded by phenoxy radical leads to oxidation at α carbon or cleavage of bond between α and β carbon. This oxidation results in an O_2 centered free radical which can be converted into second enzyme catalyst reaction to quinine[3]. Laccase also oxidizes the non-phenolic substrates by the inclusion of organic compounds which are low molecular weight organic compounds which when oxidized by laccase can further oxidize non-phenolic compounds. Most common organic compounds that act as mediators are N- hydroxyphthalimide (NHPI), 3-hydroxy anthranilic acid [17].

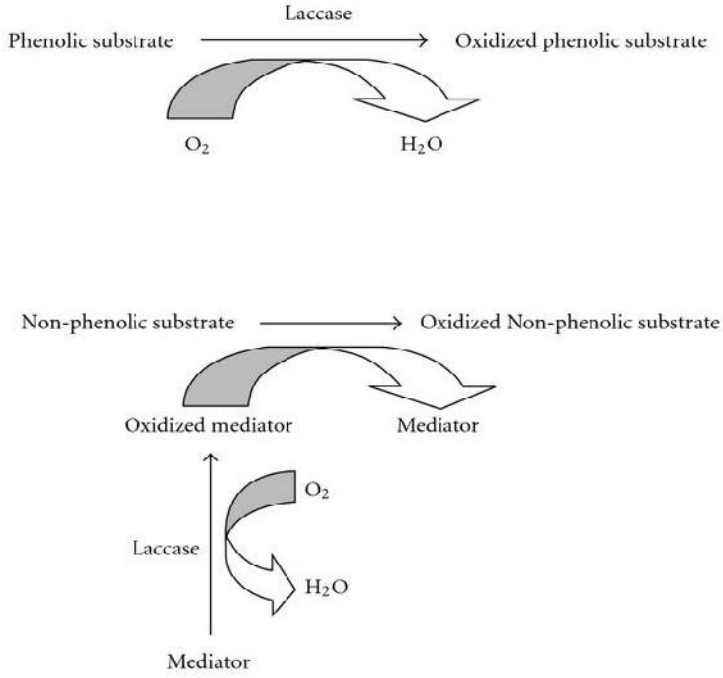

Figure 1: Mechanism of laccase action for both phenolic and non-phenolic substrates.

Isolation of laccase producing fungi

Water samples were collected from the Yamuna bank near Kalindi kunj and from a pond in anangpur village Faridabad. The samples were inoculated on petriplates containing potato dextrose agar for the isolation of fungal species (Figure-2) present in water samples. The cultures were purified by repeated transfer to agar plates and grown at 25°C for 10 days. These cultures were then screened for laccase production.

Screening

All isolated organisms were cultured on petriplates containing potato dextrose agar (PDA) supplemented with 0.04% guaiacol. In addition to this 0.01% (w/v) chloramphenicol was also added to the media to avoid bacterial growth and the pH of the medium was adjusted to5.5 with 1 N HCL. These petriplates were screened for the formation of reddish brown zones (Figure-3).

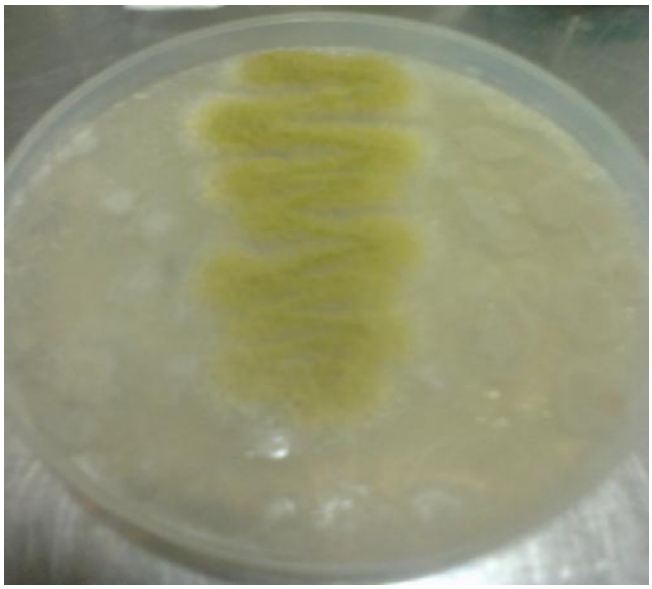

Figure 2: Colony morphology of isolated strain; green mycelia growth can be visualized.

