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Production and Bio Application of Laccase Enzyme for Seed Germination

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Abstract: Laccases are multi–copper containing oxidases (EC1.10.3.2) widely distributed in fungi, higher plants and bacteria. The mycelium of Trichodermaviridecan produce an enzyme called laccase. The laccaseenzyme has been screened for liquid cultivation monitored with enzyme activity measurement. The cultures were purified by repeated transfer to agar plates and grown. All isolated microorganism are cultured on media supplemented with 0.04% guaiacol. Guaiacol as a substrate, indicated by the formation of reddish brown hallows. It has the capability to oxidize a wide range of toxic and environmentally problematic substrate. The present paper delineates about the production and bioapplication of laccase.
Keywords: laccase, guaiacol, reddish brown, dye degradation, seed germination

I. INTRODUCTION

Enzymes are protein molecules in cells which work as catalysts. Enzymes speed up chemical reaction in the body, but don't get used up in the process. Almost all biochemical reactions in living things need enzymes. With an enzyme, chemical reactions happen much faster than they would without the enzyme. Laccase (benzenediol:oxygenoxidoreductase; EC 1.10.3.2) exists widely in nature.They are predominantly found in higher plants and fungi (Thurston 1994; Mayer and Stapes 2002). Laccases oxidize a surprisingly wide variety of organic and inorganic compounds, including diphenols, polyphenols, substituted phenols, diamines and aromatic amines, with concomitant reduction of molecule oxygen to water (Thurston*et. al.*,1994).Discovery of novel laccases with different substrate specificities and improved stabilities is important for industrial applications. Microbes that produce laccases have been screened for either on solid media containing coloured indicator compounds that enable the visual detection of laccaseproduction (Nishida *et. al.*,1988; De Jong *et. al.*, 1992; Barbosa *et. al.*, 1996) or with liquid cultivations monitored with enzyme activity measurement (Szklarz*et. al.*,1989; Pelaez*et. al.*, 1995; Lutere*et. al.*, 1997).

The traditional screening reagents, tannic and gallic acid (Harkin and Obst1973) have nowadays mostly been replaced with synthetic phenolic reagents, such as Guaiacol and syringaldazine (Nishida *et. al.*, 1988; De Jong *et. al.*, 1992). With Guaiacol a positive reaction is indicated by the formation of reddish - brown halo (Nishida *et. al.*, 1988), while with tannic and gallic acid the positive reaction is a dark - brown coloured zone (Harkin and Obst 1973). Fungal laccase are monomeric, dimeric or tetrameric glycoproteins. Upon purification, laccase enzymes demonstrate considerable heterogeneity. Glycosylation content and composition of fungal glycoproteins can vary with growth medium composition, for this reason data can be heterogeneous. The molecular mass of the monomer ranges from about 50 to 100 kda. An important feature is covalently- linked carbohydrate moiety, which may contribute to the high stability of the enzyme. Recently some microbial laccases have been characterized from *Azospirillumlipoferum* (Givaudan*et.al.*, 1993), *Bacillus substilis*(Martins *et. al.*, 2002), *Streptomyceslavenduals* (Suzuki *et. al.*, 2003) and *Sterptomycescyaneus* (Arias *et. al.*, 2003). Most of the laccases studied thus far are of fungal origin, especially from white - rot fungi such as *Phlebiaradiata* (Niku-Paavola*et. al.*, 1988) and *Pleurotusostreatus* (Palmieri *et.al.*, 2000). *Trichodermaviride* is a mold which produces spores asexually by mitosis. It is the anamorph of *Hypocrearufa,*itstelomorph is the sexual reproduction stage of the fungus and produces a typical fungal fruiting body.

The mycelium of *Trichodermaviride* can produce a variety of enzymes including laccases and chitinases. Guaiacol has been reported as an efficient substrate for laccase assay. The intense brown colour due to oxidation of Guaiacol by laccase can be correlated to its activity often read at 450 nm.Guaiacol in sodium acetate buffer was used as the substrate. Guaiacol is a phenolic natural product first isolated from Guiac resin and the oxidation of lignin. It is yellowish aromatic oil that is now commonly derived from Guaiacum or wood creosote. It is used medicinally as an expectorant, antiseptic and local anaesthetic. Germination is usually the growth of a plant contained within a seed; it results in the formation of the seedling, it is also the process of reactivation of metabolic machinery of the seed resulting in the emergence of radicle and plumage. The seed of the vascular plant is a small package produced in a fruit or cone after the union of reproductive cells. All fully developed seeds contain an embryo and in most plant species, some store food reserves wrapped in a seed coat. Under proper conditions, the seed begins to germinate and the embryonic tissues resume growth, developing towards a seedling. Seed germination depends on both internal and external

conditions. Disposal of untreated dyeing effluent in water bodies, from industries, cause serious environmental and health hazards. The chemical structures of dye molecules are designed to resist fading on exposure to light or chemical attack, and they prove to be quite resistant towards microbial degradation. Laccases are currently studied intensively for many applications and they are already used in textile industry (Tza-nov*et.* al., 2003).

