International Journal of Research and Review
www.ijrrjournal.com E-ISSN: 2349-9788; P-ISSN: 2454-2237

*Original Research Article*

# Biowaste as Substrate for Laccase Production

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*Received: 25/08/2016* *Revised: 02/09/2016* *Accepted: 07/09/2016*

## ABSTRACT

Approximately 700 million tons of organic waste such as food waste, livestock manure, vegetable waste and wastewater sludge is produced in India each year. Only 0.5% of these wastes are appropriated as inputs for various processes while the rest is regarded as landfills or dumped. Laccases are multicopper enzymes belonging to the group of blue oxidases. Laccase is widely distributed in higher plants and fungi isolated from soil litter, humus and wood. Potato dextrose media and standard media were used for the production of laccase and the same were incorporated with bio-waste- cauliflower waste and wood apple waste. The production was carried out at 120rpm at 28$^0$C for 5 days. The concentration of protein, enzyme activity and % dye decolorization was measured. From this study we can conclude that media incorporated with bio-waste form potential substrates for production of laccases. Out of the two bio wastes used cauliflower waste and wood apple waste, media incorporated with cauliflower waste showed maximum concentration of protein, enzyme activity and % decolorization of dye. Media incorporated with both cauliflower waste and wood apple waste also showed a fairly good amount of protein, enzyme activity and % dye decolorization. The process with optimized fermentation parameters could be used for scaling up of the process to a pilot scale or commercial fermenter level.

***Key words:*** Laccase, Fusarium, screening, biowaste, production.

## INTRODUCTION

Soil litter or leaf litter is a dead plant material, such as leaves, needles, bark and twig, that has fallen to the ground. The dead organic material and its constituent nutrients are added to the top layer of soil, commonly known as the litter layer or O horizon ("O" for "organic"). Litter has been in use by various ecologists for various reasons like that it is an instrumental factor in ecosystem dynamics, is indicative of ecological productivity, and may be useful in predicting regional nutrient cycling and soil fertility. Litter provides a proper habitat for a variety of organisms. Many organisms that live on the forest floor are decomposers, such as fungi. Organisms whose diet consists of plant detritus, such as earthworms, are termed detritivores. The community of decomposers in the litter layer also includes bacteria, amoeba, nematodes, rotifer, springtails, cryptostigmata; pot worms insect larvae, mollusks, mites, woodlice, and millipedes. [1] The consumption of the litter fall results in the breakdown of simple carbon compounds into Carbon dioxide ($CO_2$) and water (H2O) and releases inorganic ions (like nitrogen and phosphorus) into the soil where the surrounding plants can then reabsorb the nutrients that were shed as litter fall for their metabolic process. In this way, litter fall becomes an important part of the nutrient cycle that sustains forest environments. Microorganisms play a major in litter decomposition. Fungi typically

dominate soil microbial biomass during the early stages of litter decomposition. [2]

Fungi play a significant role in human life, besides their utilization in industry, agriculture, medicine, food industry, textiles and bio remediation. [3] Fungi are a source of large number of secondary metabolites, enzymes, penicillin, ergotrate and statins being some of the well-known compounds. Among enzymes laccases are the most commonly produced ones. [4]

Laccases are multicopper enzymes belonging to the group blue oxidase which exists widely in nature and are defined in the Enzyme Commission (EC) nomenclature as oxidoreductases which oxidize diphenols and allied substances and use molecular oxygen as an electron acceptor. [5-8] they are predominantly found in higher plants and fungi. [5,6,9] Laccases act on a surprisingly broad range of substrates, including diphenols, polyphenols, different substituted phenols, diamines, aromatic amines, benzenethiols and even some inorganic compounds such as iodine. [5,10] Laccases from fungi have been implicated in lignin degradation, in differentiation and in protection from toxic phenolic monomers of polyphenols. Laccases are used for many industrial purposes such as paper processing, prevention of wine discoloration and detoxification of environmental pollutants, oxidation of dye, production of chemicals from lignin. [5] Laccases can transform toxic compounds into safer metabolites and may be useful to control environmental pollution. [5,11] Laccases are also useful for the decomposition of azo dyes by oxidative methods. [5,12] Screening of a large number of microbes is, therefore, necessary to select strains that are able to produce high titers of laccases with novel characters. Microbes that produce laccases have been screened for either on solid media containing colored indicator compounds that enable the visual detection of laccase production [13-16] or with liquid cultivation monitored with enzyme activity measurements. [13,17-19] Laccase can be produced at varying rates by using a wide range of organisms grown on different substrates and by using several methods of fermentation, such as solid state, semisolid state, and submerged. [20-24] However, for effective laccase production, it is very important to use efficient laccase-producing organisms, suitable fermentation methods, and cheap and widespread sources. Accordingly, one of the most suitable approaches for the production of this enzyme is to use the most efficient biowaste for increasing the production of the ligninolytic enzymes. [20,25]

