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Degradation of Agroresiduces with Value Added Products by Solid State Fermentation with Calocybe Indica

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INTRODUCTION

Mushrooms are biotechnological produced food products from ligninocellulosic, the economic value of these has expanded all over world in the last two decades.

The utilization of the insoluble lignocellulosic substrates by edible mushrooms depends upon the production of a wide array of lignocelllulolytic enzymes cellulose, hemicellulose, ligninase by the fungal mycelium which is a crucial part of the colonization process and is an important determinant of mushroom yield. Therefore, to exploit the potential of locally *in vitro* studies were carried out on the effect of supplementation on mycelial growth and enzymes production of *Calocybe indica* during solid state fermentation of wheat straw. (Shammi kapoor *et al.,* 2009).

The cultivation of edible mushroom is a prime example of how low-value waste which is produced primarily through the activities can be converted to a higher value commodity useful to mankind.

India is blessed with varied agroclimate, abundance of agricultural wastes and manpower making it most suitable for the cultivation of all the types of temperate, subtropical and tropical mushrooms (FAO, 1997).

Lignocelluloses wastes (LCW) refer to plant biomass wastes that composed of cellulose, hemicellulose and lignin. Lignin degradation by white rot fungi has been extensively studied.

The huge amounts of residual plant biomass considerable as “waste” can potentially be converted into various different value added products including biofules, chemicals cheep energy source for fermentation improved animal feeds and human materials (Howard *et al.,* 2003).

A number of agro industrial residues are thrown away as wastes or under utilized; more than half of the total production of plant residues mainly straw, leaves and forest wastes remain unused. The waste materials are partially burnt or disposed on land and composed (Kirk and Moore, 1972; Zadrazil, 1977, 1980). Some of the agricultural wastes are used as animal feed and raw materials in industries. One of the useful outlets for these raw materials is to utilize them as a substrate for growing edible mushroom which are in great demand in India.

Ligninolytic enzymes are primarily involved in lignin degradation in oxidative reactions that are mainly free radical driven in the presence (or sometimes absence) of mediators. The main enzymes involved are lignin peroxidase, manganese peroxidase and laccase (Hao *et al.,* 2006; Mtui and Nakamura, 2007, 2008; Mtui and Masalu, 2008).

Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues. Biodevelopment of biowastes provide a wide range of affordable renewable value-added products from LCW (Pandey *et al.,* 2000; vanWyk, 2001; Howard *et al.,* 2003).

Lignocellulosic enzymes, mainly from fungi and bacteria, are important commercial products of LCW bioprocessing used in many industrial applications including chemicals, fuel, food brewery and wine, animal feed, textile and laundey, pulp and paper and agricultural (Howard *et al.,* 2003).

The benefits of using waste residues as lignocellulosic feedstocks will be to introduce a sustainable solid waste management strategy for a number of lignocellulosic waste materials; contribute to the mitigation in greenhouse gases through sustained carbon and nutrient recycling; reduce the potential for water, air and soil contamination associated to broaden the feedstock source of raw materials for the bio-ethanol production industry (Champagne, 2007).

The current supplies from LCW based oil crops and animal fats account for only approximately 0.3%, biodiesel from algae is widely regarded as one of the most efficient ways of generation biofuels and also appears to represent the only current renewable source of oil that could meet the global demand for transport fuels (Schenk *et al.,* 2008).

Biohydrogen production from agricultural residues such as olive husk pyrolysis (Ca lar and Demirba, 2002); conversion of wheat straw wastes into biohydrogen gas by cow dung compost (Fan *et al.,* 2006); bagasse fermentation for hydrogen production (Singh *et al.,* 2007) generate up to 70.6% gas yields. System optimization for agrowaste-based hydrogen production seems to be the ideal option for clean energy generation. Hydrogen generation from inexpensive abundant renewable biomass can produce cheaper hydrogen and achieve zero net greenhouse emissions (Zhang *et al.,* 2007).

Bioconversion of lignocellulosic agro-residues through mushroom cultivation and single cell protein (SCP) production offer the potential for converting these residues into protein-rich palatable food and reduction of the wastes. Mushroom cultivation provides an economically acceptable alternative for the production of food

of superior taste and quality which does not need isolation and purification (Israilildes and Philippoussis. 2003; Philippoussis *et al.,* 2007).

LCW provides a suitable growth environment for mushrooms that comprise a vast source of powerful new pharmaceutical products. In particular, *Lentinula edodes, Tremella fuciformis* and *Ganoderma lucidum* contain bioactive compounds such as anti-tumor, anti-inflammatory, anti-virus and anti-bacterial polysaccharides. Moreover, they contain substances with immunomodulating properties as well as active substances that lower cholesterol (Israilides and Philippoussis, 2003; Philippoussis *et al.,* 2007; Zhang *et al.,* 2007). Future prospects for research in bioactive compounds from fungi grown on such cheap and ubiquitous substrates look bright and could lead to breakthroughs in the search for antibacterial, antiviral and anticancer chemotherapies.

The pretreatment systems and the concomitant release of bio-products from LCW have been greatly improved by new technologies; there are still challenges that need further investigations. These challenges include development of more efficient pretreatment and production technologies, bioprospecting and development of stable genetically engineered microorganisms, improved gene cloning and sequencing technologies and enhancement of productions based on ecomoes of scale for more efficient and cost effective conversions LCW into value-added products.

So far, lignocellusosic biomass has been the most promising economically viable and renewable source of biohydrogen and biodiesel. However, the second generation microalgal systems seem to be more advantageous in that they: (1) have a higher photon conversion efficiency (as evidenced by increased biomass yields per hectare): (2) can be harvested batchwise nearly all-year-round, providing a reliable and continuous supply of oil: (3) can utilize salt and waste water streams, thereby greatly reducing freshwater use: (4) can couple CO_2 – natural fuel production with CO_2 sequestration: (5) produce non-toxic and highly biodegradable biofuels (Schenk et al., 2008). Therefore, extensive research is now being directed toward that ends. Plant fibers as fillers and reinforcements for polymers are currently the fastest-growing type of polymer additives. Nanobiotechnology seems to take charge as far as the use of LCW nanofibres in plastic composites is concerned (Alemdar and Sain, 2008). It is envisaged that nano materials from renewable biowastes will be the main focus of future research.