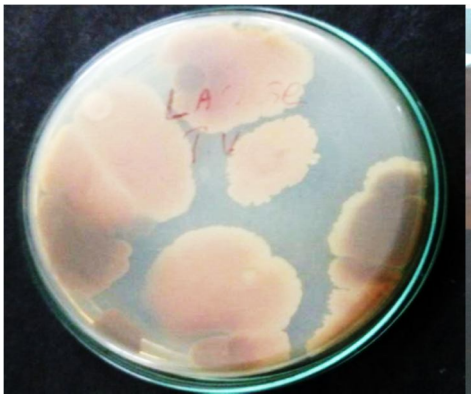
II. MATERIALS AND METHODS

A. Chemicals

Potato dextrose agar, agar-agar, guaiacol, peptone, glucose, potassium dihydrogen phosphate,$MgSO_4.7H_2O$,$MgSO_4$, sodium molybdate, calcium chloride, sodium chloride, ferric chloride, $MnSO_4$, $FeSO_4$, Lowry reagent, follins reagent, potassium phosphate buffer, malachite green dye, Tris, Sodium Dodecyl Sulfate (SDS), acryl amide, Ammonium Per Sulfate (APS), Tetramethylethylendamine (TEMED), Single Stranded Binding Protein (SSB), distilled water.

B. Screening test

Guaiacol is a sensitive substrate which enables the detection of laccase secretion. *Trichodermaviride*from the slant was streaked on the screening media containing 0.02% guaiacol and inoculated into plates and incubated at 30˚C. Screening media consists of 1.95g potato dextrose agar, 1g agar and 20µl of guaiacol in 50 ml distilled water. The growth of fungal species is shown in Figure (1). The screening media is shown in Figure (2).



Figure(1)Trichoderma species

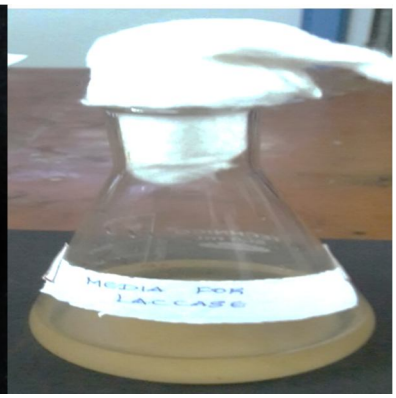


Figure (2) Laccase media

C. Laccase enzyme production

At first culture media was prepared in 250ml Erlenmeyer flask containing 0.6g of peptone, 2g of glucose, 0.04g of KH_2PO_4, 0.002g of sodium molybdate, 0.04g of $MnSO_4.5H_2O$, 0.01g of $MgSO_4.7H_2O$, 0.0026g of $CaCl_2$, 0.1g of NaCl, 0.2g of $FeCl_3$ dissolved in 250ml of distilled water and it was sterilized at 121˚C for 20 minutes and kept undisturbed for 24 hours. Then 10ml of culture media was inoculated into production media and incubated for four to five days. Production media contains 0.75g of peptone, 2.5g of glucose, 0.15g of KH_2PO_4, 0.1g of K_2HPO_4, 0.0125g of $MgSO_4$, 0.0125g of $MnSO_4$, 0.0125g of $FeSO_4$ in 200 ml distilled water. It was then sterilized at 121˚C for 20 minutes at 15lbs pressure. The production media is shown in Figure (3).

Figure (3) Production media for laccase

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D. Partial purification of enzyme

After five days, the medium was centrifuged at 10,000rpm for 15minutes where the supernatant was collected and the pellet was removed.170ml of supernatant was collected from the medium and add70%(120g) of Ammonium sulphate and incubated at 4°Covernight to get precipitated. Again the collected supernatant was centrifuged after one day at 10,000 rpm for 15minutes and now the pellet was collected which is the crude enzyme.

