SCREENING OF WOOD ROT FUNGI FOR PRODUCTION OF EXTRACELLULAR ENZYMES AND BIODEGRADATION OF TEXTILE DYES

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INTRODUCTION

Fungi

Fungi in general are diverse, eukaryotic, multicellular organism consisting of a dense hyphal network called mycelium which ramifies through the substrate. The fungal cell wall is made up of complex polysaccharides called chitins. The heterogeneity in lifestyle of fungi is reflected by their complex reproduction, which may be either sexual or asexsual depending on the environmental conditions. Fungi play a vital role in maintaining the environmental equilibrium, and their ubiquitous nature, not only enables them to colonize all matrices *viz*. air, soil and water, but also adapt their metabolism to varying carbon and nitrogen sources (Saratale *et al.* 2011; Anastasi *et al*. 2013).

Saprophytism is most essential lifestyle of most of them while others prefer parasitism. The procurement of nutrients during the course of evolution and development, both becomes a great challenge to their survival. Therefore, to cope up with these challenges, fungi developed certain physiological and cellular mechanisms to suffice energy need, they invade live or dead organisms/wood/plant biomass and decompose it (Anastasi *et al*. 2013). Fungi are the most important component of forest ecosystems and play a crucial role in wood decay (Pekka 2000) to recycle carbon stored in cell walls in the form of complex organic matter.

Wood degrading fungi

Wood is featured as the most profuse, natural and renewable source of energy. The wood cell wall is mainly composed of a mixture of lignocellulosics and comprises about ca. 40 % cellulose, ca. 20-30 % hemicellulose, and ca. 20-30 % lignin (Sjostrom 1993; Tuomela *et al.* 2000). Wood rot fungi are able to deconstruct lignin, cellulose, hemicellulose and further metabolize them (Kirk and Cullen 1998) with the help of their innate ability to produce specific extracellular enzymes capable of hydrolyzing recalcitrant organic compounds (Kaushik and Malik 2009; Idris *et al.* 2014). During the course of wood degradation, different taxonomic group of fungi shows a characteristic decaying pattern; due to the chemical and physical changes occurring in the wood. On the basis of the chemistry and morphology of these decay patterns, wood degrading fungi are classified into: Brown- rot, Soft-rot and White-rot fungi.

Brown-rot fungi cause rapid depolymerisation of cellulose and hemicellulose components of wood cell walls, but can modify lignin to a limited extent (Eriksson *et al.*1990; Green and Highley 1997; Schwarze 2007). The fungi that cause soft-rot have generally been attributed to Ascomycetes and Deuteromycetes group of fungi (Blanchette 1992). They differ from other wood decay types in their pattern of development, which involves T-branching or L-bending and hyphal tunnelling inside lignified cell walls particularly in S_2 later. This distinctive mode of attack was described in the mid-19th century by Schacht (1863). Further, it was elucidated by Savory (1954), who proposed the term soft rot. White-rot fungi belongs to the subdivision Basidiomycetes which either attack hardwood or softwood, while Ascomycetes probably degrade only hardwood (Kirk and Farrell 1987). Compared to other organisms, white-rot fungi degrade lignin extensively with a very fast pace, which apparently facilitates enzyme access to carbohydrates (de Koker *et al*. 2000; Mohamed *et al*.2013).

Lignin degradation by white rot fungi

Lignin is a major and an integral constituent of the cell walls of mechanical tissues of the vascular plants. Lignin are heterogeneous class of compounds (Fengel and Wegener 1989) and is the second most (after cellulose) abundantly present natural polymer and the most abundant aromatic material accounting about 40 % of the solar energy stored in plants (Leonowicz *et al*. 1999). The lignin matrix surrounds and protects the cellulose and hemicelluloses. Thus, removal of lignin enables the subsequent use of plant carbohydrates to microorganisms and this degradation of lignin proves to be a key step for closing the carbon cycle.

Increasing research interest on the degradation of lignin also accounts the importance of wood and other lignocellulosics as a renewable resource for the production of paper products, feeds, chemicals, and fuels (Ruqayyah *et al.* 2011). The only known organisms that can completely break down lignin into carbon dioxide and water are the white-rot fungi, and indeed gains access to their actual source of carbon and energy, i.e. cellulose and hemicelluloses (Leatham 1986; ten Have and Teunissen 2001). White rot postulates a unique ability to degrade the most intractable polymer (lignin) along with other components of the cell wall by means of either i). Selective delignification and ii). Simultaneous decay (Blanchette 1984; Maloy and Murray 2001, Schwarze 2007; Koyani *et al*. 2010; Sanghvi *et al*. 2013; Koyani *et al*. 2013).

