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Production and Partial Purification of Extracellular Laccase from Mushroom Pleurotus sp. and its Application in Green Synthesis of Silver Nanoparticle

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Abstract: ***Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2.) is a type of copper containing polyphenol oxidases. It is present primarily in higher plants and filamentous fungi, but also has been found in several species of bacteria. It is widely used in the food and paper industry, synthetic chemistry, bioremediation and biodegradation of phenolic pollutants or in analytical applications. The aim of the present work is to extract and partially purify the extracellular laccase enzyme from mushroom (Pleurotus ostreatus, Pleurotus florida) and synthesize silver nano particle using the isolated laccase enzyme. For this the fungi that believed to have the laccase producing ability were cultured on a standardized medium (PDA). Isolates were then screened for laccase production by growing them on guaiacol incorporated agar medium. The isolates showing maximum activity were evaluated by submerged fermentation for maximum enzyme production. The extracellular laccase enzyme was then extracted and partially purified by ammonium sulphate precipitation and dialysis. The purity of enzyme preparation was assessed by SDS PAGE analysis. The purified enzyme preparation was then used for the synthesis of silver nano particle using silver nitrate and the presence of silver nanoparticle was confirmed by SEM & FTIR analysis. The SEM analysis revealed the shape of the nanoparticle whereas FTIR results yielded the predominant functional groups present in the molecule.***
Key words: Laccase, Pleurotus, Guaiacol, Dialysis, SEM, FTIR, SDS- PAGE

I. INTRODUCTION

Enzymes are the biocatalysts produced by living cells. Enzyme technology broadly involves production, isolation, purification and use of enzymes (in soluble or immobilized form) for the ultimate benefit of humankind. Fungal fermentations in the commercial production of a wide range of secondary metabolites have been extensively exploited in recent times. For example; fungal metabolism have been exploited in the manufacturing of ethanol, citric acid, steroids, antibiotics and other substances with applications in the food, fuel, chemical and pharmaceutical industries. Lignocellulose is one of the renewable organic materials that have an important structural role in plants. Lignin consists of three aromatic radicals including Coniferyl, Sinapyl and Coumaryl. Further, complex of lignin with hemicellulose and cellulose makes lignin as a resisting barrier against decomposition. 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[1].

Laccases are copper containing 1, 4 – benzenediol oxygen oxidoreductases (EC 1. 10. 3. 2) found in higher plants and microorganisms. These enzyme contain 15 – 30 % carbohydrate and have a molecular mass of 40 – 90 kDa [2]. These are glycosylated poly phenol oxidases that contain 4 copper ions per molecule that carry out one electron per oxidation of phenolic and its related compounds and reduce oxygen to water. When substrate is oxidized by laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or nonenzymatic reactions including hydration, disproportionate and polymerization. These enzymes are polymeric and generally contain one each of type 1, type 2, and type 3 copper center / subunit where the type 2 and type 3 are close together forming a trinuclear copper cluster. The oxygen molecule binds to the trinuclear cluster for asymmetric activation and it is postulated that the oxygen binding compartment appears to restrict the access of oxidizing agent. The native isolates of Pleurotus sp. was found to have an excellent ability of laccase production [3]. These enzymes have less substrate specificity and have the ability to degrade a wide range of xenobiotics. It is possible to reduce the cost of enzyme production by optimizing the fermentation medium and is a basic research strategy for industrial application of laccase enzyme. Nanoparticles are the simplest form of structures with sizes in the nm range. In principle, any collection of atoms bonded together

with a structural radius of < 100 nm can be considered a nanoparticle. The integration of nanoparticles with biological molecules has led to the development of diagnostic devices, contrast agents, and important tools in cancer therapy [4].

II. MATERIALS AND METHODS

A. *Sample collection*

Three different mushroom spawns were collected randomly from Vegetable and Fruit Promotion Council (VFPCK), Kakkanad, Kerala.

1) *Pleurotus ostreatus*
2) *Pleurotus florida*
3) *Calocybe indica*

These spawns were stored in ambient condition in microbiology laboratory.

B. *Media*

Different types of solid and liquid media were used for the study[5].

1) Potato Dextrose Agar- For fungal culture
2) Guaiacol incorporated agar- For screening of fungi which produces laccase enzyme
3) Potato dextrose broth and Guaiacol incorporated liquid medium- For submerged fermentation of fungi

C. *Cultivation of fungus*

The PDA medium was prepared and autoclaved along with forceps, plates, filter papers and distilled water. The medium was allowed to cool, streptomycin was added at a concentration of 1 mg/ml and the medium was poured in to sterile petriplates. All the plates were allowed to solidify and each of them were inoculated with the mushroom spawn (P. ostreatus, P. florida and Calocybe indica) using sterile forceps and filter papers. The plates were incubated at 37^0c for 6-7 days[6].