The technical advances made during recent decades have, along with myriad other implications, resulted in edible mushroom cultivation attaining global dimensions, since cultivated mushrooms can be grown under different climatic conditions on cheap, readily available waste materials, they represent a solution to many of the world's current problems, including protein shortages, resource recovery and re-use, and environmental management.

Many varieties of mushrooms are valued greatly as nutritious food sources, as tonic foods, and as important sources of medicinal compounds anti-tumor/anti-viral agents and other pharmaceutically-active components. A food, are marketed currently and the demand for such product is expected to increase.

Milky mushroom has a fruit body similar to button mushroom. The sporophores are robust, attractive and milky white in colour. These mushrooms grow well at a temperature range of 25-35ºC (Hindu 2004).
Most of the edible fungi have strong enzyme system and are capable of utilizing complex organic compounds which occur as agricultural wastes and industrial by-product. These can be used as bedding material for mushroom cultivation. (Khan *et al.,* 1987)

OBJECTIVES

- To assess the degradative capability of Milky mushrooms.
- To analysis biodegradation of agro wastes mainly Paddy straw and Wood saw.
- To study the profile of enzyme pattern of Milky mushroom.
- To optimize the value added products in Solid State Fermentation (SSF)

REVIEW OF LITERATURE

White-rot fungi are of particular interest due to their ability to degrade lignin (Rayner *et al.,* 1998). The potential biotechnological use of white-rot fungi has attracted considerable attention during the past few decades, but among the species tested so far, only a few are potentially useful in contaminated soil.

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The plant and wood wastes are mainly composed of three biopolymers. Cellulose, Hemicellulose and Lignin (Jim *et al.,* 1993). Among these biopolymers cellulosic plants and their by products are widely recognized as a viable source of cellulose. But their uses have been limited because of their poor digestibility.

The straw is mostly used as a fuel and for manufacturing compost and paper and cardboard (Arora *et al.,* 1992). The digestibility rate depends upon the amount and quality of natural polymers, the amount of readily soluble substance and amino acid content of the feed (Zadrazil, 1977). Because of these reasons the ruminants could not digest these agro residues effectively (Bisaria *et al.,* 1987). Bagasse is another main agricultural wastes produced from sugar cane industries, In cuba sugar cane crop residues constitute traditional animal feed the cane residues contain 75% dry matter as cellusose and hemicellusose (Martinez *et al.,* 1990).

The coir is one of the major ligninocellulose material from coconut fiber consistently lignin as major component and is having low bio degradability so the disposal and utilization process is a typical problem. One way to overcome this problem is to use microbes degrade coir. Totally Tamilnadu products 1423 million coconuts with an average of 10,000 nuts/hectares from which 1 ton of coir fiber and another 1 ton of coir pith become available. Accumulation of this waste leads to environmental disharmony, Theradimani and Marimuthu (1992) developed a technique for decomposing coconut coir into useful organic manure using *Pleurotus* spp., (Nallathambi and Marimuthu, 1993; Ragunathan *et al.,* 1996).

Plant cell wall:

The cell wall contains cellulose, hemicellulose and lignin. Lignin is the second most abundant biopolymer in nature. The complex structure of ligninocellulose prevents the economical use of lignin derivatives as chemical feed stocks or ruminant feed (Zohar Kareem *et al.,* 1992; Cramford, 1981; Freundenberg 1965).

Cellulose:

Cellulose is a linear polymer of glucose units kinked by β 1-4 linkage. In nature cellulose chains are closely held together and are termed as micelles and such a structure imparts crystallinity to the cellulose; wherever the chains are more loosely arranged the term amorphous is used to designate the structure. Hydrogen bonding and vanderwall's forces hold the micelles together making native cellulose impervious to high molecular weight macro molecules like enzymes.

Hemicellulose:

Hemicelluloses are a complex group of cell wall polysaccharides which constitute 30 to 40% of dietary carbohydrate intake of the ruminants (Wilkie, 1979). Hemicellulose consists of pentose or hexoses as well as uronic acids. Hemicellulase are glycon hydrolases which specifically degraded only the hemicellulosic polysaccharide (Dekkar and Richards, 1976) and this definition excludes the related glucosidases which may hydrolyze not only the oligosaccharide products of the polysaccharides but also the short side chain moieties attached to the main polymer backbone. The glucosidase activity is thus essential if the complete breakdown of the hemicellulosic polysaccharide is to be achieved.

Lignin:

Lignins are complicated macromolecules both structurally and behaviorally and have a molecular weight of Da 11000. Lignin is a amphorphous three dimensional aromatic polymer composed of oxy phenyl propane units (C_6-C_3). It is formed in plant cells by the enzyme mediated polymerization of three substituted cinnamyl alcohol viz.B coumaryl and sinapyl alcohol (Sarkanen and Ludwig, 1971).

Enzymology of Lignin Biodegradation:

The structural features of the ligninocellulosic material dictate that their degradation must be enzymatic extracellular, non-hydrolytic (Kirk and Farrel 1987).

The groups of enzymes that catalyze the oxidative depolymerization of lignin are collectively known as ligninases. Ligninases are extracellular and are produced by white rot basidiomycetes. The peroxidases are Lignin peroxidase (LiP) and Manganese dependent peroxidase (MnP) and Laccase.

Peroxidases:

The wood rot fungus *Calocybe indica* reduces during the secondary metabolism two families of extracellular heme proteins designated as lignin peroxidase (LiP) and Manganese peroxidase (MnP) as key components of lignin degrading enzyme system.

LiP appears to oxidize non-phenolic electron at the low optimum pH (Schoemaker, 1990). Characteristic reactions include depolymerisation, aromatic ring opening, demethoxylation, hydroxylation, decarboxylation and phenol coupling reaction (Schoemaker and Leisla 1990).