Approximately 700 million tons of organic waste such as food waste, livestock manure, vegetable waste and wastewater sludge is produced in India each year. [26, 27] Owing to the inherent biodegradable characteristics of fruits and vegetables, the total wastes generated from them accounts for about 50 million tons per annum. Only 0.5% of these wastes are appropriated as inputs for various processes [28] while the rest is regarded as landfills or dump yards. [26]

The main aim of this study was to investigate the feasibility of using biowaste - cauliflower waste and wood apple shell waste as natural low cost substrates for laccase production using laccase producing fungi isolated from various sources.

## MATERIALS AND METHODS

### Fungal isolation

Soil Litter was collected from the garden soil at Begumpet, in a sterile beaker and transported to laboratory, mixed with 100ml sterile water and inoculated on petriplates containing Potato Dextrose Agar medium amended with pinch of streptomycin to inhibit bacterial growth. After incubation at 25$^{o}$C for 3-4 days, the plates were observed for growth of fungal colonies. The cultures were purified by repeated transfer to agar plates and grown at 25°C for 4 days. These cultures were then screened for laccase production. The morphological analysis was done based on Lacto phenol cotton blue staining.

**Screening test**

The ability of the fungal strains to secrete extracellular laccase was visualized according to the method of Kiiskinen *et al.* [2004]. The assay plate contained 15 ml of 4% potato dextrose agar amended with 0.01% of guaiacol. The plates were incubated at 30 C for 1-3 days. The presence of brick red color around the mycelium was considered as guaiacol oxidizing laccase secreting organism. [13]

Production of laccase using Synthetic media (Songulashvili *et al.*, 2006) incorporated with bio-waste.

**Preparation of bio-waste**

The bio-waste used dried in air at room temperature for 5 days and then the air-dried wastes were mechanically pretreated by chopping or pulverization to reduce the size of the bio-waste and thus facilitate laccase production. [20]

**Production by submerged fermentation**

Four flasks with 50ml of synthetic media were taken. One of the flasks without the incorporation of bio-waste was used as control. Into the next two flasks 1g of cauliflower waste and 1g of wood apple waste were added. Into the fourth flask 0.5g of cauliflower waste and 0.5g of wood apple waste were added to check the cumulative effect. [29] The flasks were sterilized at 10lbs and actively growing culture was inoculated. The flasks were incubated in orbital shaker at 120rpm at $28^0$C for 5 days.

**Estimation of total protein content**

The protein content present in the sample was estimated by [30] method. Bovine serum albumin (BSA) was used as a standard (660) nm. The color development was read at 660nm in spectrophotometer. [31]

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 activity was calculated as shown in Equation 1. The enzyme activity was expressed in enzyme unit (U) per ml (U ml-1). [32,33]

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A470\ \text{nm/min} \times 4 \times VT \times \text{dilution factor}}{€ \times Vs}$$

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

**Application of crude enzyme for dye removal**

Crude Enzyme was used for removal of various dye like 1mM Malachite green and 4.9 mM Coomassie blue R-250. 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 and 546nm for Malachite green, Coomassie blue R-250, respectively after incubation with enzyme and before incubation with enzyme. [32] Decolorization activity was calculated using given formula [34]

$$\text{Decolorisation Activity} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100$$

## RESULT AND DISCUSSION

**Isolation and Screening of Fungal culture for laccase production**

A total of 7 fungal colonies F1-F7, were isolated from the soil as shown in figures 1,2,3,4. The cultures F1 to F7 were maintained on PDA plates for further experiments. They were screened for their potential to produce laccase using guaiacol. Based on the observed colony morphology and the microscopic characteristics, the fungi were identified by comparing the characters with those specified in the Descriptions of Fungi-Second Edition and Diana S. et al, 2014.

Based on the colony morphology (fig 1,2,3,4 and table 1) and the microscopic observation (fig 5,6,7 and table 1) the samples F1 and F3 are identified to be *Mucor sps*, F2 and F6 are identified as *Aspergillus sps*, F5 and F7 are identified as *Rhizopus sps* and F4 is identified as *Fusarium sps.*