D. *Screening for laccase production*

Screening was done to identify the cultures producing laccase. For this required volume of PDA was prepared incorporating 0.1 % guaicol. The media was sterilized by autoclaving at 121^0 c for 15 minutes. Allowed to cool and was poured in to sterile petriplates. The media was inoculated with a loopful of the cultured fungal mycelia and incubated at 37^0 C for 6-7 days[3].

E. *Submerged fermentation*

For submerged fermentation, about 1000 ml fermentation medium (both PD broth and guaiacol medium) was prepared and was transferred to 10 conical flasks. Each of the flasks was autoclaved at 121^0c for 15 minutes and allowed to cool. The flasks were then inoculated with a loopful of mycelium of P. ostreatus and P. florida. The flasks were incubated at 37^0c for 14 days[6].

F. *Assay for enzyme activity – guaiacol oxidation method*

The activity of laccase enzyme produced by *Pleurotus ostreatus* and *Pleurotus florida* was assayed using guaiacol oxidation method. The assay was carried out at room temperature by using 2 mMguaiacol in 0.2 mM acetate buffer. The reaction mixture contained 3ml acetate buffer, 1ml guaiacol and 1ml sample solution (Centrifuged supernatant of each culture). The change in the absorbance of reaction mixture containing guaiacol was monitored at 470 nm for 5 minutes of incubation using UV- visible spectrophotometer. Enzyme activity is measured in U/ ml which is defined as the amount of enzyme catalyzing the production of 1 micromole of colored product per min per ml[3].

The activity was calculated using the given formula;

$$\text{Enzyme activity (U/ml)} = \frac{A470nm / min \times 4 \times Vt \times Dilution\ factor}{€ \times Vs}$$

G. *Enzyme purification*

Enzyme purification was done partially by ammonium sulphate precipitation and dialysis

Ammonium sulphate precipitation: The nine- day old culture was filtered using Whatmann No. 1 filter paper. The filtrate was centrifuged at 10, 000 rpm for 20 minutes at 4^0c. The pellet was discarded and supernatant was collected into a sterile beaker. Solid

ammonium sulfate was added to this supernatant up to 80% saturation. The mixture was then kept overnight at 4^0c in order to complete the precipitation. The precipitate was removed by centrifugation at 10,000 rpm for 20 min. The pellet was redis solved in minimum volume of 50 mM Sodium phosphate buffer, pH 6, and was used for further purification[7].

Dialysis: Dialysis was done to selectively remove small molecules from sample containing mixture of small and large molecules. Dialysis membrane of size 10 kDa cutoff was used for purification of isolated enzyme. The dialysis membrane supplied by the manufacturers contain about 10 % glycerol and can be removed by soaking in distilled water. The dialysis bag was cut into required size and soaked in distilled water to remove the glycerol content. The dialysis tube was then sterilized by boiling in 10 mM EDTA, 2 % $NaHCO_3$ and washed using distilled water. The tubes were stored in methanol at 4^oC and again washed with distilled water. One end of the dialysis membrane was closed and 2/3 rd of the bag was filled with the sample using a pippet. The bag was then kept in required volume of 50mMsodium acetate buffer of pH 6. The solution was dialysed overnight at 4^oC for proper purification. The buffer after dialysis was removed and the sample was collected into a fresh beaker. It was then stored at 4^0 c and used for further analysis[7].

H. Protein estimation – Lowry's method

The amount of protein present in the purified enzyme was estimated using Lowry's method. This method is based on the conversion of protein in to a Cu- protein complex under alkaline medium [8]

I. Determination molecular weight of protein

Molecular weight of the enzyme was determined using SDS- PAGE method. This was done to demonstrate the separation of protein based on the molecular weight. This method utilizes a synthetic gel called acrylamide gel which is a polymer of acrylamide[9].

J. Synthesis of silver nano particle

The nano particle of purified laccase enzyme was synthesized to reduce the size of enzyme to nano scale which makes it easy to use and apply in different fields. A solution of 3mM AgNO3 was used for the synthesis of silver nano particle. For this, 15 ml of purified enzyme was taken in a clean beaker. To this, 150 ml of 3mM AgNO3 solution was added. Mixed well and incubated overnight at 60^0 c. The color change of the solution as well as the formation of brown colored precipitate was observed[10].