E. Dialysis

Dialysis was done by preparing a dialysis buffer called the potassium phosphate buffer (0.1M) for 200ml of pH 6.5 contains 18g of potassium hydrogen phosphate. To the buffer the pellet was mixed in small amount and here the dialysis membrane was used to get the clear enzyme. Before undergoing this process, the membrane was pre-treated in the buffer for 15minutes. After pre-treatment the pellet was injected to the membrane through the needle. Both the ends of the membrane were tightly tied in order to prevent the leakage and now the membrane with pellet was inserted into the beaker containing buffer and the beaker was closed with glass plate. This setup was kept for 2days and the buffer was changed every 24 hours. During dialysis, the pure enzyme will get attached to the membrane and the other waste particles are removed. After two days the pure laccase enzyme was produced and was collected. The dialysis setup is shown in Figure (4).

Figure (4) Dialysis process

III. RESULTS AND DISCUSSION

From this study, it has been shown that laccase enzyme was produced and the characterization (protein estimation, enzymatic activity, dye degradation, SDS PAGE) of laccase enzyme was described by the following process.

A. Protein estimation

In this process, protein concentration was estimated by using FollinLowry method. It was observed that the protein concentration was higher in the enzyme treated mixture than the blank (non-enzyme treated mixture). Blue colour indicates the presence of protein in the sample and is shown in Figure (5).

Figure (5) Protein estimation

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Table-1Concentration of protein

Test	Protein concentration(µl/ml)
Enzyme treated	0.456
Enzyme untreated (blank)	0.440

B. Enzyme Activity

Laccase activity was measured using guaiacol as substrate. This method clearly points out the enzyme activity. For determination of the enzyme activity, 3ml of potassium phosphate buffer, 1ml of guaiacol, 1ml of sample are taken in the conical flask and mixed well together and incubated at 60˚ C for 15 minutes. After the period of incubation the OD value was observed at 450nm in spectrometer and noted in the Table (2). It wasobserved that the enzyme treated mixture produced good result compared to the non-enzyme treated mixture.

Table-2Measurement of enzyme activity

Sl.no	Test	Enzyme activity(Units/ml)
1.	Enzyme untreated (blank)	0
2.	Enzyme treated	2.081

C. SDS-PAGE

SDS-PAGE was performed to determine the molecular weight of the enzyme.

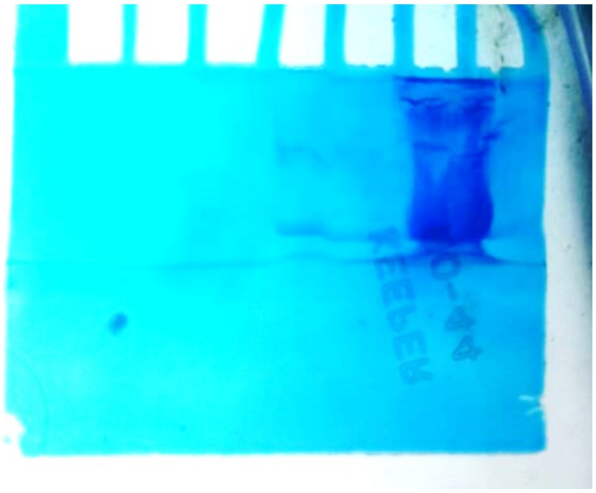

Figure (6) SDS PAGE

The sample was mixed with loading buffer. In the first well, the protein marker was loaded where in the other wells, the samples were loaded and then the entire setup was connected to power supply at the appropriate voltage. This setup was made to run for one hour and then the gel was removed and observed under UV transilluminatorand protein bands were observed and shown in Figure (6).

Enzyme loaded in SDS page contains 20µlSSB and 10µl enzyme and they were heated at 60˚ C for 10 minutes.

D. Dye degradation

The dye used for degradation process is malachite green. The dye is treated and untreated with enzyme was shown in the Figure (7) and Figure (8). Then OD value was measured at 720nm. The OD values were measured at 24 hours intervals.

Formula we use to calculate the amount of dye degradation is,

= ((Day 1 OD value - Day 2 OD value) / Day 1 OD value) *100

Figure (7) Dye degradation with enzyme

Figure (8) Dye degradation without enzyme

Table-3Measurement of dye degradation

Sl.no	Test	Without enzyme	With enzyme
1	Day 1	1.703	1.312
2	Day 2	1.702	0.922

E. Seed germination

Germination is the process of seeds developing into new plants. First the seed grows a root to access water underground. Next, the shoots begin to appear. The seed sends a shoot towards the surface, where it will grow leaves to harvest energy from the sun. The leaves continue to grow towards the light source, this process is called photo morphogenesis.

