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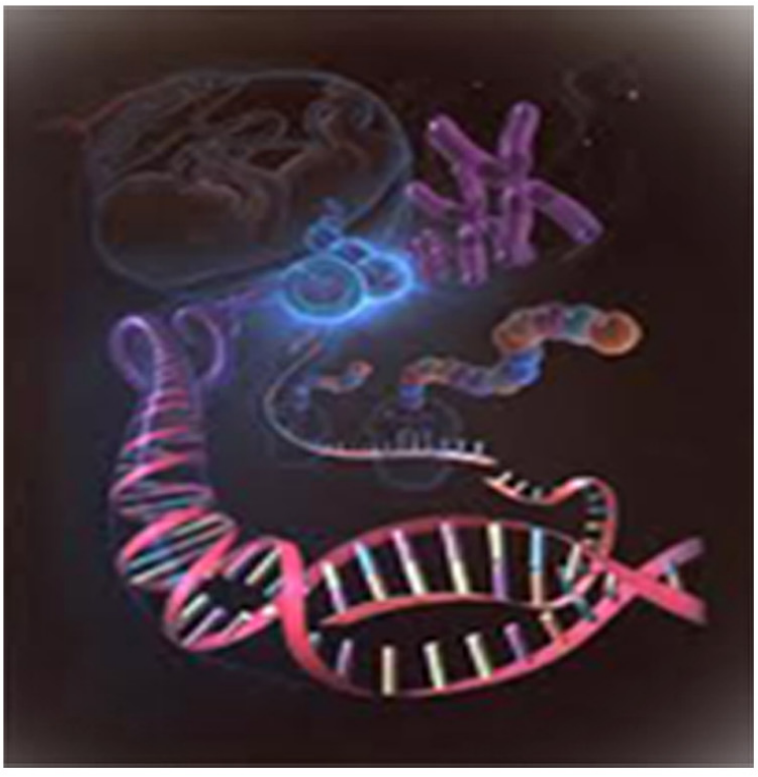

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Research Paper

CHOOSING OF GOOD WHITE ROT FUNGI FOR BIOBLEACHING OF UNBLEACHED KRAFT PULP

D Seshikala[1]*

*Corresponding Author: D Seshikala ✉ dr.shashidsk@yahoo.com

Considering 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). The selected 2 organisms were further studied for biobleaching of unbleached kraft pulp (UKP) where in cu^{+2} and Mn^{+2} influence was also studied.

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

INTRODUCTION

Lignin is the most abundant renewable aromatic Polymer and is known as one of the most recalcitrant biomaterials on earth (Crawford R L, 1980). Its degradation plays a key role in the carbon cycle of the biosphere (Tein M, 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 (types1 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 deploymerisation and carbon dioxide production (Kirk T K and Farrell R L, 1987). Microorganisms do not gain energy from lignin degradation but the degradation enables efficient utilization of carbohydrates (Erikson K E L *et al*., 1990). Thus microorganisms which utilize polysaccharides

[1] Department of Environmental Science, University College of Sciences, Osmania University.

often possess lignolytic capability lignin is finally degraded to CO^2, water and humus.

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

Manganese peroxidases (EC 1.11.1.13) belong to the family of oxidoreductases (Hammel K E and Cullen D, 2008). Manganese peroxidase (MnP) oxides Mn2+ to Mn3+, which oxides phenolic structures to phenoxyl radicals (Hofrichter M, 2002). The product Mn3+ is highly reactive and complex with chelating organic acid, as oxalate or malate, which are produced by the fungus (Mäkelä *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 K E and Cullen D, 2008). LiP is an extracellular hemeprotein, dependent of H_2O_2, with an unusually high redox potential and low optimum Ph (Erden E *et al*., 2009). LiP is capable of oxidizing a variety of reducing substrates including polymeric substrates (Oyadomari M*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 E *et al*., 2009). LiP shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules (Barr D P and Aust S D, 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 $C\alpha$-$C\beta$ 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 3x3 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, dimidiate (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 LCC, 1967; Suhirman, 2005). The pure cultures of white rot fungi were sub cultured for every one month and were stored at 4^{o} 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 on to 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 (1 gm Peptone, 20 gm Malt extract and 20 gm Dextrose and 1 L Distilled water) along with 10% Black liquor which contain 90% Lignin was supplied by Kamalapur Rayon's Factory, A.P , India. Thus prepared broath (100 ml) 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 M P *et al*., 1993), MnP (Paszczynski A*et al*., 1985) and LiP (Sarkanen K V and Ludwig C H, 1970) were measured using guaiacol as substrate. For theses enzymes, one activity unit was defined as the amount of enzyme necessary to oxidize 1µ mol of substrate per minute.