K. UV-vis spectrometry

This tool has proven to be very useful for analyzing of different nanoparticles such as gold and silver nanoparticles. Observation of strong broad surface plasmon peaks at visible region (400-600 nm) has been well documented for various metal nanoparticles, with size ranging from 2 to 100 nm. The UV-Visible spectra of silver nanoparticles were recorded as a function of wavelength using UV–Vis spectrophotometer (Helios Gamma, Thermo Corporation, England) operated at a resolution of 0.5 nm. The absorbance of sample was noticed at wavelengths ranging from 250 to 500 nm using UV- Visible spectrophotometer at regular intervals to confirm the formation of nano particle [11]

L. FTIR (Fourier Transform Infrared Spectroscopy)

A small quantity of the sample was added to KBr in the ratio 1:100 approximately. The matrix was ground for 3-4 minutes using mortar and pestle. The fine powder was transferred into 13 mm diameter die and made into a pellet using a hydraulic press by applying a pressure of 7 tonnes. The fine pellet was subjected to FTIR analysis using universal pellet holder. (a single drop of oil is poured on the KBr pellet in case of liquid samples). Infrared spectral data were collected on Thermo Avtar 370 FTIR spectrometer. Spectra were collected over a range of 4000–400 cm 1at 4 cm 1 resolution with an interferogram of 32 scans [11].

M.SEM analysis

The sample was smeared on a small piece of adhesive carbon tape which was fixed on a brass stub. The sample, then subjected to gold coating using sputtering unit (model: JFC1600)for 10 sec at 10mA of current. The gold coated sample placed in chamber of SEM (Jeol, JSM 6390LA) and secondary electron/Back Scattered electron images were recorded[11]

III. RESULTS

A. Culturing of fungus

Mycelial growth were observed in PDA agar plates inoculated with P. ostreatus, P. floridaand C. indica.

B. Screening for laccase production

The appearance of reddish brown colored zone around the mycelial growth helps in the detection of laccase production. From this it was observed that P. ostreatus and P. florida showed laccase production while C. indica lacks the ability Figure (1)

C. Submerged culture

Submerged culture was done to enhance the ability of enzyme production. Two different types of liquid media (PD broth and Guaiacol incorporated liquid medium) were used. Both mycelial growth and turbiditywere observed in PD broth while mycelial growth and colour change (from off white to reddish brown) were observed in guaiacol incorporated liquid medium. Both P. ostreatus and P. florida had shown positive results while C. indica didn't show enzyme production. From the results it is clear that guaiacol incorporated liquid medium is most effective for the submerged culture Figure (2)

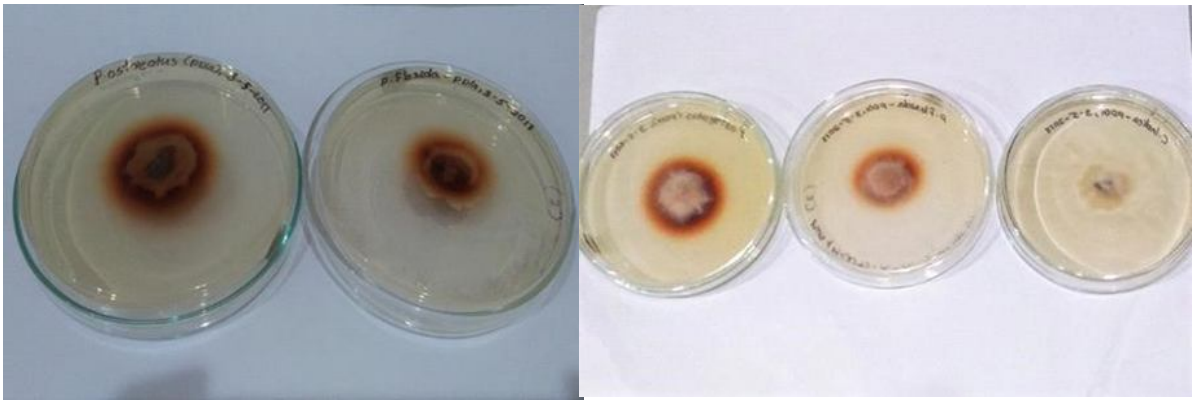
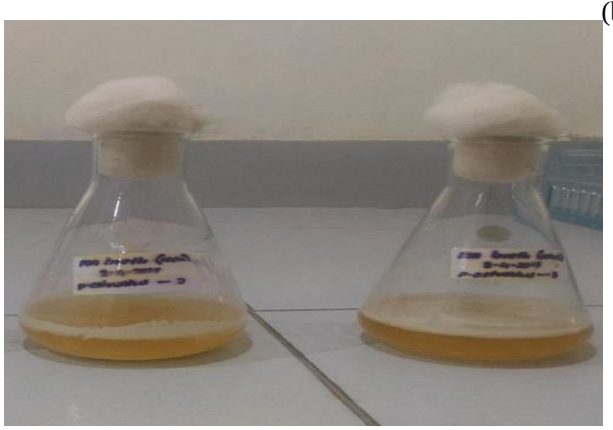


Fig. 1 Reddish brown zone around mycelial growth of P. ostreatus and P. florida

(a)



(b)

(c)

