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Research Paper

COLLECTION AND SCREENING OF BASIDIOMYCETES FOR BETTER LIGNIN DEGRADERS

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Considering the potentialities of white rot basidiomycetes in biobleaching process, 37 white rot fungi were collected from different forest areas of Andhra Pradesh, India. All of them were screened for lignolytic enzyme production. Out of them 25 different organisms were with lignolytic capacity. Then they were quantitatively and qualitatively analyzed for Laccase, LiP and MnP enzymes. Among the studied organisms *Stereum ostrea* (Laccase 40.02U/L, MnP 51.59U/L, LiP 11.87U/L), *Tremella frondosa* (Laccase 35.07U/L, MnP 29.12U/L, LiP 5.95U/L) *Tremates versicolour* (MnP and LiP production i.e 56.13 U/L, LiP 23.26 U/L) could show maximum enzyme production. All the 25 organisms could produce Laccase but few failed to produce MnP and LiP. The organisms which produced both enzymes were grown in the liquid cultures. That culture filtrate was used for qualitative (SDS PAGE) and quantitative (enzyme assay) analysis.

Keywords: Basidiomycetes, White rot fungi, Lignolytic enzymes, Laccase, MnP.

INTRODUCTION

Lignin is the most abundant renewable aromatic polymer and is known as one of the most recalcitrant biomaterials on earth Crawford (1980). Its degradation plays a key role in the carbon cycle of the biosphere Tein (1987). Only white rot fungi are found to be responsible for the complete mineralization of this polymer. Lignin degrading fungi are classified into three types according to their decay pattern: brown rot, white rot (i.e., selective delignification and simultaneous rotting) and soft rot (types 1 and 2). The most efficient lignin degraders in nature are white rot

fungi and their ability to degrade complex and recalcitrant organic molecules also makes them attractive microorganisms for bioremediation of soil contaminated by organic pollutants. Fungal attack is an oxidative and non-specific process, which decreases methoxy, phenoxy and aliphatic content of lignin cleaves aromatic rings and creates new carbonyl groups Hatakka A. (2001). These changes in the lignin molecule result in depolymerisation and carbon dioxide production Kirk TK and Farrell (1987). Microorganisms do not gain energy from lignin degradation but the degradation enables efficient utilization of

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carbohydrates Eriksson KEL *et al.* (1990). Thus microorganisms which utilize polysaccharides often possess lignolytic capability lignin is finally degraded to CO₂, water and humus.

Laccases (benzenediol: oxygen oxidoreductase EC 1.10.3.2) belong to multicopper oxidase family Alcalde (2007). These copper-containing enzymes catalyze the oxidation of various substrates with the simultaneous reduction of molecular oxygen to water Yaropolov *et al.* (1994). Laccases oxidizes phenolic rings to phenoxy radicals.

Manganese peroxidases (EC 1.11.1.13) belong to the family of oxidoreductases Hammel and Cullen(2008). Manganese peroxidase (MnP) oxidizes Mn²⁺ to Mn³⁺, which oxidizes phenolic structures to phenoxy radicals (Hofrichter, 2002). The product Mn³⁺ is highly reactive and complex with chelating organic acid, as oxalate or malate, which are produced by the fungus Mäkela *et al.* (2002). The redox potential of the Mn peroxidase system is lower than that of lignin peroxidase and it has shown capacity for preferable oxidize *in vitro* phenolic substrates.

Lignin peroxidases (EC 1.11.1.14) belong to the family of oxidoreductases Hammel and Cullen,(2008). LiP is an extracellular heme-protein, dependent of H₂O₂, with an unusually high redox potential and low optimum pH Erden *et al.*(2009). LiP is capable of oxidizing a variety of reducing substrates including polymeric substrates Oyadomari *et al.* (2003). Due to their high redox potentials and their enlarged substrate range LiP have great potential for application in various industrial processes (Erden *et al.*, 2009). LiP shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules Barr and Aust (1994).

Lignin peroxidases non-phenolic units of lignin by removing one electron and creating cat ion radicals, which will then decompose chemically. Lignin peroxidase preferentially cleaves the C α -C β lignin molecule but is also capable of ring opening and other reactions.