MnP appear to oxidize the phenolic lignin and phenolic reaction products. The MnP appear earlier than LiP in ligninolytic cultures of the fungus (Wariishi *et al.,* 1988, 1989).

Laccase:

Laccase is another kind of peroxidase that has been found to be associated with lignin degradation. Many white rot fungus produce the extracellular laccase (Buswell and Odier, 1987).

Laccase causes free radical formation of cinnamyl alcohol and this non-enzymatic polymerization may include cleavage of bonds between aromatic rings and propane side chain as well as the formation of carboxyl

group in the side chain would enhance the rate of lignin degradation.

Ligninocellulose is main source of natural cellulose generate in agricultural number, food processing and municipal service (Bisaria *et al.,* 1981).

The carboxymethlcellulose filter paper assay activity and cellobiose were assayed by method of (Glose *et al.,* 1983). Xylenase was estimated by method of (Resse *et al.,* 1966).

The reducing sugar was estimated by the method of Miller (1959). The extracellular protein content of the crude enzyme filtrate was determined using bovine serum albumin as standard (Lowry *et al.,* 1951).

L.Polychrous cultivation on solid substrate using para rubber as a carbon source, rice brawn as a nitrogen vitamin source plus define minerals on these types of medium colonized fully in 35 days and yielded the higher level of extracellular proteins (Kannikar *et al.,).*

For ligninolytic enzyme the level of laccase activity was higher then MnP maximam at colonized stage and rapidly declined during fruting stage the pattern was similar to *Lentinula edodes (*Ohga *et al.,* 2000).

The enzyme catalysin biotransformation reaction was extracellular and quite stable. The enzyme obtained from *P.anceps* was identified as a laccase on comparison biochemical taste with tyrosine and hydroquinone as substrate and by comparison with *R.vernicifera* laccase (Richard *et al.,* 1980).

Based on the enzyme production patterns of an assay of white rot fungi three categories of fungi are suggested (i) Lignin-manganese peroxidase group (ii) Manganese peroxidase, Laccase group (iii) Lignin peroxidase- Laccase group (Annele *et al.,* 2006).

Tien et al., 1993 prepared the elution profile of the ligninolytic enzyme from the DEAE – Bio Gel A column. The major protein band was collected with H_2O_2 requiring oxidative activity against veratyl alcohol and H_2O_2 requiring cleavage activity.

Two methods are described in increasing the production of ligninase by culture of *Pheanerocheate crysoporium* grown in a nitrogen limiting medium. The first method involves addition of veratyl alcohol (0.4) and excess trase metal to stationary flask culture. The second method entails scale up using a disc fermentor with a mutant strain which adheres well to the plastic discs in contrast to the wild type and which in addition produces high titers of ligninase (Tkat kirk *et al.,* 1985).

Manganese peroxidase was found to have a pH of 3.4 and a pH optimum of 5.4 to 5.5 with maximal activity during the initial stage of fruting (pin stage). The activity declined considerably with fruit body maturation (Bonnen *et al.,* 1994).

The cellulose production was more in solid state fermentation (SSF) than in submerged fermentation. The changes in extracellular enzyme activity of cellulose and lactase are directly correlated with growth and fruit body formation.

LiP is characterized by the oxidation of high redox potential aromatic compounds (including veratyl alcohol) where as MnP require Mn^{2+} to complete the catalytic cycle an forms Mn^{3+} chelates acting as diffusing oxidases. There are3 peroxidase involved in lignin degradation and that have been characterized by the crystal structures and molecules of the white rot fungi *P.eryngii* and *Phanerochaetes chrysosporium* (Periasamy *et al.,* 2003).

The effect of N addition on enzyme production was studied in medium containing rice straw and the data showed an increase of 3 up to 4 fold in the laccase production compared to that obtained in SSF on wheat brawn. The laccase presented optimum pH at 3-3.5 and were stable at neutral pH values. Optimum for MnP and LiP activity was at 3.5 and between 4.5 and 6.0 respectively. All the strains produced laccase with optimum activity between 55ºC - 60ºC while the peroxidases presented maximum activity at temperature of 30-55ºC (Elai et al., 2009).

Phanerochaete crysosporium is one of the most widely studied white rot fungi with regards to lignin degradation enzymes (Tein *et al.,* 1987). It h as drawn considerable attention as an appropriate host for the production of lignin waste material consists of lignin 20-40%, cellulose 40-50%, hemicellulose 45-35% and protein 2.04% (Sjosnom 1993). Coir is produced from the fibrous of coconut (Cocos micifera).

Solid State Fermentation is an attractive process to produce fungal microbial enzymes (Chahal *et al.,* 1996; Halfrich *et al.,* 1996; Jech 2000). SSF is characterized by the complete (or) almost complete absence of free liquid (or) water, which is essential for microbial activities. The water is present in an absorbed in complexes from with the solid matrix and the substrate (Cannel and Moo-Young, 1980).

These cultivation conditions are especially suitable for the growth of fungi, known to grow at relatively low water activities. As the microorganisms in SSF grow under conditions closer to their natural habitats and they are capable of producing enzymes and metabolites that will not be produced (or) will be produced only in low yield in submerged conditions (Jech 2000). SSF are considered practical for complex substrate fermentation including agricultural, forestry and food processing residues and wastes which are used as the carbon source (Hattich *et al.,* 1996).

During the post few years, the Kradang mushroom *Lentinus polychriys*, is recognized as one of the widely commercial cultivated edible mushroom of the northeast and northern part of Thailand. The value of the

known as kradang mushroom crop accounted to 4.0 million baht in the year 1995. Kradang mushrooms considered nutritious contains high quantities of protein, carbohydrate, calcium and phosphorus. Moreover, it also contains some medicinal substances such as eritadenine, germanium, and ergosterol (Pegler, 1983). Para rubber is one of the most popular agro-industrial residues used as substrates in bioprocess for production of commercial mushroom (Petcharat, 1995).