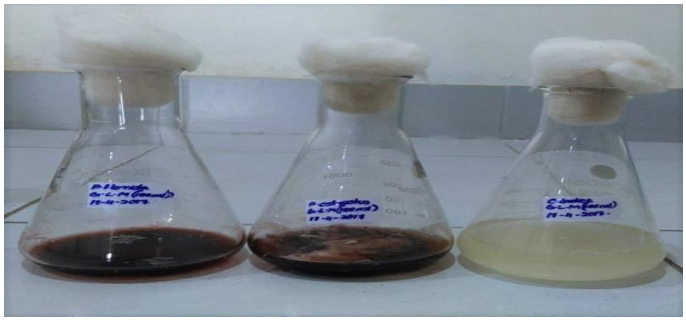


Fig. 2 (a)Submerged culture of P. ostreatus and P. florida in PD Broth (b) Submerged culture of P. florida and C. indica in PD broth (c) Submerged culture of P. ostreatus, P. florida and C. indica in guaiacol incorporated liquid medium

D. Enzyme assay

Enzyme activity is measured in U/ ml which is defined as the amount of enzyme catalyzing the production of 1 micromole of colored product per min per ml.

Enzyme assay during submerged fermentation: It was observed that the culture of *P. ostreatus* at 9th day of incubation in guaiacol incorporated liquid medium have the maximum enzyme activity (40.23× 10^{-5} U / ml) when compared to other days, Table(1).

Enzyme assay during purification**:** It was found that the enzyme solution after dialysis had higher activity (23.00 × 10^{-5} U / ml) when compared to the crude as well as ammonium sulphate precipitated enzyme solution, Table (2)

Table. 1 Enzyme assay during submerged fermentation

Sample	Days of incubation	Enzyme activity (U/ ml)
POGM5	5	0.178 × 10^{-5} U / ml
POGM7	7	23.85 × 10^{-5} U / ml
POGM9	9	40.23× 10^{-5} U / ml
POPB3	3	0.237× 10^{-5} U / ml
POPB6	6	0.178× 10^{-5} U / ml
POPB7	7	2.37× 10^{-5} U / ml
POPB9	9	0.118× 10^{-5} U / ml
PFGM3	3	0.296× 10^{-5} U / ml
PFGM5	5	0.118× 10^{-5} U / ml
PFGM7	7	0.237× 10^{-5} U / ml
PFGM9	9	0.712× 10^{-5} U / ml
PFPB3	3	0.712 × 10^{-5} U / ml
PFPB5	5	0.356× 10^{-5} U / ml
PFPB7	7	0.652× 10^{-5} U / ml
PFPB9	9	0.059 × 10^{-5} U / ml

Table .2 Enzyme assay during purification

Sample	Enzyme activity (U/ml)
Crude sample(C)	1.24 × 10^{-5} U / ml
Supernatant after precipitation (S)	0.89 × 10^{-5} U / ml
Pellet dissolved in distilled water (P.W)	1.78 × 10^{-5} U / ml
Pellet dissolved in phosphate buffer (P.B)	4.68 × 10^{-5} U / ml
Dialysed sample (D)	23.0 x 10^{-5} U / ml

E. Enzyme purification

Enzyme purification was done by 4 steps ie, Filtration, Centrifugation, Ammonium sulphate precipitation and dialysis

1) *Filtration*: Filtration was done by using What mann No. 1 filter paper. As a result, large derbies like media components, fungal mycelium, and all other precipitates were removed from the liquid medium that contain the extracellular enzyme.
2) *Centrifugation:* Centrifugation helped in removing fine particle present in the medium. This was done at 10,000 rpm for 20 minutes. The supernatant that contain the enzyme was stored at -20^{0}c while the pellet was discarded. The enzyme activity after centrifugation has been found to be 2.48×10^{-5}IU/ ml.
3) *Ammonium sulphate precipitation:* The precipitation was done by using ammonium sulphate at a concentration of 80 % saturation. As a result, the enzyme contents were precipitated out from the medium after overnight incubation at 4^0 c. The precipitate formed was collected and stored at -20^{0}c**.** The enzyme activity after ammonium sulphate precipitation was found to be 3.56×10^{-5} IU/ ml.
4) *Dialysis:* Dialysis was done using dialysis bag having cut off size of 10 kDa. This helps to remove particles having a size less than 10 kDa. All the impurities present in the crude sample was separated into the buffer by keeping the sample overnight at 4^{0}c in the buffer. The sample remaining in the dialysis bag with an enzyme activity 46×10^{-5} IU/ ml was then collected and stored.

Table. 3 Determination of purification factor

Purification stage	Volume (ml)	Protein mg/ ml	Enzyme activity (IU/ ml)	Enzyme activity/ mg protein (L)	Purification factor
Crude	10	85.32	2.48×10^{-5}	0.0290	_
80 % precipitation	5	66.66	3.56×10^{-5}	0.0534	1.84
Dialysis	5	74.64	46×10^{-5}	0.616	11.53

F. SDS PAGE

The SDS PAGE separation showed that the purified enzyme have a molecular weight of approximately 60 kDa when compared with BSA standard, Figure (3).