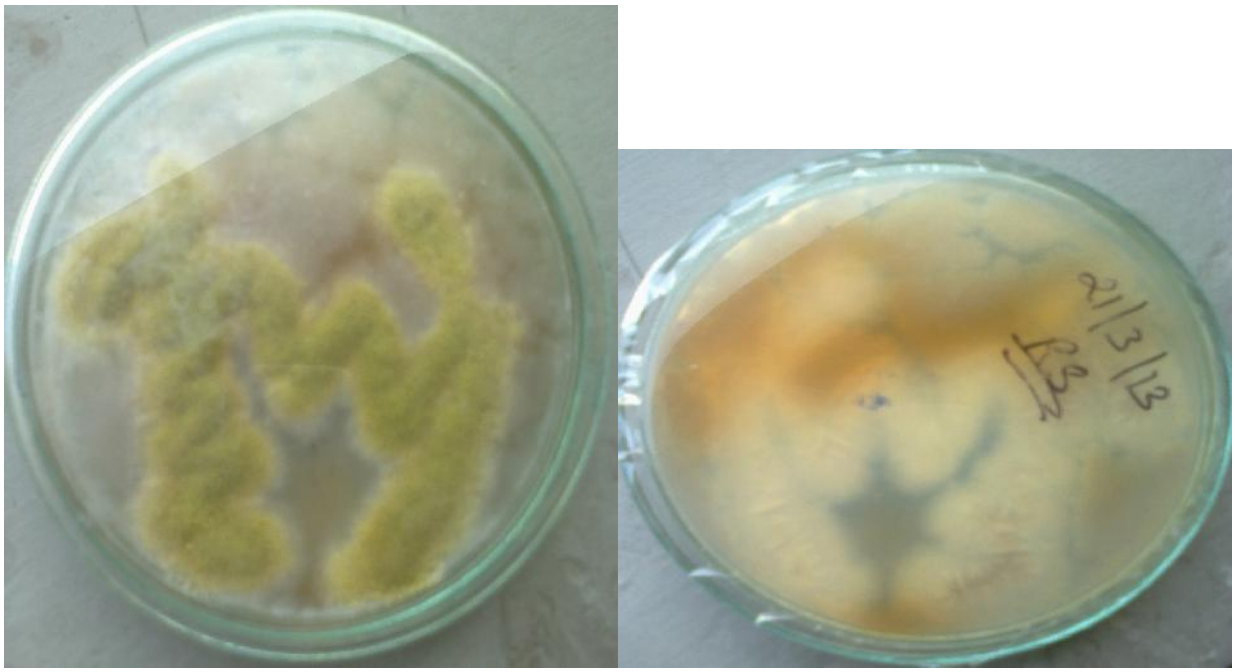

Figure 3: Reddish–brown colonies/ zones apparently due to laccase catalyzed polymerization of Guaiacol

Selection of production medium

Submerged fermentation

Fungal strain was cultivated in liquid medium containing 3g peptone,10g glucose,0.6gmKH_2PO_4,0.001g $ZnSO_4$, 0.4g K_2HPO_4, 0.0005g $FeSO_4$, 0.05g $MnSO_4$ and 0.5g $MgSO_4$ per L, and kept in incubator shaker at 150 rpm, 30°C for 12 days. 3 ml broth was collected from the fourth day, fungal mycelium was separated from the broth by filtering it through Whatman No.1 filter paper. The filtrate was used for enzyme assay.

Solid state fermentation

In this method rice bran is used as solid support for the growth of fungus. 5 petriplates containing rice bran moistened with distilled water were incubated with selected fungal strain at 30°C in an incubator for 12 days. Autoclaved water (10 ml) was dispensed into a PDA slant (incubated with selected organism at 30°C for 7 days) properly mixed and equal amount of water was then used for inoculating individual plate. The enzyme activity was measured from the fifth day of incubation. Crude culture and filtrate was obtained by mixing 15 ml distilled water to plates and filter it with the help of muslin cloth. Again it was centrifuged at 10,000 rpm for 10 min. The supernatant was used for enzyme assay.