During the past 15 years chemistry and biochemistry of lignin biodegradation by white rot basidiomycetes have considerably advanced by means of chemical analysis of the biodegraded lignin and studies of the degradation mechanism of lignin substructures model compounds by lignolytic cultures of these basidiomycetes and their enzymes.

Such interesting findings lead the author to take up the project of collecting the different local white rot fungi to explore their potentialities for the different industrial use, especially in paper and pulp industries. The organisms were collected from different timber depots and forests of Warangal, Karimnagar and Khammam districts of Andhra Pradesh, India. Nearly 97 organisms were collected but few of were spoiled soon after collection but few of them could not grow. Out of 97 organisms 37 were survived and their slants were maintained and organisms were identified. The organisms which were found positive in screening test they were further studied for qualitative and quantitative enzyme analysis.

MATERIALS AND METHODS

Collection of White Rot Fungi

Fungi in the form of fruit bodies were collected from forests, timber depots in Warangal, Andhra Pradesh. They were placed into plastic bags. Fruit bodies of fungi were cleaned with disinfectants and approximately 3 x 3 mm was placed on MEA agar medium in petri-dishes. Later on, when the

mycelium had grown on the medium in the vicinity of the tissues, the sample was transferred to fresh agar media in tubes. This was repeatedly carried out until pure cultures could be obtained as single cultures or so called fungal isolates. The samples were marked with information such as number, procurement location, growth site and specific characteristics.

Identification and Maintenance of White Rot Fungi

White rot fungi were identified based on their characteristics of the fruit bodies i.e. corticioid (effused), stereoid (effuso reflexed), corolloid, dimidiata (shelf's or brackets) cyphelloid (capulate), polyporoid, agaricoid and boletoid. Macro morphological characters like color, shape, size and odor of sporocarps and micro morphological features like spore characteristics, pileus anatomy were observed for identification Krieger (1967), Suhirman (2005). The pure cultures of white rot fungi were sub cultured for every one month and were stored at 4° C until for further use.

Selection of White-Rot Fungi for Lignolytic Activity

The selected 37 white-rot fungi were tested for the production of lignolytic enzymes. These isolates were inoculated aseptically onto presolidified malt extract agar medium containing 0.05 % guaiacol and then incubated at room temperature for 7 days. Lignolytic positive organisms developed colored zone.

Liquid Cultures

The 12 organisms which produced Laccase and MnP were cultivated in Treveor's media (1gm peptone, 20gm malt extract and 20gm Dextrose and 1L distilled water) along with 10% Black liquor which contain 90% Lignin was supplied by

Kamalapur Rayon's Factory, AP, India. Thus prepared broath (100ml) was taken in conical flasks sealed with cotton plugs and sterilized at 15 lbs for 10 min. Then each organisms (12) were aseptically inoculated in triplicates. After 7 days of incubation culture filtrate was separated and used it for different analysis.

Enzyme Assays

Enzyme Activities of Laccase Coll *et al.* (1993), MnP Paszczyński *et al.* (1985) and LiP Sarkanen *et al.* (1970) were measured using guaiacol as substrate. For these enzymes, one activity unit was defined as the amount of enzyme necessary to oxidize one μ mol of substrate per minute.

SDS PAGE

Sample Preparation

Sample buffer was prepared by adding 5ml of 50% glycerol, 2ml of 10% SDS 0.5ml of 2-mecapto ethanol, 1ml 1% Bromophenol Blue, 0.9 ml of distilled water and 1 ml of 20% Sucrose.

Staining Solution

1 gm comassie blue R-250, 450 ml methanol, 450ml of DW and 100ml of glacial acetic acid was added in this same order.

Destaining Solution

Destaining solution was prepared by adding 100 ml methanol, 100 ml glacial acetic acid and 800ml DW.

10% gel was prepared and poured on a presealed gel plates. Immediately comb was inserted and then allowed the gel to polymerize. Comb was removed and ammonium sulphate precipitated culture filtrate 60 μ l was added on to the slots (in the first line marker protein was loaded). Loaded sample plates were mounted on the unit, both the tanks were filled with tank buffer

(pH 8.8) and 100 volt Direct Current was supplied. The current supply was seized when tracking dye just touched the bottom. Plates were separated from the unit and gel was separated carefully and placed in staining solution. For 30 min with constant shaking. Then the gel was transferred into Destaining solution. Then we can visualize the bands. The gels were scanned and placed in self locked polyethane covers.