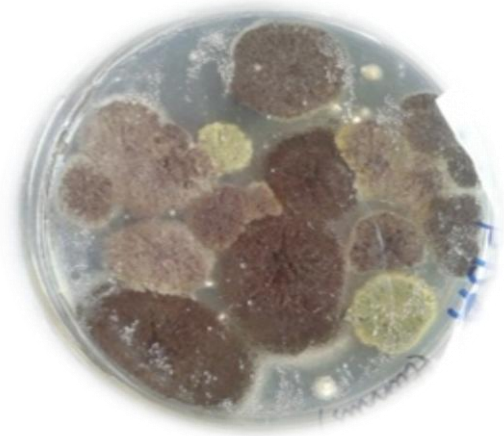

**Fig1: Fungi isolated from Humus**

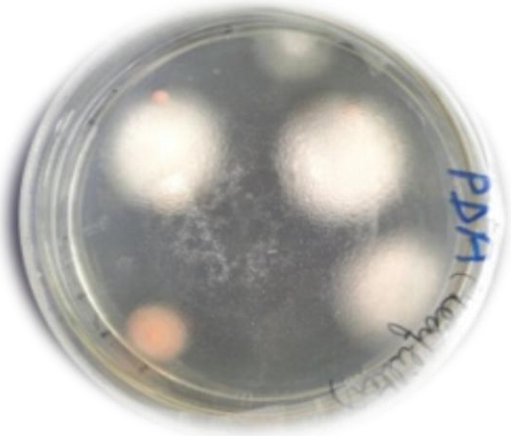

**Fig 2: Fungi isolated from leaf litter F4**

**Fig 3: Fungi isolated from wood**

**Fig 4: *Fusarium* pure culture**

**Table: 1 Colony morphology and Microscopic observation of Fungi isolated from soil and litter**

| S. No. | Sample | Colony morphology | Microscopic observation |
|---|---|---|---|
| F1 | Humus | 3cm, flat, dark brown colonies radiating out, no reverse pigmentation | Conidia heads are columnar, conidiophore stipes are short, conidia are globose and are produced in basipetal succession |
| F2 | Humus | 4cm, flat granular yellowish green colonies, with radial grooves, showing no reverse pigmentation | Radiate conidial heads, biseriate or having some heads with phialides borne directly on the vesicle (uniseriate), hyaline conidiophore stipes bear pale green and echinulate, globose conidia |
| F3 | Humus | 3cm, raised light brown colony with no reverse pigmentation | Erect sporangiophores forming large spherical multispored sporangia |
| F4 | Soil litter | 4cm, slightly raised, colonies with white hyphae | Microconidia are usually abundant, cylindrical to oval, one- to two-celled and formed from long lateral phialides, 8-16 x 2-4.5 µm. Chlamydospores are hyaline, globose, smooth to rough-walled, borne singly or in pairs on short lateral hyphal branches |
| F5 | Soil litter (direct inoculation) | Lawn growth showing white mycelia, which becoming brownish black due to sporulation, no reverse pigmentation | Sporangiophores are smooth-walled, non-septate, simple or branched, arising from stolons opposite rhizoids in groups of three or more, globose sporangia with a flattened base, greyish black, powdery in appearance containing many spores |
| F6 | Wood sample | 3cm, flat, granular, yellowish green colony, white hyphae, no reverse pigmentation. | Radiate, Black, biseriate conidial heads, conidiophores are smooth, globose to subglobose conidia |
| F7 | Wood sample | 1.5cm White cottony colony, turning blackish grey due to sporulation | Sporangia are smooth walled, non septate, branched arising from stolons, globose with flattened base |

**Table 2: Concentration of Protein in Synthetic Media (SM)**

| S No | Sample | Concentration of protein mg/ml |
|---|---|---|
| 1 | SM | 0.24 |
| 2 | SM+CW | 0.32 |
| 3 | SM+WA | 0.28 |
| 4 | SM+CW+WA | 0.25 |

**Table 3: Enzyme activity**

| S. No. | Sample | Enzyme activity U/ml |
|---|---|---|
| 1 | SM | 0.00076 |
| 2 | SM+CW | 0.0033 |
| 3 | SM+WA | 0.0009 |
| 4 | SM+CW+WA | 0.0031 |