MATERIALS AND METHODS

Source of Inoculum:

The primary inoculum of Milky mushroom was prepared from fleshy healthy fruit body. The middle portion of the mycelium was removed with a sterile scalpe inoculated the block in Malt Extract agar slants and incubated at room temperature for 4-6 days for mycelial growth and spore formation.

Estimation of Protein:

The protein content of the substrate was estimated by the method of Lowry et al., (1951).

Reagents:

- Solution A: Sodium carbonate 2% in 0.1N NaOH
- Solution B: Copper sulphate 0.5% in 1% sodium tartarate.
- Solution C: To 50ml of solution A, 1ml of solution B was added.
- Folin phenol reagent:

  The commercial folin's phenol reagent was diluted in the ratio 1:1 (V/V) with glass distilled water. This reagent was always prepared fresh whenever needed.

Procedure:

- To 1ml of the sample, 2ml of 10% trichloroacetic acid was added and incubated for 1h. After that, the sample was centrifuged at 15,000 rpm for 30 min.
- The supernatant was discarded and to the pellet 5 ml of solution C was added. After 10 min, 0.5 ml of diluted folin's phenol reagent was added and the tubes were shaken vigorously and kept for colour development.
- After 30 min the intensity of colour developed was read at 500 nm in a spectrophotometer.
- Distilled water was taken as blank with the same treatment as that of the sample. Standard was prepared with bovine serum albumin.

Substrate Analysis:

The two chosen for the present study as carbon agro-residues and source are (Paddy straw and Wood saw). They were used as substrate for Milky Mushroom degradation studies. For biochemical analysis, portions of the substrate were chopped into bits powdered in a blender. The chemical component of these powdered samples were analysed by the fraction method of Thornber and Northcote (1961).

Determination of Ash: (Thornber and Northcote 1961)

The weighed sample (500 mg) was maintained at dull red heat (200ºC) for 2hrs in a silica crucible and after cooling, the ashed material was reweighed.

$$\text{Ash} = \frac{\text{Weight of ashed material}}{\text{Weight of the fresh material}} \times 100$$

Estimation of Crude Fiber:

The crude fiber was estimated by Maenad (1970) method.

The weighed (500mg) material was extracted with ether for removal of fat, and then boiled with 0.25N H_2SO_4 for 30 min filtered, again boiled with 0.313 NaOH for min, filtered through muslin cloth and serially washed with 1.25% H_2SO_4, distilled water and alcohol. The residue was transferred to reweighed ashing dish (W_1) died at 130ºC for 2h and weighed (W_2). Finally the dried sample was ignited at 600ºC for 30 min and reweighed (W_3).

$$\text{Crude fiber Content (\%)} = \frac{(W_2-W_1)-(W_3-W_1)}{\text{Fresh weight of the sample}} \times 100$$

Total Soluble Substances (TSS):

The weighed material (500mg) was extracted 3 times with distilled water at 100ºC for 3h. After extraction, the material was dried and further extracted with ethanol-benzene (1:2 v/v) at 70ºC for 6h. The extracted free material was dried and weighed.

$$\text{Total soluble substances (\%)} = \frac{\text{Initial weight of the material} - \text{Weight after extraction}}{\text{Initial weight}} \times 100$$

Estimation of Lignin: (Thornber and Northcote 1961)

The extracted material (100mg) was treated with 2 ml of Conc. H_2SO_4 for 1h. After one hour, the residue was centrifuged and thoroughly washed by suspension and centrifugation in distilled water. The dried residue represented lignin and it was weighed.

Estimation of Cellulose: (Upegroff, 1969)

Weighed amount of (500mg) powdered samples were acetolysed with acetic/nitric reagents (150ml of 80% acetic acid and 15ml of Con. Nitric acid) in a boiling water bath for 30 minutes. After cooling, the content was centrifuged and washed until there was no acetic smell in the residue. The residue was hydrolysed with 67% H_2SO_4 for 1h at room temperature. The hydrolysed solution was made upto 100ml with distilled water. To 0.1ml or 0.5ml of the diluted solution, 5ml of anthrone reagent was added and heated in a boiling water bath for 10 min. After cooling, the colour developed was measured at 630nm (Updergroff, 1969).

Enzyme Activity:

Estimation of Cellulase Enzyme: (Dension *et al.*, 1977)

Principle:

Initiation of hydrolysis of native cellulase is effected by C_1 enzyme. The enzyme is an exo-β-1,4 glucanase . Exo- glucanase splits alternate bonds from the non- reducing end of cellulase chain yielding cellobiose. The endo-gluconase is distinguished by the mechanisms of their attack on carboxy methyl cellulose.

Only organisms producing C_1-cellulase are capable of hydrolysing native cellulase. The production of reducing sugar glucose due to cellulolytic activity is measured by dinitro salicylic acid method.

Reagents:

- Sodium citrate buffer 0.1M (pH 5.0)
- Carboxymethyl cellulose 1%
  Dissolve 1g carboxymethyl cellulose in 100ml Sodium citrate buffer 0.1M (pH 5.0)
- Dinitro salicylic acid (DNSA) reagent
- 40% Rochelle salt solution (Potassium Sodium Tartrate)

Procedure:

- Pipette out 0.45ml of CMC solution at a temperature of 55ºC and 0.05ml of enzyme extract.
- Incubate the mixture at 55ºC for 15 min.
- Immediately after removing the enzyme substrate mixture from the bath add 0.5ml DNSA reagent.
- Heat the mixture in a boiling water bath for 5min.
- Cool to room temperature. Then add water to make 5ml volume.
- Measure the absorbance at 540nm.
- Prepare a standard graph with glucose in the concentration range 50µg to 100µg/ml.