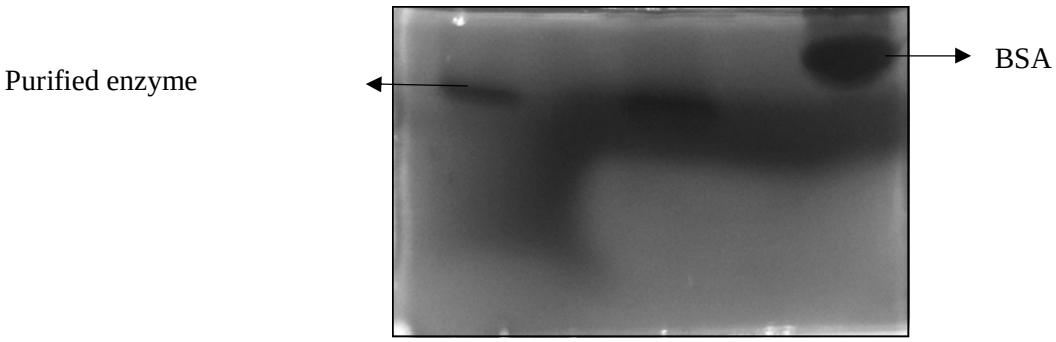


Fig. 3 Determination of molecular weight using SDS- PAGE

G. Synthesis of silver nanoparticle

In this study, AgNPs were synthesized using reduction of aqueous Ag+ with the culture supernatants of Pleurotus ostreatusat room temperature. It was generally recognized that AgNPs produced brown solution in water, due to the surface plasmon resonances (SPR) effect and reduction of AgNO3. After the addition of AgNO3 solution, the cell filtrate of Pleurotus ostreatus changed from light yellow to brown within a few hours, while no color change was observed in the culture supernatant without AgNO3. Thus, color change of the solution clearly indicated the formation of AgNPs, Figure (4). The color intensity of the cell filtrate with AgNO3 sustained even after 24 h incubation, which indicated that the particles were well dispersed in the solution, and there was no obvious

aggregation. All these reactions were monitored by ultraviolet-visible spectroscopy of the colloidal AgNPs solution. The formation of nanoparticle was then confirmed by SEM and FTIR.

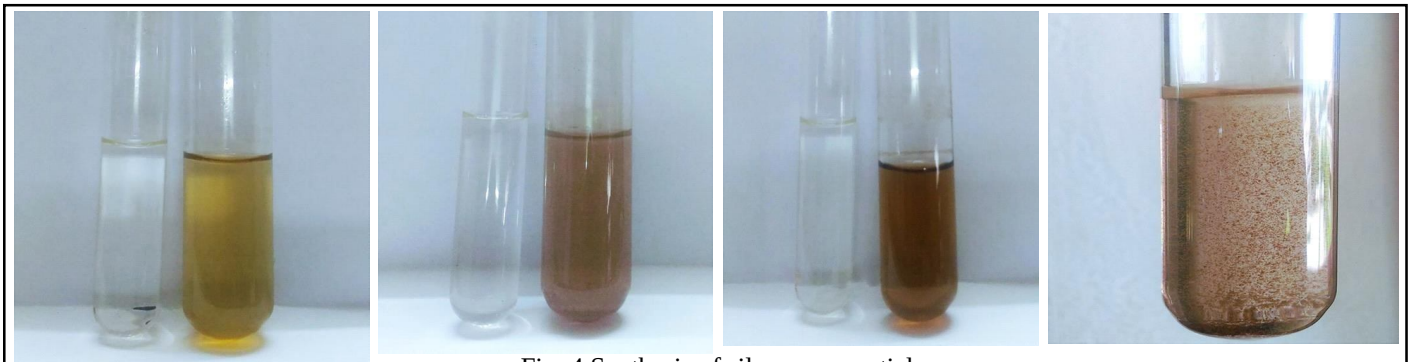


Fig. 4 Synthesis of silver nanoparticle

H. UV-VIS spectroscopy

The surface plasmon absorption bands at range 300-450 nm were observed with 3mM concentrations of AgNO3 solutions. The ultraviolet-visible spectra of the cell filtrate with $AgNO_3$ showed a strong broad peak at 440 nm which is due to surface Plasmon resonances (SPR band) of AgNPs, Figure (5).