Solid State Fermentation

Biobleaching of the pulp in the solid state fermentation system was followed with.15 mL of M2 medium supplemented with Cu^{++} (2 mgL^{-1}) was added to each 250 mL conical flask containing 50 g of unbleached kraft pulp (Supplied by Kamalapur Rayons Factory A.P India) Flasks were sterilized by autoclaving at 15 lbs pressure for 10 min. Flasks were inoculated with of fresh fungal mycelium from 10 days precultured malt agar plates. An uninoculated flask containing pulp was served as blank. Rate of decolourization was measured in terms of increase in brightness. After incubation with fungi, pulp samples were washed with water and pulp sheets were prepared with a porceline Buchner funnel (diameter, 11 mm) and air dried. Kappa number was determined according to Tappi (Technical Association of pulp and paper industry) test methods T 236. Kappa number defined as the ml of 0.1 N KMnO4 solution

consumed per gram of moisture-free pulp under standard conditions. Brightness was determined with Aimil glass reflectance meter (Cat.No.070, S.No.95358). Viscosity of the pulp was measured by the method developed by department of quality control, A.P. Rayon factory, using capillary viscometer. 0.255 g of pulp was dissolved in 25 mL of cuprammonium solution and 2-3 drops of pyrogalla (1:3 diluted) was added and stirred for 15 min. on magnetic stirrer. This solution was used to measure the flow time of the solution. The flow time was converted into centiopoises (cp) using standard chart. All pulp bleaching experiments were conducted at least in triplicates. Results presented are the average of these values.

FUNGAL MYCELIUM

From the studies it was observed that two organisms were good in producing lignolytic enzymes they were selected for solid state fermentation studies.

EXPERIMENTAL SETUP

Unbleached Kraft Pulp (UKP) is considered as B1, modified Trevors media (3% malt Extract, 0.01%NH4Cl) as BT, Modified Trevors media added with 10ppm Cu^{+2} is BTC, Media with 7ppm Mn^{+2} is coined as BTM all these are considered as Blanks. These were Inoculate with *Stereum ostrea* and *Tremates versicolor* separately. Quantitative standardization of $Cu^{+2}$and Mn^{+2} was performed but results are shown.

SDS PAGE

Sample Preparation

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

Staining Solution

1 g comassie blue R-250, 450 ml methanol, 450 ml 0f D.W and 100ml of glacial acetic acid was added in this same order.

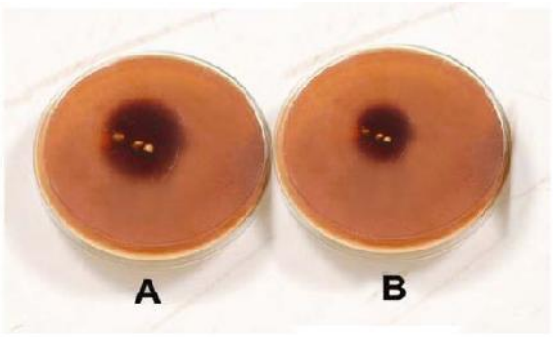
Destaining Solution

Destaining solution was prepared by adding100 ml methanol, 100 ml glacial acetic acid and 800ml D.W 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 µl 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 D.C 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

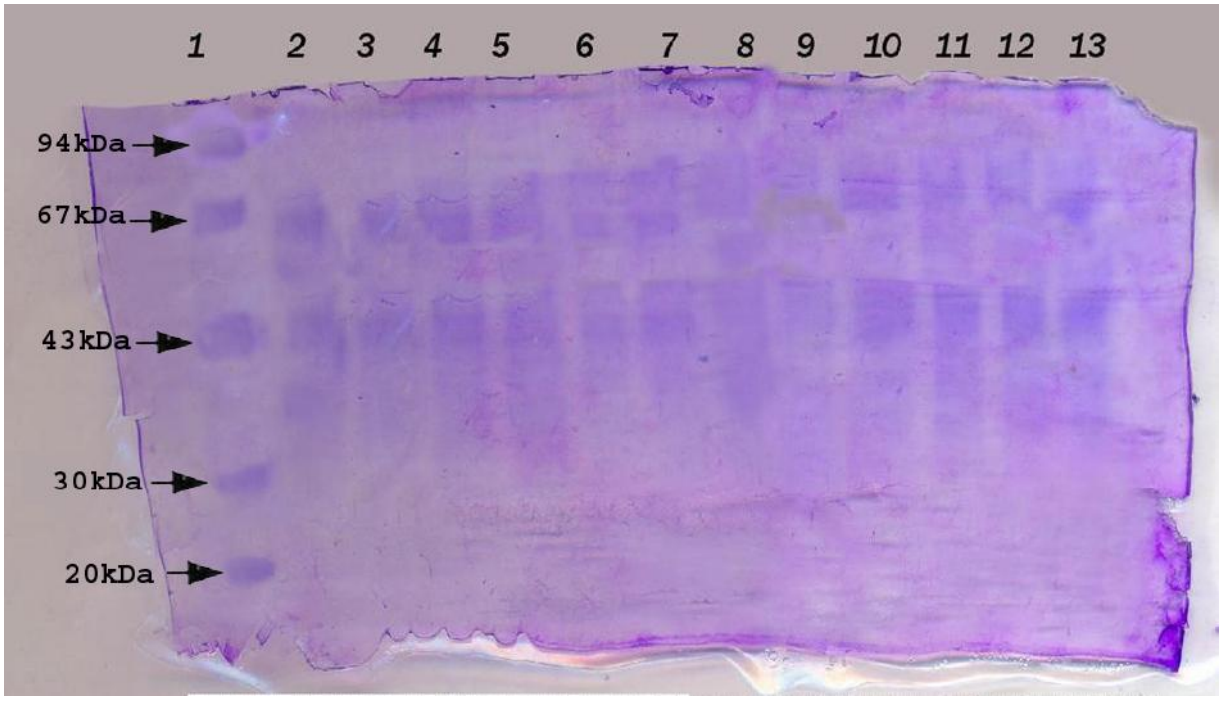
After collection, identification cultures were maintained 7 day old cultures of each organism(12) were taken inoculated into the pre solidified malt extract agar petri plate containing 0.05% guaiacol aseptically (in triplicates). Observations were made from 24hr to 7 days. The organisms which were lignolytic,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