Estimation of Laccase Enzyme:

Reagent:

- 0.3mM of ABTS
- 100mM of Sodium acetate (pH 3.5)
- Enzyme solution

Procedure:

- The reaction mixture, containing 0.1ml of 0.3mM ABTS in 100mM of sodium acetate (pH 3.5) and 0.1ml of crude enzyme solution.
- The reaction mixture was incubated at 4ºC for 1 min.
- The ABTS oxidation was monitored by the increase in absorbance at 420nm (e=36,000 M^{-1} cm^{-1}).

Estimation of Manganese Peroxidase:

Reagent:

- 50mM of Sodium lactate buffer (pH 4.0)
- 0.3mM of Manganous ions (Mn^{2+})
- Enzyme solution

Procedure:

- The reaction mixture containing 0.9ml of 50mM of sodium lactate buffer (pH4.0) containing 0.3mM of manganous ions (Mn^{2+}) and 0.1 ml of crude enzyme solution at 40ºC.
- The reaction was started by addition of 40µm H_2O_2 and absorbance at 270nm (e=8100 M^{-1} cm^{-1}) was monitored.

Assay of Protease: (Folin & Ciocalteau, 1929)

Principle:

The assay involves quantitative estimation of lysosome released during the hydrolysis of protein folin ciocalteau reagents retails with lysosome released to produce a blue coloured complex which has read at 660nm. One unit of enzyme activity can be defined as amount of enzyme that liberated 1mm lysosome per minute. Under assay condition and reported in terms of protease activity per gram dry substrate.

Reagents:

10% Trichloric acetic acid:

10g acetic acid dissolved in 100 ml distilled water.

1% Casein:

1g of casein was dissolved in 100 ml of phosphate buffer since casein is spatially soluble in water dissolved it in a minimal quantity of 0.1N NaOH and then rinse the volume of 100 ml with buffer. The pH was adjusted to 7.

0.1 M Phosphate buffer:

0.1M Monobasic sodium phosphate (1.38 in 100ml)

0.1M Dibasic sodium phosphate (2.69 in 100ml)

0.44N sodium carbonate

Folin ciocalteau reagent

Procedure:

- 100µl of crude enzyme extract, 1ml of 1% casein and 900 µl of 0.1M phosphate buffer (pH 8) was added.
- The reaction mixture then incubated at 60ºC for 10 minutes and arrested by adding 1ml of 10% TCA.
- The reaction mixture was then centrifuged at 3000rpm for 15 minutes.
- The supernatant was collected and 5 ml of 0.44N sodium carbonate and 1ml of 3 fold diluted folin ciocalteau reagent was added.
- The resulting solution was incubated for 30 min at 30ºC in dark and absorbance was read at 660nm.
- Control tubes were also kept and steps were followed without adding the crude enzyme extract.

Assay of Amylase: (Bernfield, 1995)

Principle:

α-amylase requires C_l-ion for activation of optimum pH 6.7 (range 6.6TO 6.8). The enzyme hydrolyses α-1→ 4 glycosidic linkagase at random deep inside polysaccharides molecules like starch, maltose, glucose and trisaccharides maltotriose α-amylase action stops in stomach when pH falls to 3.0.

Starch, glycogen,

$$\text{Dextrin} \xrightarrow{\alpha\text{-amylase}} \text{Glucose, Maltose, Maltotriose}$$

Amylase is measured by allowing the enzyme to act upon the starch and measuring the rate of hydrolysis of starch. As the reacts proceeds the concentration of starch in the reaction system decreases and that of maltose increases.

The reduces sugar produced by the action of α-amylase reacts with di nitro salicylic acid in alkaline medium and reduced to orange red color compound nitro amino salicylic acid. The orange red color developed is read at 540nm.

Reagents:

1. Phosphate Buffer pH = 7

   Solution A =0.2M Monobasic sodium phosphate

   Solution B =0.2M Dibasic sodium phosphate

   39ml of solution A and 61ml of solution B was mixed and made up to 200ml with distilled water.

2. Buffered starch substrate:

   1g of starch was weighed dissolved and made up to 200ml with phosphate buffer in SMF.

3. 2M NaOH

4. DNSA reagent

   Solution A= 20g of sodium potassium tartrate dissolved in 500ml 0f distilled water.

   Solution B=10g of 3,5 dinitro salicylic acid dissolved in 200ml OF 2M NaOH.

   50ml of solution A and 20ml of solution B was mixed and made up to 100ml with distilled water.

5. Working standard solution:

   100mg of maltose was dissolved and made up to 100ml with distilled water in SMF.

6. Inhibitor:

   2M NaOH

7. Enzyme

   1ml of enzyme solution was diluted to 10ml with water.

Procedure:

- In to a series of test tubes (0.2, 0.4, 0.6, 0.8 & 1ml) working standard solution were taken and marked as S1, S2, S3, S4, S5.
- The volume was made up to 3ml with distilled water.

- The blank was prepared with 3ml of distilled water.
- 2.5ml of buffer was added to two test tubes containing 2.5ml of starch substrate act as control and test.
- Then 1ml of enzyme sample to test alone were added and incubated at 37C for 15 minutes.
- The reaction was arrested by adding 0.5ml of 2M NaOH.
- 1ml of enzyme sample was added to control tube.
- 0.5ml of DNSA reagent was added to all the test tubes both working standards test and control tubes.
- All the tubes were kept in boiling water bath for 10minutes.
- The intensity of color developed was proposed to maltose concentration.
- The color developed was read calorimetrically at 540nm.
- The standard graph was plotted concentration against optical density or absorbance concentration was taken in x-axis and absorbance in y-axis.
- The amount of maltose released by amylase activity was calculated from graph.

Estimation of Peroxidase:

Principle:

Peroxidase is a hydrogen donor one among the many found in plants. The enzyme peroxidase is specific to hydrogen peroxide. Hydrogen peroxide acted up on by the enzyme peroxidase and release water molecule and oxygen. Up on cleavage by the enzyme the product oxygen oxidizes the phenolic compound guaiacol (1-hydro 2 methoxy benzene).