During selective delignification, lignin is degraded earlier than cellulose or hemicelluloses, which occurs at a distance from fungal hyphae (Blanchette *et al*. 1997), resulting in defibration by dissolution of the middle lamella. The cells tend to separate in the early stage of decay, and remarks attack on hemicelluloses and lignin (Worrall *et al.* 1997; Srivilai *et al.* 2013), with limited attack on cellulose (Guerra *et al*. 2003). In simultaneous decay, decomposition of lignin takes place close to the hyphae and results in erosion of the cell wall from the lumen surface. Delignification is prominent due to the simultaneous removal of all three components cellulose, hemicelluloses and lignin (Blanchette and Reid 1986; Anagnost 1998, Luna *et al*. 2012).

Thus, white-rot fungi completely depolymerize and mineralize the plant cell wall polymer (lignin) by using the de-lignifying extracellular ligninolytic enzymes.

Ligninolytic enzymes of white rot fungi

Ligninolytic enzymes produced by white rot fungi include hemeperoxidases and phenol oxidases, which are responsible for generating highly reactive free radicals that undergo a complex series of spontaneous cleavage reactions which is a key step in lignin degradation (Reddy and Mathew 2001). Heme peroxidases include Manganese peroxidases (MnP), Manganese independent peroxidase (MIP) and Lignin peroxidase (LiP). On the other hand, phenol oxidases include Laccase (Lac) that catalyse the oxidation polyphenols and methoxy-substituted phenols, as well as aromatic amines.

Manganese Peroxidase (MnP)

Manganese peroxidase (Mn(II): hydrogen-peroxide oxidoreductase, EC 1.11.1.13) are extracellular glycoproteins with an iron protoporphyrin IX (heme) prosthetic group. This heme protein has a highly specific Mn^{2+} binding site acting as a mediator for MnP and belongs to the class II peroxidase group in basidiomycetous fungi (Jarvinen *et al*. 2012).

Lignin Peroxidase (LiP)

Tien and Kirk (1983), discovered lignin peroxidase (LiP) which is characterized by its low pH optima 2-5, high redox potential and molecular mass varying from 37 to 50 kDa for different white rot fungal species (Hirai *et al*. 2005; Asgher *et al.* 2006). LiPs consequently oxidize not only the usual peroxidase substrates such as phenols and

anilines.However, a variety of non-phenolic lignin structures and other aromatic ethers that resemble the basic structural unit of lignin. Therefore, they are considered powerful oxidants than typical peroxidases (Kersten 1990).

Laccase (Lac)

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belonging to the copper oxidase family. It is mainly characterised by the presence of copper atoms in the catalytic centre usually called multi-copper oxidase. Fungi belonging to the classes of Ascomycetes, Deuteromycetes and Basidiomycetes mainly account the presence of laccase (Gnanasalomi and Gnanadoss 2013). Laccases can degrade lignin in the absence of lignin peroxidase and manganese peroxidase (Mayer and Staples 2002).

The fungal ligninolytic enzymes have recently become the focus of much attention for their possible biotechnological applications (Lee *et al*. 2014). Ligninolytic enzymes work extracellularly allowing the access for many of the non-polar, non-soluble toxic compounds and bind non-specifically. This is one of the plausible evolutionary origins for the degrading pathway to mineralize a wide range of highly recalcitrant compounds and textile dyes that contain carbon skeletons similar to those found within the lignin polymer (Tien and Kirk 1983 and 1988). White rot fungi attributed with nonspecific extracellular enzymes that fascinates for their considerable industrial applications such as bio-pulping of paper, bioremediation of xenobiotic compounds such as poly-aromatic hydrocarbons and textile dyes (Kirk and Farrell 1987).

Fungi have been acknowledged as the most effective microorganisms in biodegradation of polyaromatic compounds, including lignin and synthetic dyes and wood decay. Therefore, the main objectives of the present study are:

OBJECTIVES OF THE STUDY

- Isolation, purification and characterization of rot fungi.
- Optimization of growth media.
- Optimization of its degrading and decolourizing ability.
- Screening of pure cultures for various enzymes.
- Production of enzymes by Solid State Fermentation.
- Isolation, purification (complete/partial) and characterization of different enzymes.

- To study enzyme kinetics (effect of temperature, pH, etc.).
- Molecular characterization of enzymes through electrophoresis.
- *Invitro* testing of isolated fungi for wood decay.