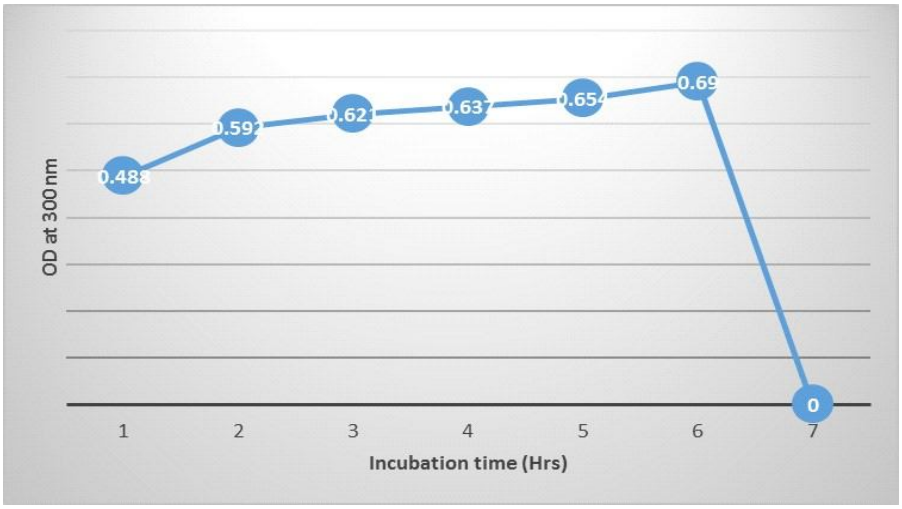


Fig. 5 UV- Vis spectroscopy

I. Characterization of silver nano particle

Characterization was done by using a variety of techniques, mainly drawn from material science.To broaden the application scope, the $AgNO_3$ obtained were systematically characterized using SEM and FTIR analysis.

1) *SEM Analysis:* The Scanning Electron Microscope (SEM) image of the silver nanoparticle synthesized indicates well dispersed particles that are more or less spherical, Figure (6).
2) *FTIR analysis:* FTIR spectroscopy is a useful technique to study the core–shell morphology of AgNPs. FTIR measurements of silver nanoparticles and their bands were observed at 3331- 557cm-1. Numerous absorbance bands were observed for various functional groups present in the silver nanoparticles. The specific absorbance bands of the crude sample corresponding to wave numbers 3331cm^{-1}635cm^{-1}, 1544 cm^{-1},1442 cm^{-1},1384 cm^{-1},1265 cm^{-1},1225 cm^{-1},1072 cm^{-1},1033 cm^{-1},771 cm^{-1}, 551 cm^{-1} were obtained. The two broad bands at ~1635.93 and ~3331.42 were observed and can be attributed to NH and -OH stretch vibrations in the amide linkages of the protein correspondingly. The specific absorbance bands of the purified sample corresponding to wavenumbers 3,373cm1650cm^{-1}, 1450 cm^{-1},1393 cm^{-1}, 1258 cm^{-1},1078 cm^{-1},984 cm^{-1},858 cm^{-1},617 cm^{-1},7 514 cm^{-1} were obtained Figure (7).

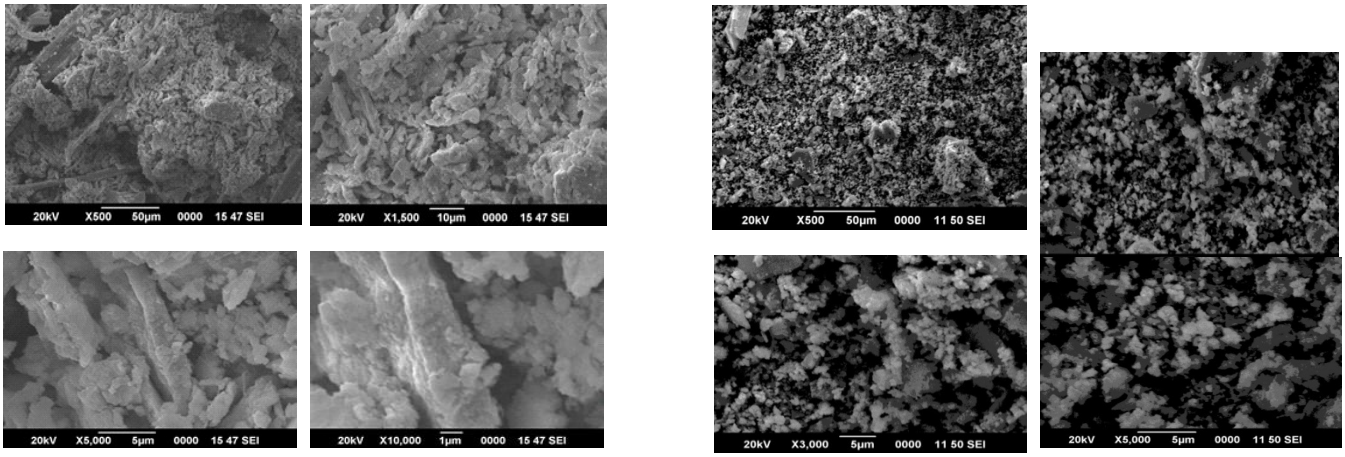


Fig. 6 SEM analysis of nanoparticle (Crude) SEM analysis of nanoparticle (Purified)