Guaiacol assay method for laccase assay

Oxidation of guaiacol has been reported for laccase assay. The reddish brown colour developed due to oxidation of guaiacol by laccase is used to measure enzyme activity at 450 nm. The reaction mixture can be prepared as-

a) Guaiacol (2mM) 1 ml
b) Sodium acetate buffer (10 mM) 3 ml
c) Enzyme source 1 ml

A blank was also prepared that contains 1 ml of water instead of enzyme. The mixture was incubated at 30°C for 15 min and the absorbance was read at 450 nm blank using UV spectrophotometer. Enzyme activity was expressed as International Units (IU), where 1 IU is the amount of enzyme required to oxidize 1 µmol of guaiacol per min. The laccase activity in U/ml is calculated by the formula-

$E.A = A \times V / t \times e \times v$

E.A = Enzyme activity

A = Absorbance
V = Total mixture volume (ml)
v = enzyme volume (ml)
t = incubation time
e = extinction coefficient

RESULTS AND DISCUSSION

Production of laccase under solid-state condition

SSF process shows higher enzyme activity than SmF. In SSF isolated microorganism was cultivated on rice bran. Assay was done from third day of incubation and further readings were taken after equal intervals of three days. Highest activity of organism was 11.7 U/ml (Figure 4).

Production of laccase in submerged condition

Isolated species were cultivated on basal medium and enzyme activity was observed from third day of incubation and mean after 3 days interval. Enzyme activity was higher on sixth day (Figure 5). Highest activity measured was 8.67 U/ml.

Effect of pH and Temperature on enzyme activity

Effect of temperature can be studied by incubating the enzyme mixture containing enzyme, guaiacol and sodium-acetate buffer at different temperatures, i.e. 10°, 20°, 30°, 40°, 50°and 60°C for 15 minutes. After incubation for 15 minutes record the absorbance of enzyme catalyzed reaction. Temperature at which the enzyme showed maximum activity was noted as the optimum temperature of the enzyme(Figure 6) Similarly the effect of pH was studied by mixing the substrate with buffers having different pH values (Figure 7).

In this study, the laccase producing fungi have been isolated from different water samples using guaiacol as substrate that forms reddish brown zones in the agar plates. The isolated fungus belongs to *Trichoderma* genus. Laccase is produced with the help of both submerged and solid-state fermentation but higher production was achieved under solid-state conditions where rice bran was taken as support substrate. Higher levels of laccase were obtained from SSF (11.7 U/ml) in comparison to SmF (8.67 U/ml). For enzyme activity, optimum temperature was found to be 45°-50°C and optimum pH to be 4.5-5.5.

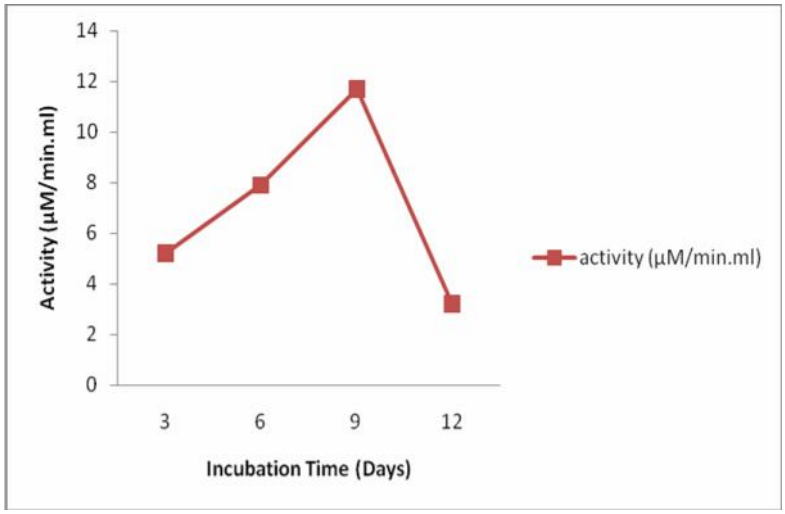

Figure 4: The activity of enzyme produced after particular days of incubation under solid state condition [activity (U/ml) v/s incubation time (days)].