RESULTS AND DISCUSSION

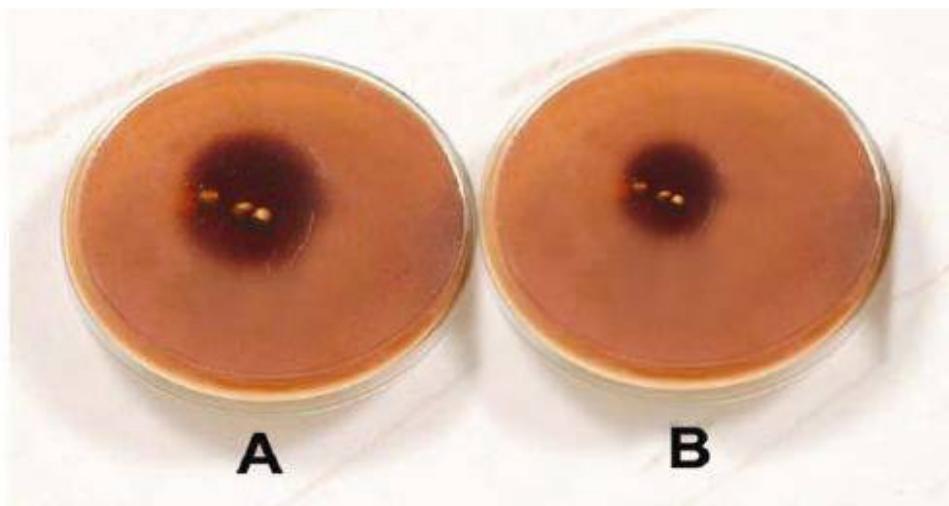
After collection, identification cultures were maintained 7 day old cultures of each organism (12) was taken inoculated into the pre solidified malt extract agar petri plate containing 0.05% guaiacol aseptically (in triplicates). Observations were made from 24 hr to 7 days. The organisms which were lignolytic they produced coloured zone (Figure 1). Such organisms were considered for further qualitative and quantitative study. Among the 25 organisms *Stereum ostrea* and *Trematus versicolor* developed bigger zones.

Vijaya and Malikarjuna reddy (2012) made similar attempts with edible mushrooms for the biodegradation of Agro wastes and found that Oyster mushroom produced 50mm diameter coloured zone. Premjet *et al.* (2009) studied 62 fruiting bodies were screened for peroxide activity and found maximum of 90mm diameter in RBBR dye containing agar petri plates. Similarly Mtui (2007) studied the biodegradation capacities of white rot fungi (*c. variabilis*) RBBR decolourization.

From the Figure 2 it was observed that all the 12 organisms produced Laccase and peroxidases. As the culture filtrates were crude the bands were not clear, they were effused between 67 kDa to 30 kDa. A clear band was seen in 67 kDa it can be laccase. Near 47 kDa all the organisms showed the band it can be peroxidases.

The filtrates of *C. variabilis* concentrated by ultrafiltration and subjected to spectrophotometric analysis at 280 and 260nm had overall protein content of 4.3 mg/ml. SDS-PAGE analysis showed distinct bands at relative molecular