Guaiacol → oxidized form of guaiacol
(colourless compound) (coloured compound)

Reagents:

- Phosphate buffer:
  Solution A: 0.2M monobasic sodium phosphate
  Solution B: 0.2M dibasic sodium phosphate
  51ml of solution A, 49ml of solution B diluted to a total of 200ml.
- 0.5M Guaiacol:
  Prepare 0.5M guaiacol and dissolving completely with the help of magnetic stirrer.
- Hydrogen peroxide
- Enzyme sample
  1g of fresh turning sample was ground with 10ml phosphate buffer the slurry was filtered and centrifuged at 1000 rpm for 10 minutes the supernatant was transferred to SMF and upto 100ml with phosphate buffer. It was used as the enzyme source.

Procedure:

- Into a test tube 2.5ml of buffer 1ml of guaiacol and 0.5ml of enzyme sample was added.
- It was mixed well prepared 0.5ml of substrate was added.
- A blank was prepared 2.5ml of buffered 1ml of guaiacol 0.5ml of distilled water and 0.5ml of substrate.
- After addition of substrate the mixture was transferred into cuvette immediately and kept in colorimeter.
- Absorbance was measured at 460nm for 10-100 seconds at 10 second interval.
- The results were tabulated and activity was expressed at OD/min/mg of tissue.

Value added Products:

Estimation of Glucose by Anthrone Method:

Principle:

Carbohydrate are first hydrolysed into sample sugars using dilute hydrochloric acid. It hot acidic medium glucose is dehydrated to hydroxy methyl furfural. This compound forms with anthrone reagent colored product with an absorption maximum at 630nm.

Reagents:

- Anthrone reagent:
  Dissolve 200mg of anthrone in 100ml of ice-cold 95% H_2SO_4. Prepare fresh use.
- Stock standard glucose (1mg/ml)
  Dissolve 100mg of glucose in 100ml of distilled water.
- Working standard solution:
  10ml of stock is dilute to 100ml of distilled water.

Procedure:

- Pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml of working standard solution in the respective tubes S1, S2, S3, S4 and S5 corresponds to 20, 40, 60, 80 and 100µg of concentration.
- Made up the volume of all the tubes to 1ml with distilled water.
- 1ml of distilled water added to a test tube marked as blank.
- Then add 4ml of anthrone reagent.

- Heat for 8 minutes in a boiling water bath.
- A standard graph was drown by plotting the concentration in the x axis OD in the y axis.

Estimation of Ethanol:

Reagents:

- Alcohol
- Chromic acid
- Distilled water
- Enzyme

Procedure:

- Pipette out 0.1, 0.2, 0.3, 0.4 and 0.5 ml of alcohol in respective tubes S_1, S_2, S_3, S_4 and S_5 corresponds to 2, 4, 6, 8 and 10% of concentration.
- Made up the volume of all the tubes to 5ml with distilled water.
- 5ml of distilled water was added to a test tube marked as blank.
- Then added 12.5ml of chromic acid to all the tubes.
- Made up the volume of all the tubes to 25ml with distilled water.
- Then incubate the tubes at 80ºC for 15 minutes.
- After incubation the absorbance was measured at 620nm.

RESULTS

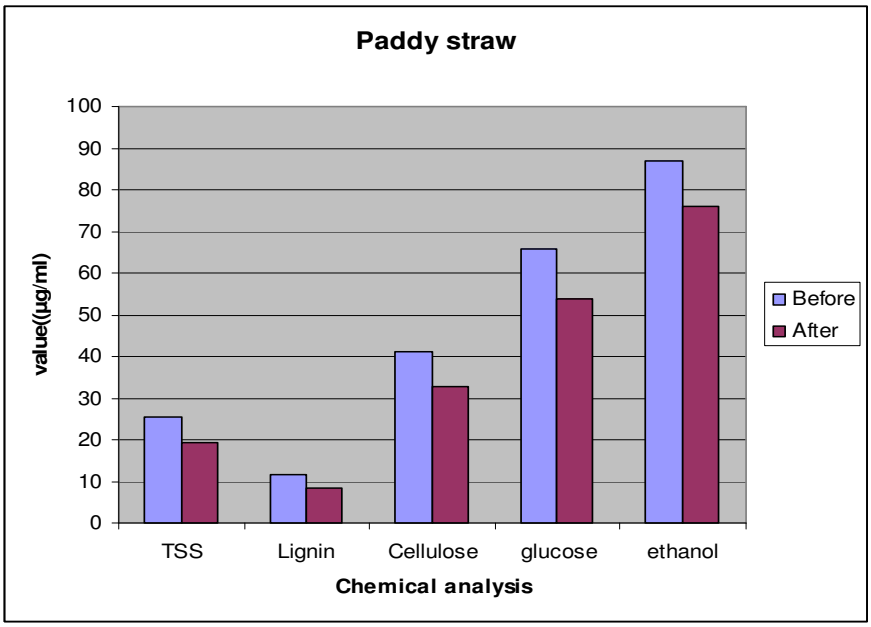
The ligninocellulose content of the substrates were calculated. Before treatment the totals soluble substancees were present in high amount. After treatment the substrates were utilized by fungai . Before and after treatment a minimum lignin was found in wood saw (12.5µg/ml & 8.2µg/ml) and Cellulose was high in paddy straw (41.2 µg/ml & 32.6µg/ml), Ash (26.4µg/ml & 20.4µg/ml), Crude fiber (28.1µg/ml & 22.8µg/ml), Total Soluble Substance (25.4µg/ml & 19.4µg/ml) in paddy straw were analysed and compared with wood saw (Table 1).

The ligninocellulolytic enzymes were calculated in culture filterates of milky mushroom. Amylase, Cellulase, Protease, Laccase, Manganese peroxidases were assayed in the culture filtrate supplemented with paddy straw and wood saw as carbon source. The maximum enzyme activity of Amylase (0.014µ mol/min), cellulase (80µg), Protease (38µg), Laccase (00.035µg), Manganese peroxidase (01.128) was found in paddy straw and the minimum enzyme activity of Amylase (0.011µ mol/min), cellulase (40µg), Protease (26µg), Laccase (00.033µg), Manganese peroxidase (01.120) was found in wood saw (Table 2).