***Microscopic Observation:***

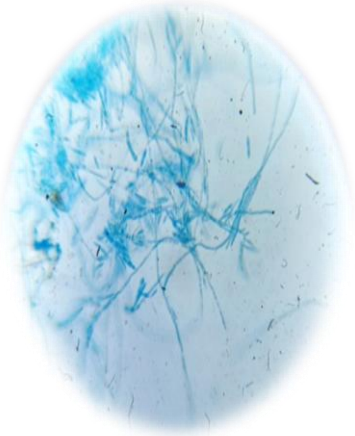

**Fig 5: Fusarium sps**

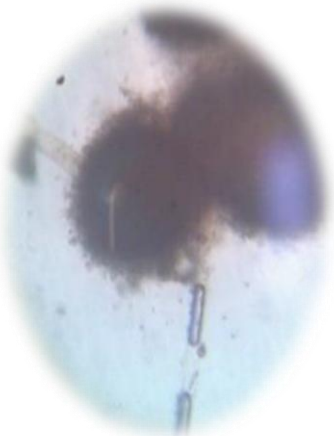

**Fig6: Aspergillus Niger**

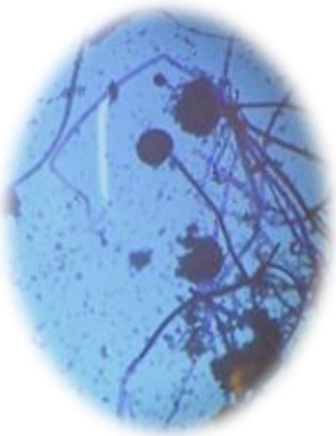

**Fig 7: Rhizopus sps**

**Screening of laccase producers**

All the isolates F1-F7 were tested for laccase activity according to the method of Kiiskinen *et al* which was confirmed with guaiacol assay. Among the 7 isolates, isolate F4 -*Fusarium sps* was positive for laccases (fig. 8). This was visualized by presence of brick red color around the mycelium and was considered as guaiacol oxidizing laccase secreting organism and hence it was used for further study.

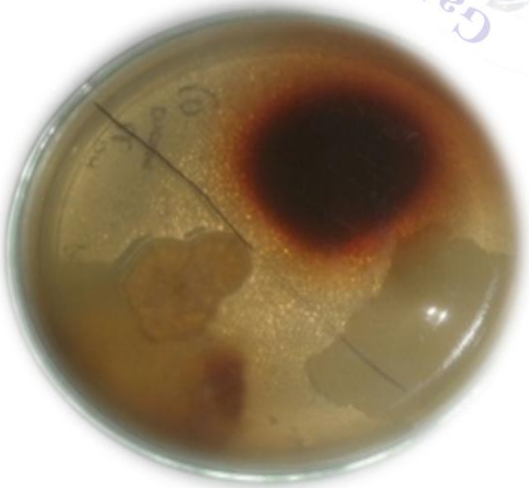

**Fig.8 screening for laccase producer**

**Protein estimation:** The protein reacted with Folin Ciocalteu reagent (Lowry's reagent) and formed a complex which is blue purple color complex which was read at 660nm in spectrophotometer. Bovine serum albumin (BSA) was used as a standard

From Table 2, it can be inferred that there is a significant increase in Protein concentration when the media was incorporated with Cauliflower and wood apple shell waste when compared to the media with glucose as the carbon source. The media incorporated with the cauliflower waste showed a significant increase in the protein concentration.

**Laccase activity**

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. Laccase activity was calculated as shown in the Equation.

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

The enzyme activity and protein concentration was maximum in media incorporated with cauliflower waste (Table 3). Significant increase in protein concentration and enzyme activity was also observed when the media was incorporated with cauliflower waste and wood apple together when compared to media incorporated with only wood apple waste. The results of enzyme activity correlate with that of protein. The protein concentration and enzyme activity is maximum for the media incorporated with cauliflower waste.

Application of crude enzyme for dye decolourisation In order to study the dye removal ability of the laccase produced by

the fungi, a dye removal experiment was performed with different dyes. The dye decolourising activity of lacasses was performed using Malachite green and Coomassie Brilliant blue R- 250. Decolourisation activity was calculated using given formula.

$$\text{Decolourisation Activity} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100$$

**Table 4: % decolourisation of Malachite Green**

| S no | Media | % removal |
|---|---|---|
| 1 | SM | 25 |
| 2 | SM+CW | 66 |
| 3 | SM+WA | 33 |
| 4 | SM+CW+WA | 41.6 |

SM- Synthetic Media, CW- cauliflower waste, WA- Wood Apple

Table 4 represents the dye decolorization ability of laccase produced using different media incorporated with biowaste. The dye decolorization and enzyme activity was maximum for laccase produced using the media incorporated with biowaste which corresponds with the protein concentration.

## CONCLUSION

Fungal laccases are involved in the degradation of lignin or in the removal of potentially toxic phenols arising during lignin degradation. From this study we can conclude that media incorporated with biowaste form potential substrates for production of laccases.

Out of the two biowastes used - cauliflower waste and wood apple waste, media incorporated with cauliflower waste showed maximum concentration of protein, enzyme activity and % decolourisation of dye. Media incorporated with both cauliflower waste and wood apple waste also showed a fairly good amount of protein, enzyme activity and % dye decolourisation.

The process with optimized fermentation parameters could be used for scaling up of the process to a large scale production.

## ACKNOWLEDGEMENTS

The authors thank Management of St. Francis College for Women, Begumpet, Hyderabad, Telangana for permitting us to carry out the work and also Dr. M. Shailaja Raj for her support and Guidance.

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How to cite this article: Malhotra S, Roselin P. Biowaste as substrate for laccase production. Int J Res Rev. 2016; 3(9):46-53.

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