Here the plant *TrigonellaFoenumGraecum*used for seed germination process. Seed germination was carried out with the help of 3 different parameters. For control, only water is sprinkled on the plant. Degradation of dye with enzyme treated is sprinkled on the plantdaily. Degradation of dye without enzyme treated mixture is sprinkled on the plant daily. The height of the plants are then measured.

IV. CONCLUSION

From this study, it can be concluded that the fungi culture (Trichodermaviride) possesses great laccase activity and a small amount oflaccase enzyme was produced byTrichodermaviride on lab scale. Partial purification was carried out by precipitation method followed by dialysis method and its activity, protein concentration, application were estimated. This study observed that the enzyme treated dye leads to better plant growth.

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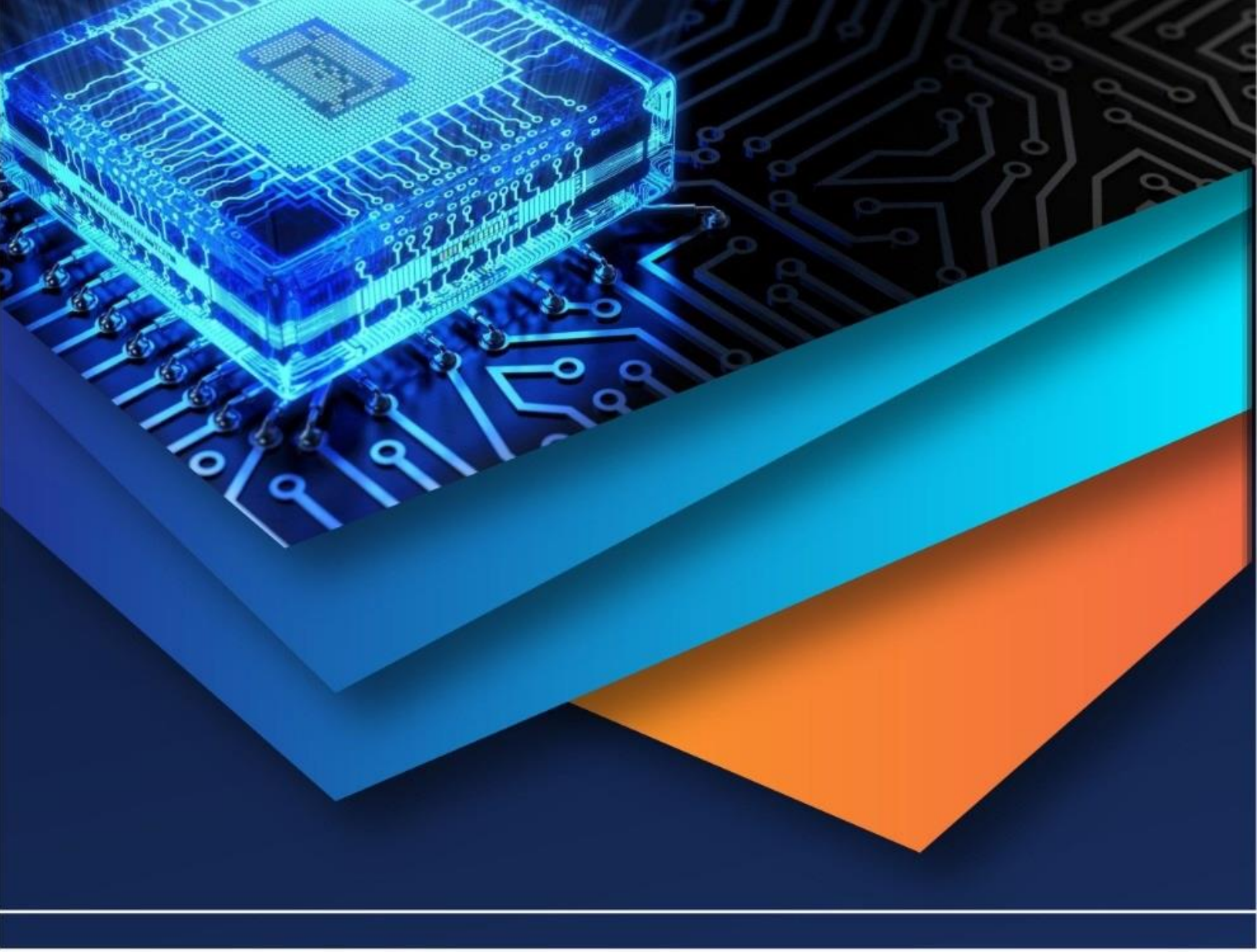

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