This utilization of carbon sources also yielded some by products that were economically useful. The value added products were assayed to be Glucose and Alcohol in the spent medium of solid state fermentation. The maximum yield of Glucose (66µg), Alcohol (87µg) per ml of spent medium was found in paddy straw. A comparatively low yield of Glucose was 54µg, Alcohol was 62µg per ml of spent medium was found in wood saw (Table 3).

Table 1: Chemical analysis of ligninocellulosics

S.No.	Components	Paddy straw		Wood saw	
		Before treatment (µg/ml)	After treatment (µg/ml)	Before treatment (µg/ml)	After treatment (µg/ml)
1.	Ash	26.4	20.4	25.2	18.7
2.	Crude fiber	28.1	22.8	23.1	16.4
3.	TSS	25.4	19.4	21.4	14.6
4.	Lignin	11.5	8.5	12.5	8.2
5.	Cellulose	41.2	32.6	40.7	31.2


Paddy straw
100
90
80
70
60
50
40
30
20
10
0
value((µg/ml)
TSS
Lignin
Cellulose
glucose
ethanol
Chemical analysis
Before
After

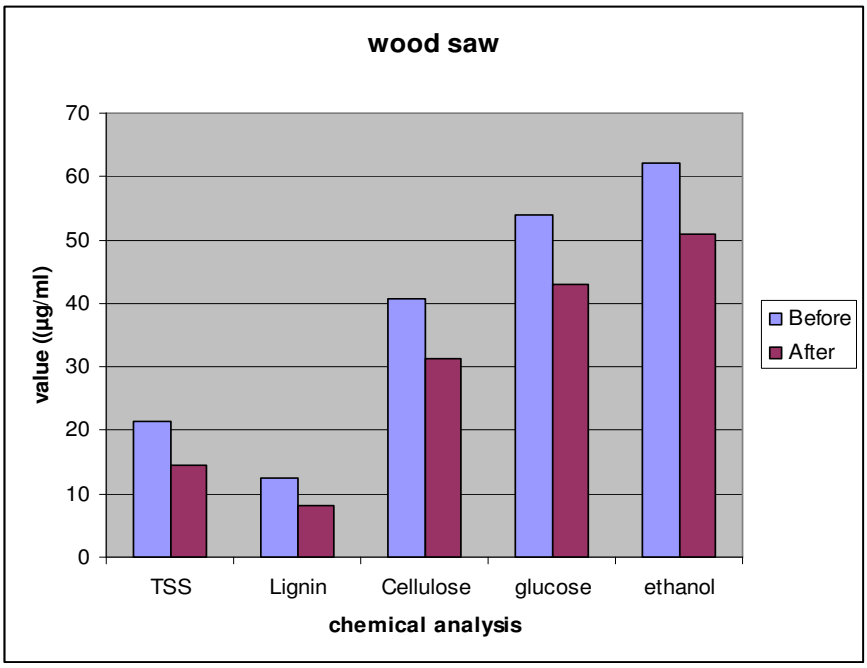

wood saw
70
60
50
40
30
20
10
0
value ((µg/ml)
TSS
Lignin
Cellulose
glucose
ethanol
chemical analysis
Before
After

Table 2: Enzyme activity in Solid State Fermentation

S.No.	Enzymes	Paddy straw Moles/min	Wood saw Moles/min
1.	Amylase	0.014	0.011
2.	Cellulase	80	40
3.	Protease	38	26
4.	Laccase	00.035	00.033
5.	Manganese peroxidase	01.128	01.120

Table 3: Assay of Valuable end products

S.No.	Value added products	Paddy straw	Wood saw
1.	Glucose	66µg	54µg
2.	Alcohol	87µg	62µg

DISCUSSION

In the natural environment, white-rot fungi interact with different microorganisms and complete with them for resources and space. The interactions among wood-rotting fungi have attracted considerable attention of fungal ecologists (Boddy, L. 2000) and some studies have focused on the effect of interaction on the production of extracellullar enzymes by fungi.

The quantification of lignocellulolytic enzymes produced by the *L.edodes* mycelium during the growth on the supplemented straw was carried out to provide a positive indication to the potential solid state fermentation being carried out by this fungus.

A broad spectrum of aromatic substances that were partially mineralized by the MnP system of the white rot fungus *Nematoloma frowardii* (Martin *et al.,* 1998).

Diafiltered and purified preparation of MnP from white rot fungus *N.forwardii* were capable of degrading a broad spectrum of aromatic and aliphatic substances directly to carbon dioxide and polar fission products. On the basis of this finding the use of the term enzymatic combastion, used for the depolymerization of lignin, has to be evaluated again (Kirk *et al.,* 1987).

The white-rot fungi belonging to the basidiomycetes are the most efficient and extensive lignin degraders (Akin *et al.,* 1995; Gold and Alic, 1993) with *P.chrysosporium* being the best-studied lignin-degrading fungus producing copious amounts of a unique set of lignocellulytic enzymes.

Phanerochaete chrysosporium is one of the most widely studied white-rot fungi with regard to lignin degrading enzymes (Tien and Tu, 1987). It has drawn considerable attention as an appropriate host for the production of lignin degrading enzymes or direct application in lignocellulose bioconversion processes (Ruggeri and Sassi, 2003; Bosco *et al.,* 1999).

Coir pith is a lignocellusosic waste material consists of lignin 20-40%, Cellulose 40-50%, Hemicellulose 35-45% and protein 2.04% (Sjostrom 1993). Coir is produced from the fibrous of coconut (Cocos micifera). The estimated annual production of coir pith in coir industries of India is about 7.5 million tons (Kamaraj, 1994) and accumulates every year, which leads to pollution of the environment.

Agricultural wastes such as wheat brawn and rice straw have been used for enhanced ligininase production in submerged and solid state fermentation (Arora *et al.,* 2001; Nyanhongo *et al.,* 2002).