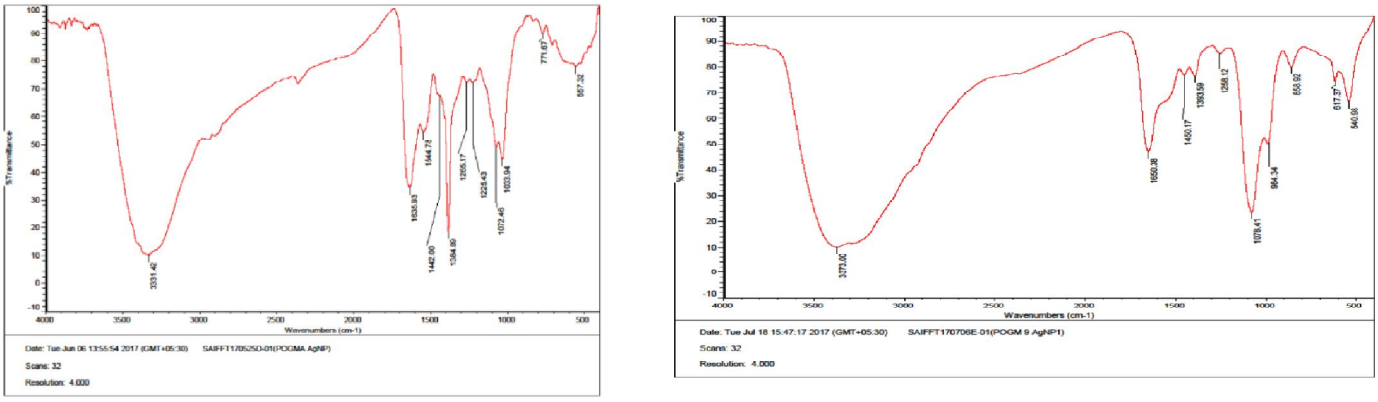


Fig. 7 FTIR Analysis of nanoparticle (crude) FTIR Analysis of nanoparticle (Purified)

IV. DISCUSSION

Laccases have been studied with respect to their biological function, substrate specificity, copper binding structure, and industrial applications [2].This research has focused on screening the three fungi (P. ostreatus, P. florida and C. indica) for the production of industrially important laccase enzymes. The three fungi used in this study are locally used as additives in both traditional and local foods. Here an attempt is made to isolate, purify and characterize the laccase enzyme from Pleurotus species.

Two different agar media were tested for the culturing of these fungi ie, PDA and SDA. The initiation of mycelium growth was recorded on 2nd day of inoculation and among the two media used in the studyPDA favoured maximum growth for all the three cultures. All the three fungi showed maximum growth on this medium and were used for the submerged fermentation. The production of extracellular laccase enzyme was monitored using another medium ie, PDA incorporated with 0.02% guaiacol. The enzyme production was characterized by the formation of reddish brown zone around the mycelial growth. The submerged cultures P. ostreatus and P. florida showed reddish brown zone indicating laccase production after6 days of incubation.

Submerged fermentation was carried out using two different liquid media called PD broth andGuaiacol incorporated liquid medium. Among these, Guaiaccol incorporated medium was the best to produce maximum enzyme activity as indicated by laccase enzyme activity. P. florida and P. ostreatus showed maximum laccase activity on 9th day after inoculation. Previous reports by Palmeiri et. al also suggests that P. ostreatus exhibited maximum laccase production on 8th day of incubation using 0.5% malt extract based nutrient medium. Laccase production by white rot fungi has been shown to depend on the composition of the cultivation medium such as carbon, nitrogen sources and inducer [13, 14]. Similar observation was made with Pycnoporus cinnabarinus and Phlebia radiate, a white rot fungus [15, 16] demonstrated that higher production of laccase in agro waste based liquid medium. The enzyme activity of the fungi was determined using the guaiacol oxidation method. The enzyme present in the sample oxidizes the guaiacol in presence of acetate buffer to yield a colored complex that can be measured spectro photometrically at 470 nm. It was found that P.

ostreatus in guaiacol incorporated liquid medium of 9th day culture showed the maximum laccase activity (POGM9) ie, 40.23 × 10-5 U / ml (Table 1)

Protein with laccase activity was then purified to homogeneity from the basal medium. During the first step,laccase was separated from the fungal mycelium andmedia components by filtration and further by centrifugation. Ammonium sulphate precipitation helped to precipitate the protein content from filtrate at a concentration of 80 % saturation. Laccase enzyme was further purified using dialysis bag with molecular weight cut off 10 kDa. After the dialysis laccase enzyme was found to be purified about 11.53 folds when compared to the crude enzyme and the molecular mass was found to be 60 kDa as determined by SDS-PAGE. The purified laccase from P. ostreatus was found to be homogeneous with a single protein band. Previous reports of molecular mass of laccases from white-rot basidiomycetes had reported to be ranging from 55 to 65 kDa. Further, the relative molecular mass of laccase purified from Lentinula edodes and Phoma sp. reported to be 58.5 and 75.6 kDa, respectively [17].