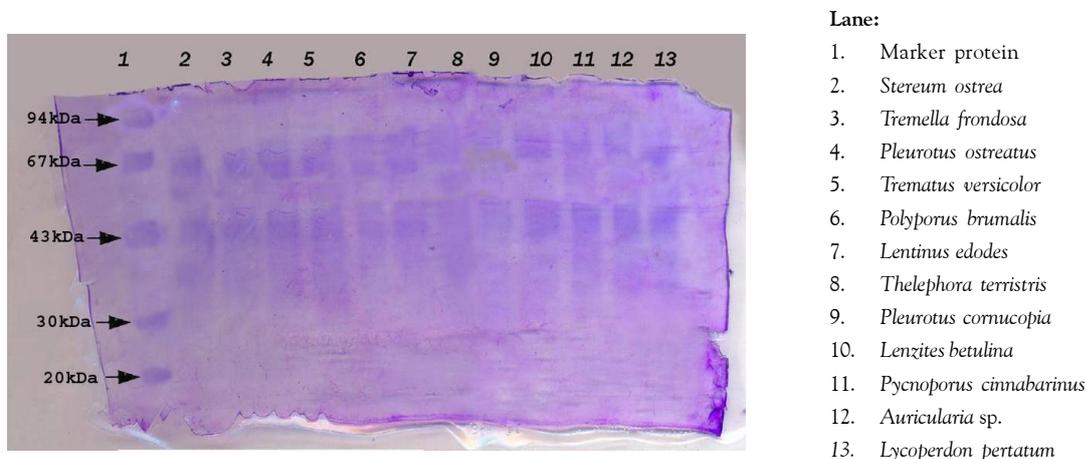
Figure 1: Screening of Organisms in Malt Extract Agar Media Which Contain 0.05% Guaiacol. Photograph was Taken After 48 hrs



A: *Stereum ostrea*

B: *Polyporus brumalis*

**Figure 2: SDS-PAGE Analysis of Enzymes Filtrate from *different organisms*.
Main bands appear at 67 kDa (laccases), and 47 kDa (peroxidases).
Lane 1: Standard Molecular Marker proteins: Phosphorylase b (94 kDa),
Bovine Serum Albumin (67kDa), Ovalbumin (43 kDa) and Carbonic Anhydrase (30 kDa)**



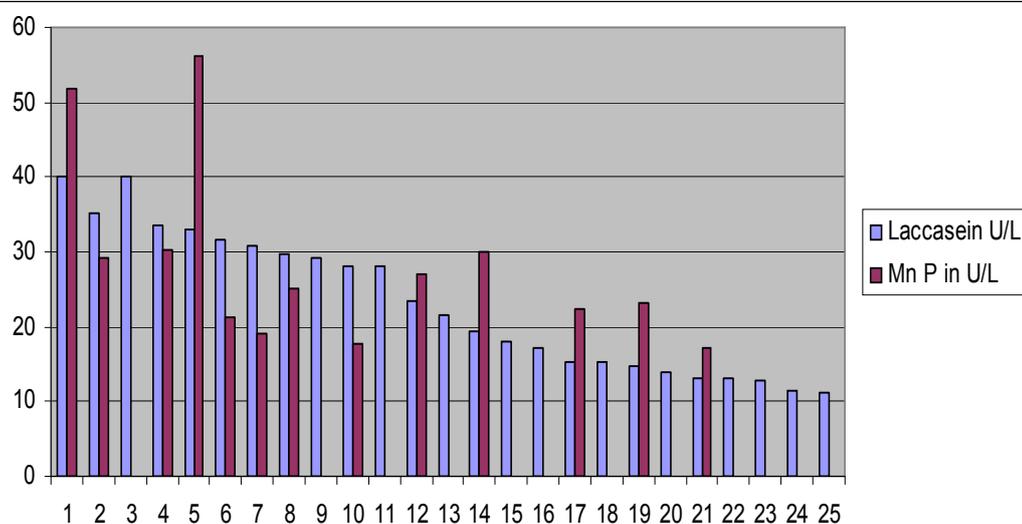
weights of 67 kDa and 47 kDa, which corresponds to laccases and peroxidases, respectively. A minor band resolved at 55 kDa could be an isoform of laccase. The results are comparable to studies by Karhunen *et al.* (1990), and Heinfling *et al.* (1998) who observed the molecular weights of peroxidases from *Phlebia radiata*, IZU 154, *Phanerochaete chrysosporium* and *Bjerkandera adusta* to be 49 kDa, 43 kDa, 42 kDa and 45 kDa, respectively. Fungal laccases have been shown to have various relative molecular weights as follows: PM1 (CECT2971 Strain - 64 kDa (Coll *et al.* 1993); *Phenero-chaeteflavido alba* - 96 kDa (Perez *et al.* 1996), *Panaeolus sphincrinus* and *Trametes gallica*-60 kDa (Heinzkill *et al.*, 1998, *Trametes trogii* -70 kDa (Garzillo *et al.*, 2004) and *Ganoderma lucidum*-75 kDa (Wang and Ng, 2006).