MATERIALS AND METHODS

The present work aims to explore the potential of white rot fungi in terms of ligninolytic enzyme production and biodegradation, focused on textile dye decolourization and degradation. The work also endeavors to understand prospective of these white rot fungi in biological delignification and study the cell wall degradation/decay pattern exhibited by the fungi in the wood species. Therefore, to cover both these aspects, the present work is divided into:

I. Enzyme and biodegradation study

II. Histological study

I. ENZYME AND BIODEGRADATION STUDY

Collection and Isolation of fungi

Thirty eight strains of different wood rot fungi were collected from Pavagadh (central Gujarat) and Junagadh (Saurashtra region) forests of Gujarat State. Samples were collected from the fruiting bodies of the rot fungi along with decaying wood or plant debris. Samples of the dead and decaying wood blocks were excised with the help of chisel and hammer. Half of the collected wood blocks were fixed in Formaldehyde: Acetic acid: Alcohol (Berlyn and Miksche 1976) for histological study while the rest of the blocks and fungal fruiting bodies were packed in the sterile polyethylene bags for the isolation and purification of causal organisms. After arriving to the laboratory, wood blocks and collected fruiting bodies of the fungi were suitably trimmed and surface sterilized with 0.1 % $HgCl_2$ with an intermediate washing with sterile distilled water followed by a treatment of 70 % ethanol for a few seconds. Subsequently samples were inoculated on different media and incubated at 27 ^{o}C. Pure cultures were established by serial transfer and stored at 4 ^{o}C in refrigerator for further studies.

Optimization of growth media

To optimize the fungal growth conditions, experiments using different media were carried out with supplementation of 0.1 % of streptomycin to prevent bacterial

contamination. Pure cultures were maintained at 4 ˚C with the following growth media Yeast Extract Agar, Sabouraud Agar, Czapex dox Agar, Potato Dextrose Agar, Rose Bengal Agar and Malt Extract Agar.

Screening of white rot fungi

Collected fungal strains were further screened for the production of ligninolytic enzymes, which is a characteristic feature of white rot fungi. To distinguish between the white rot and brown rot fungi, all the collected fungal strains were subjected to Bavendamm's test (Bavendam 1928). Mycelial plugs of 10 mm size from seven days old culture were inoculated on Malt agar plates containing 0.1 % tannic acid. These plates were incubated at 28 ˚C in B.O.D incubator and regularly checked for browning of media as a confirmatory observation of white rot nature of the fungi.

From the total collection, fifteen strains (*Agaricus blazei*, *Bjerkandera adusta*, *Coprinellus micaceus*, *Coriolopsis caperata*, *Flavidon flavus*, *Hexagonia tenuis*, *Inonotus hispidus*, *Microporus ochrotinctus*, *Pleurotus ostreatus*, *Podoscypha petalodes*, *Polyporus tricholoma*, *Schizophyllum commune*, *Trametes hirsuta*, *Trametes versicolor*) were found to be positive for Bavandamm's test.

To compare the enzyme activity with our isolates, pure cultures of *Trametes hirsuta* (Acc No NTCC 729/C) and *Trametes versicolor* (Acc No NTCC 165/S) were procured from Forest Research Institute, Dehra Dun. When compared with our isolates, both the strains obtained from Forest Research Institute, Dehra Dun were found to be relatively more potent for the production of ligninolytic enzymes. Therefore, further study was carried out using fungal strains obtained from FRI.

Molecular identification of *Trametes hirsuta* and *Trametes versicolor*

For the extraction of genomic DNA, both the strains (*Trametes hirsuta* and *Trametes versicolor*) were inoculated in liquid media and the mycelial mat was filtered out from the 10-12 days old cultures. Extraction of DNA was carried out using Plant/Fungi DNA isolation kit (Sigma Cat# E5038) and by manually as described by Plaza *et al*. (2014). PCR was carried out using 1X final concentration of Ready Mix™ Taq PCR Reaction Mix (Sigma) and template DNA (50 ng/µl). Amplification of the DNA was performed by using Thermal cycler (Applied BiosystemsVeriti®). The ITS region was amplified by PCR machine using the primers ITS 1 and ITS 4 as described by

White *et al.* (1990). The amplified products were purified using Purelink™ Quick PCR Purification kit (Cat# K310001). Successfully purified PCR products were sent for sequencing to Eurrofins Genomics India Pvt. Ltd., Bangalore. Sequence data obtained after sequencing was subjected to sequence match analysis using Basic Local Alignment Search Tool (BLAST) on NCBI for identification of fungal species. Identification was done by 99 % base-pair match of the sequence obtained to the closest available reference sequences. After the preliminary analysis, the sequence was submitted to NCBI by using BankIt tool and also submitted to BOLD SYSTEMS according to the guidelines provided on the BOLD website (http://www.boldsystems.org/).