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



and quantitative study. Among the 25 organisms *Stereum ostrea* and *Trematus versicolor* developed bigger zones. Vijaya CH and Malikarjuna Reddy R (2012) made similar attempts with edible mushrooms for the biodegradation of Agro wastes and found that Oyster mushroom produced 50mm diameter coloured zone. Premjet Siripong *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

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)



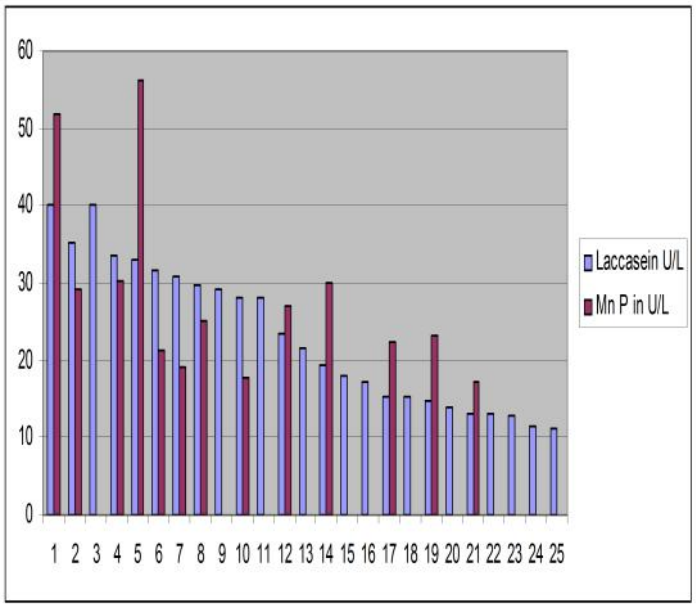
1. Marker protein
2. Stereum ostrea
3. Tremella frondosa
4. Pleurotus ostreatus
5. Trematus versicolor
6. Polyporus brumalis
7. Lentinus edodes
8. Thelephora terristris
9. Pleurotus cornucopia
10. Lenzites betulina
11. Pycnoporus cinnabarinus
12. Auricularia sp.
13. *Lycoperdon pertatum*

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 67kDa it can be laccase. Near 47kDa 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 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 E *et al*., 1990), and (Heinfling A *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.

Graph1: Quantitative Analysis of Lignolytic Enzymes
X - Axis Shows Name of the Organisms, Y - Axis show Quantity of Enzyme Produced in Units U/L



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

Fungal laccases have been shown to have various relative molecular weights as follows: *PM1* (CECT2971 Strain - 64 kDa [16]; *Phenerochaeteflavido alba* - 96 kDa (Perez *et al*., 1996), *Panaeolus sphincrinus* and *Trametes gallica*- 60 kDa (Heinzkill M *et al*., 1998), *Trametes trogii* - 70 kDa (Garzillo AMV *et al*., 2004) and *Ganoderma lucidum* - 75 kDa.

Form the Graph 1 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 are not represented in the Graph 1.

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 1.89g/flask on the 10th day of incubation as against 1.78g/flask in respect of *P. chrysosporium* (Praveen *et al*., 2011).

Similar to our present discussion (De Jong E*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 O *et al*., 1991). 25 white rot fungi were studied for the production of LiP, MnP Laccase and aryl alcohol oxidase for their ability to degrade, dehydrogenation and polymerization capacity for selective lignin degradation (Hatakka A, 1994). Erikson K E L *et al*. (1990) studied few white rot fungi and selectively degrade lignin, some of these fungi lack one of more of these lignolylic 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.