Form the Graph1 it was observed that *Stereum ostrea* was better in producing lignolytic enzymes. It could produce 40.02 U/L of Laccase, 51.89 U/L of MnP and 11.87 U/L of lignin Peroxidase. Only 6 organisms could produce all the three enzymes. All the 25 organisms could produce Laccase but

very few could produce MnP and LiP. *Coriolopsis occidentalis* is the least producer of lignolytic enzymes. Except six organisms, lignin peroxidase was not produced by the organisms so they were not represented in the Figure 3.

Biomass of cultures of *Stereum ostrea* and *Phanerochaete chrysosporium* upon growth in liquid medium under shaking conditions was determined. Growth of both cultures was initially slow for 4 days and then picked up and remained steady from 8th day of incubation. *Stereum ostrea* produced maximum biomass of 1.89 g/flask on the 10th day of incubation as against 1.78 g/flask in respect of *P. chrysosporium* (Praveen *et al.*, 2011)

Similar to our present discussion De-Jong *et al.* (1992), 20 basidiomycetes were isolated and screened on hump stem wood medium and their results showed that 18 fungi out of 20 organisms produced MnP and Laccase. Several white rot fungi which can produce manganese peroxidase and laccase but not lignin peroxidase Maltseva *et al.* (1991). 25 white rot fungi were studied for

Figure 3: Quantitative Analysis of Enzymes

- | | | |
|---------------------------------|------------------------------------|-------------------------------------|
| 1. <i>Stereum ostrea</i> | 10. <i>Pleurotus cornucopia</i> | 19. <i>Lycoperdon pertatum</i> |
| 2. <i>Tremella frondosa</i> | 11. <i>Agaricus bisporus</i> | 20. <i>Inonatus sp.</i> |
| 3. <i>Pleurotus eringii</i> | 12. <i>Lenzites betulina</i> | 21. <i>Cantherillus sp.</i> |
| 4. <i>Pleurotus ostreatus</i> | 13. <i>Geastrum triplex</i> | 22. <i>Caldesulla sp.</i> |
| 5. <i>Trematus versicolor</i> | 14. <i>Pycnoporus cinnabarinus</i> | 23. <i>Irpex lactus</i> |
| 6. <i>Polyporus brumalis</i> | 15. <i>Tyronyces sp.</i> | 24. <i>Corticium rolfsii</i> |
| 7. <i>Lentinus edodes</i> | 16. <i>Leatiporus sulphurus</i> | 25. <i>Coriolopsis occidentalis</i> |
| 8. <i>Thelephora terrestris</i> | 17. <i>Auricularia sp.</i> | |
| 9. <i>Ganoderma lucidren</i> | 18. <i>Arimellaria mellea</i> | |

Note: X axis shows name of the organisms; Y axis show quantity of enzyme produced in Units U/L

the production of lip, MnP Laccase and aryl alcohol oxidase for their ability to degrade, dehydrogenation and polymerization capacity for selective lignin degradation Hattakka (1994). Erikson et al (1990) studied few white rot fungi and selectively degrade lignin, some of these fungi lack one of more of these lignolytic enzymes even though they are good in lignin degrade. Anju (2012) selected two species of *Pleurotus*, three substrates and studied mycelial growth, extra cellular enzymatic production yield performance and biological characteristics of pleurotus species.

CONCLUSION

India is whelmed with very good biodiversity of

plants, animals, and decomposers. Macro fungi are one among them. Similarly like any other community very little was explored about macro fungi, i.e., white rot Basidiomycetes. Because, they are good lignin degraders, authors vested their interest and tried to explore the maximum possible organisms from different local areas (forests and timber depots) and could succeed in identified 3 white rot fungi as better lignin degraders.

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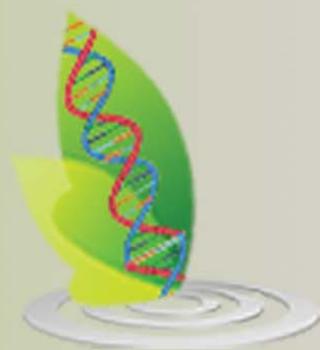
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