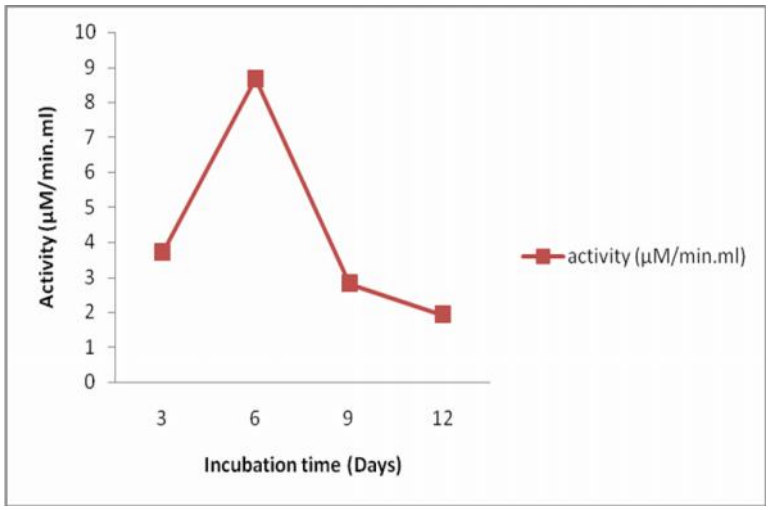

Figure 5: The activity of enzyme produced after particular days of incubation under submerged condition [activity (U/ml) v/s incubation time (days)].

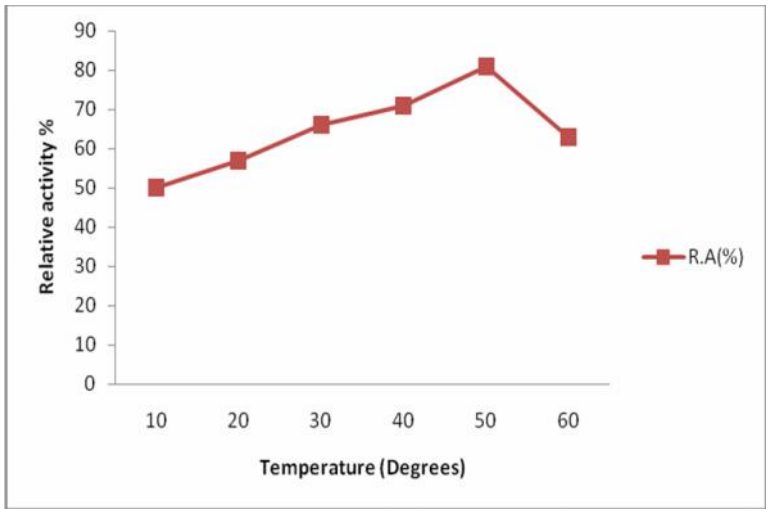

Figure 6: The enzyme activity reached the maximum at particular temperature and decreased thereafter [activity (U/ml) v/s temperature (°C)].

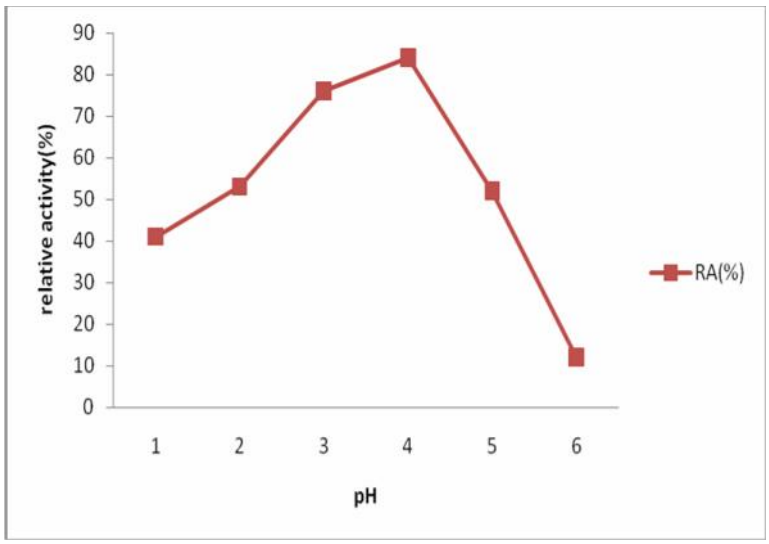

Figure 7: The enzyme activity reached the maximum at particular pH and decreased thereafter [activity (U/ml) v/s pH].

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