BIOBLEACHING STUDIES

Biobleaching is done in industries with Chlorine compounds, which let out organo chlorine compounds in to the environment through effluent and they are potential carcinogen. That is the reason biobleaching has gained importance. The imparted brown colour to the unbleached kraft pulp is due to presence of ruminants of lignin. The lignolytic enzymes delignify the pulp (Yaropolov A I *et al*., 1994; Hofrichter M, 2002;

Table 1: Biobleaching in SSF

Type	Kappa No.	Brightness	Viscosity
B1	17.1	45	36
BTC	17.1	46	35
BTM	17.1	45	35
BT Stereum	14.1	56	18
BTC Stereum	13.9	63	11
BTM Stereum	13.8	65	11
BT Tremates	14.1	56	17
BTC Tremates	13.8	66	12
BTM Tremates	13.8	65	11

Note: Brightness is expressed in %,Viscosity in entipoises(CP). Kappa no decreases brightness increases.

Oyadomari M *et al*., 2003). To improvise the biobleaching process, influence of minerals were studied. From the Table 1 it was observed that unbleached kraft pulp(UKP) is with kappa no 17.1(+/_ 0.1), Brightness 45% (+/_1%) and Viscosity 36Centipoise. The Kappa number was similar to the both organisms i.e., 14.1 (*Stereum ostrea*) and 14.0 (*Tremates versicolor*). When Cu^{+2} was added then Kappa number decreased to 0.1, Brightness was 63% and Viscosity 11 cp (*Stereum ostrea*). This difference was 0.3, when Mn^{+2} is added in case of BTM with the same organism. There was appreciable decrease in viscosity from 36 cp to 11cp when BTC with *Stereum* and with *Tremates.*

Similar to present studies, thermophilic mannanase from the *Bacillus* sp. MG33 was applied on wheat straw rich soda pulp to evaluate its bleaching potential. mannanase 3Ug 1of pulp at pH 6.5 and temperature 65ºC that enhanced the brightness by 30% and reduced the kappa number by 20% within 2 h. (Meenakshi, 2011). The biobleaching potential of a Kraft pulp by the laccase produced by *S. cyaneus* CECT 3335 was much improved by the use of some synthetic (i.e., ABTS) and natural mediators (i.e., phydroxybenzoic acid and acetosyringone). Thus, kappa number of the pulp was reduced about 20-25% by using 100 mU of laccase per gram of pulp after 3 hours of treatment. In addition, a significant improvement in kappa number and brightness were achieved when hydrogen peroxide was applied to the biobleached pulp (Manuel Hernández *et al*., 2004).

An alkalophilic and thermophilic mannanase from *Streptomyces* sp. PG-08-3 was applied to wheat straw-rich-soda pulp to check its bleaching potential. Mannanase 5 Ug-1 of pulp at pH 8.5 with temperature 55ºC that enhanced the brightness by 7.3% and reduced the kappa number by 24.6% within 4 h. of incubation (Preeti Bhoria and Gursharan Singh, 2012). A potential lignolytic fungus, *Stereum ostrea* was analysed for the decolourisation of industrial effluents discharged from rayon pulp mills. The colour removal was from 5580.85 to 2690.05 CU during 40 days of incubation time (Rama Krishna G *et al*., 2011). The biological decolourization of Cibacron black W-NN using five commercial *Pleurotus* species was reported among which *P. sajor-caju* fully decourized the dye (Halil Býyýk *et al*., 2009). Cu^{2+} (1.0 mM) and veratryl alcohol induced maximum laccase production giving 6.6 and 6.07 U/ml laccase activity, respectively (Shankar S, 2012). The effects of Mn^{2+} concentration, organic acid chelating agent, surfactant, pH, enzyme dosage and pulp concentration on the lignin degradation during biobleaching of hardwood kraft pulp with rMnP were evaluated. 10mM H_2O_2 supplied semi-continuously (pulse concentration 0.1mM) provided the best bleaching results in terms of kappa number reduction and pulp brightness improvement. Higher concentrations of H_2O_2caused a significant loss of rMnP activity during bleaching. The optimal Mn^{2+} concentration for lignin oxidation was in the range of 0.1<0.2 mM (Xu Haowen, 2007).

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 is 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 three white rot fungi as better lignin degraders.

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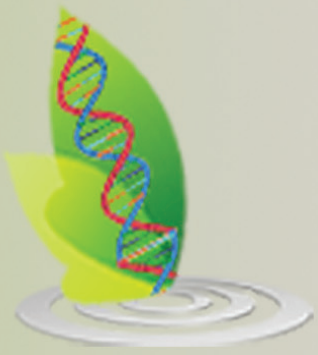

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