The silver nanoparticles of laccase enzyme was then synthesized using a reduction of aqueous Ag+ with the partially purified enzyme of P. ostreatus at 60^0c. It was generally recognized that AgNPs produced brown solution in water, due to the surface plasmon resonances (SPR) effect and reduction of AgNO3 [18]. The characteristic brown color was due to the excitation of Plasmon vibrations in the nanoparticles that provides a convenient signature of their formation. The ultraviolet-visible spectra of the cell filtrate with AgNO3 showed a strong broad peak at 300 nm, which indicated the presence of AgNPs. These results were consistent with the previous reports of Maurya et.al. The absorbance range of prepared silver nanoparticles solution was checked on 1st hour, 5th hour and 7th hourand there was no obvious in peak position for hours, depicting its stability. From this, it can be concluded that the AgNPs formed were quite stable in the laccase enzyme solution of P. ostreatus.

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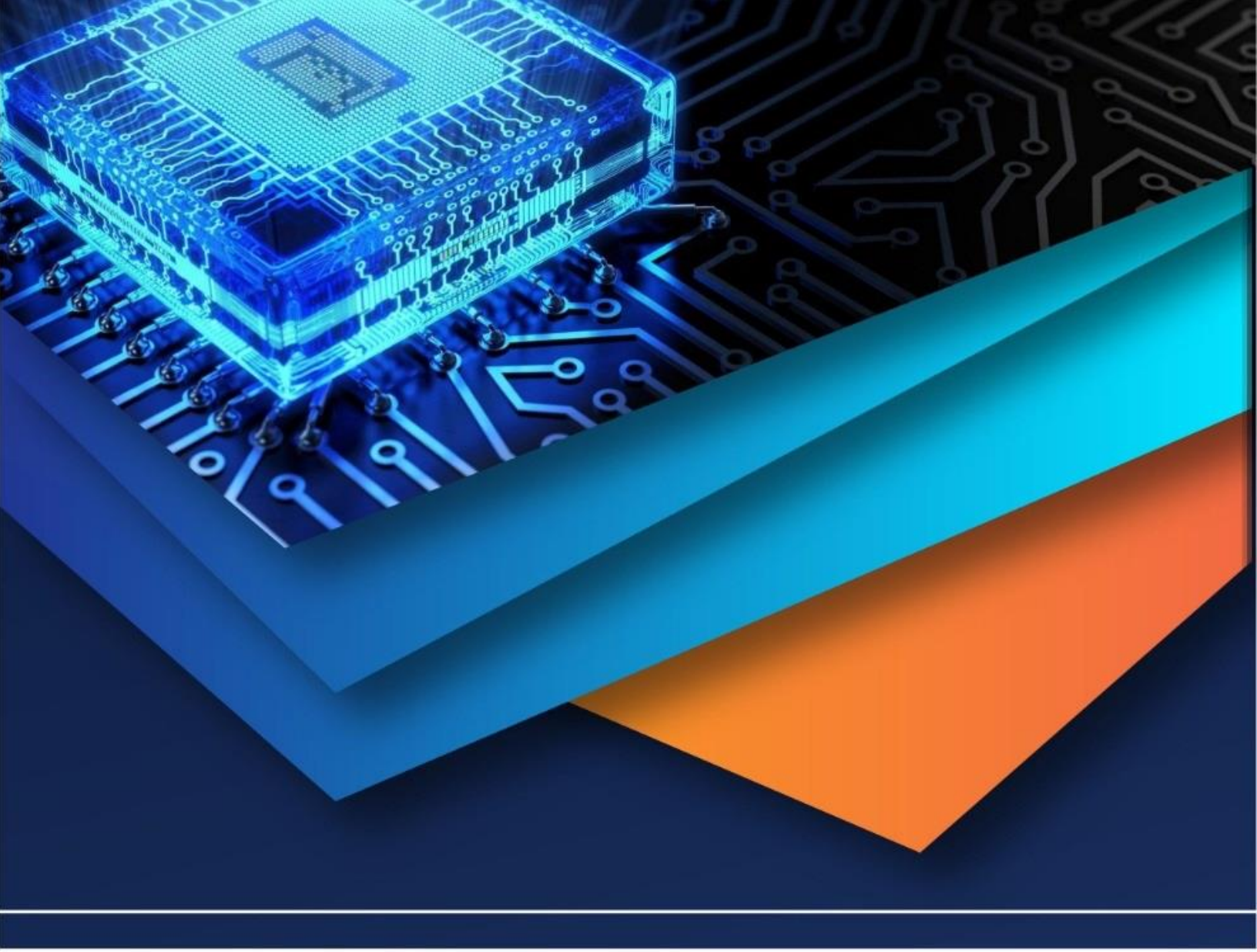

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