The white rot fungus *Pleurotus ostreatus* in a chemically defined solid sate fermentation system amended with cotton stalk extract (CSE). Treated cultures exhibited increased laccase activity as well as

enhanced lignin mineralization (Ardon *et al.,* 1998).

Data about strict effect of available N source in the substrate on Extracellular lligninolytic enzyme induction in wood-rotting basidiomycetes have been conflicting. For some species, the ligninolytic enzyme production is suppressed by high nitrogen concentration while for other, high concentration of this nutrient stimulate ligninase production (Hatvani *et al.,* 2002; Kaal *et al.,* 1995; Leatham *et al.,* 1983).

The production of the extracellular ligninolytic enzymes is strongly affected by the nature and amount of the nutrients in the substrate, specially nitrogen and microelements (Buswell *et al.,* 1995; Chawachart *et al.,* 2004; Couto *et al.,* 2005).

The laccase produced by all the fungi exhibited maximum activity at pH 3.0-3.5 and stability in acidic to alkaline pH. It is common for basidiomycetes laccase pH optima in the acidic range, although they are more stable at neutral to alkaline pH values (Machado *et al.,* 2006; Perie *et al.,* 1998; Xu *et al.,* 1996).

The laccases showed thermophilic properties with optimum activity t 55-65˚C, similar to thermostable laccase from basidiomycetes strains (POXA1) (Jordaan *et al.,* 2004) and form *Peniophora sp.* (Keyser et al., 1978) although they were more sensitive to temperatures above 40˚C when in absence of substrate when compared to that mentioned laccases.

The maximum peroxidase activities did not exceed 40˚C but MnP from majority of strains were stable up to 70˚C when compared to that mentioned laccase.

The pattern of protease enzyme production was different in the both fungi *P.chrysosporium* produced higher than *R.stolonifer*. Bt all the enzyme activity was higher in co culture studies. The protease production started at 7th day (8.81 U/ml) of incubation period. However the higher amount of protease enzyme (17.31 U/ml) production was observed by growth of co culture (Kanmani *et al.,* 2009).

The revealed that the lignocellulosec enzyme activity exhibited remarkable differences against different substrates such as paddy straw, water hyacinth, leaves of *T.angustata*, groundnut plant, coir pith and coir pith amended with paddy straw (1:1 ratio of dry weight) (Periasamy *et al.,* 2003).

Solid state fermentation is an attractive process to produce fungal microbial enzymes (Chahal *et al.,* 1996; Halfrich *et al.,* 1996; Jech 2000). SSF is characterized by the complete (or) almost complete absence of free liquid (or) water, which is essential for microbial activities. The water is present in an absorbed in complexes from with the solid matrix and the substrate (Cannel and Moo-Young, 1980). These cultivation conditions are especially suitable for the growth of fungi, known to grow at relatively low water activities. As the microorganisms in SSF grow under conditions closer to their natural habitats and they are capable of producing enzymes and metabolies that will not be produced (or) will be produced only in low yield in submerged conditions (Jech 2000). SSF are considered practical for complex substrate fermentation including agricultural, forestry and food processing residues and wastes, which are used as the carbon source (Hattich *et al.,* 1996).

Almost all the selected fungi could produce a significant level of lignin peroxidase (LiP) during the fermentation period which was comparatively higher than laccase activity. Maximum amount of LiP activity (8.1 U/ml) was observed on the 28th day of fermentation by using co culture method. But *R.stolonifer* produced very low level of activity (3.51 U/ml) on the same fermentation period (Kanmani *et al.,* 2009).

L. polychrous cultivation on solid substrate using para rubber as a carbon source, rice bran as a nitrogen and vitamin source plus define minerals on three types of medium colonized fully in 35 days and yielded the higher level of Extracellular proteins. For ligninolytic enzymes, the level of laccase activity was higher than MnP, maximal at fully colonized stage and rapidly declined during fruiting stage the pattern was similar to *Lentinula edodes* (Ohga *et al.,* 2000).

Vijaya and Singaacharya (2005) reported the cellulolytic activity of *Pleurotus ostrreatus* as carboxymethylcellulase (28 relative enzyme activities) and filterpaperase (336mg/ml) on the paddy straw.

The quantification of lignocellulolytic enzymes produced by the *L.edoeds* mycelium during the growth on the supplemented straw was carried out to provide a positive indication to the potential solid state fermentation being carried out by this fungus.

The xylanase activity was significantly enhanced by the supplementation of wheat straw with different brans/meals as compared to unsupplemented straw (Shammi *et al.,* 2009).

SUMMARY

Lignin is one of the most slowly decomposing components of dead vegetation contributing a major fraction of the material that becomes humus as it decomposes. The significance of lignin degradation is in pollutant degradation. Pollution especially water pollution is due to discharge of pollutant from industries into water bodies. The chemical compositions of the major pollutants from textile effluents resemble the structure of lignin. Hence enzyme involved in lignin degradation could also degrade the pollutants and remove the toxic target compounds. Thus study of lignin degrading enzymes will have potential application in industrial wastewater treatment. White rot fungi produce lignin modifying enzymes that have been implicated in pollutant degradation

by the sea organism and they are mentioned to be the most efficient lignin degraders. The Extracellular lignin degrading enzymes are characterized as laccase, lignin peroxidase and manganese peroxidases. Based on the enzyme production patterns of white rot fungi, three categories of fungi are suggested class-I lignin manganese peroxidase class-II manganese peroxidase laccase class-III lignin peroxidase laccase group. Calocybe indica is a new variety of edible mushroom belonging to white rot fungi and is commonly called milky mushrooms because of its pure white colour. The mushroom has long shelf life and their growth is also very simple. Hence in the present study, experiments would be carried out to screen lignin degrading enzymes and group them in the above said 3 categories. In the mushroom proves to be positive further preliminary test would be done for treatment and removal of toxic compound from industrial effluent. Numerous studies on the physiology, biochemistry, chemistry and genetics of lignin degrading systems are being carried out presently.

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