Determination of enzyme activity

Production of extracellular ligninolytic enzymes by Solid State Fermentation (SSF)

To obtain a crude extract of ligninolytic enzymes, various agro-industrial wastes were used as substrate for the solid state fermentation technique (SSF). The enzyme production by SSF was carried out separately for each of the agro-industrial waste used as a solid substrate in 250 ml Erlenmeyer flasks containing 5 gm of solid substrate moistened with 50 ml distilled water. The flasks containing the production media were sterilized by autoclaving at 121 ˚C for 45 min. Five plugs (10 mm diameter) of fungal inoculums from seven days old culture of pure isolate were inoculated in each flask containing sterilized production media and incubated at room temperature for 24 days.

Optimization of assays under SSF

Optimization of agro-industrial waste used as substrates

To determine the maximum production of ligninolytic activity by SSF, different agro-industrial waste were used as solid substrates. Rice straw, wheat straw, saw dust, sugarcane bagasse and Pigeon pea (*Cajanus cajun*) pod shells. All the substrates were inoculated individually with pure cultures of both strains and crude extract of these substrates were used for the different enzyme assay. Analysis for determining the enzyme activity was performed in triplicates.

Optimization of particle size

Among the substrates used for the study (in case of wheat straw, rice straw and sugarcane bagasse) the one giving highest enzyme activity was further assessed for

optimization of its particle size. Different particle size (B.S.S: 4, 8, 12, 16; I.S: 4, 2, 1.40 and 1 mm) were assessed to obtain high efficient enzyme activity by Jayant Scientific Sieves (India).

Optimization of incubation time

The optimization of incubation period was carried out from the 3rd day till the 24th day of fungal inoculation to check the influence of time required for the growth of fungi for enzyme production. The flask containing solid substrate covered with fungal mycelia was harvested at an interval of every 3 days, i.e. 3, 6, 9, 12, 15, 18, 21, 24 and checked for the maximum enzyme production.

Optimization of reaction time

During enzyme assay, incubation period for enzyme assay reaction mixture directly affected the enzyme activity. Therefore, in the present study, varying range of incubation period from 5 to 45 minutes was also evaluated to get the maximum enzyme activity with appropriate reaction mixture.

Harvesting and enzyme assay

To assess the maximum enzyme production, the flasks were harvested at an interval of every 3 days of fungal inoculation. Crude extract of extracellular enzymes was prepared by the addition of 10 ml acetate buffer to the harvested flask. The contents in the flask were gently beaten and incubated on a rotary shaker for 30 min. Later, the content of the flask was filtered by using Whatman filter paper No. 1 and the filtrate was used as a source of crude enzyme. Crude cultural filtrates obtained by SSF were used for estimation of extracellular activity of MnP (Manganese peroxidase), MnIP (Manganese Independent Peroxidase), Lac (Laccase) and LiP (Lignin Peroxidase).

MnP and MnIP activities were determined by spectrophotometric measurements of DMAB (3-dimethyl amino benzoic acid) and MBTH (3-methyl-2-benzothioazolinone hydrazone hydro chloride) oxidation as substrates (Vyas *et al.* 1994). Oxidation of DMAB and MBTH as chromogen was followed spectrophotometrically at 590 nm. One unit (U) of MnP/MIP or laccase was defined as the amount of enzyme necessary to produce one μmol of product per min upon DMAB-MBTH oxidation (590nm) of the substrate in the reaction mixture under the assay conditions. Laccase enzyme activity was determined by the method of

Shrivastava *et al*. (2011) based on the oxidation of the substrate 2,2'-azino–bis (3-ethylbenzothiazoline)-6-sulphonicacid (ABTS, ε= 36,000 $cm^{-1}M^{-1}$). One unit of laccase enzyme activity (U) was defined as the amount of enzyme which leads to the oxidation of 1 μM of ABTS/min. Lignin peroxidase enzyme activity was assayed using dye Azure B (ε= 48,800 $cm^{-1}M^{-1}$) as a substrate (Archibald 1992; de Souza-Cruz *et al.* 2004). The enzyme activity was calculated using the molecular extinction coefficient of MnP, MIP, Laccase and Lignin peroxidise expressed in μmol/min. All measurements were run in triplicates.

Effect of physicochemical parameters

Production and activity of different enzymes was studied at varying, pH, temperature and metal ions.

Effect of pH

The pH profile was studied at the room temperature. Four different buffers *viz*. phosphate buffer (pH 4.5 - 6.5), Na- acetate buffer (pH 3.5- 6), Na- tartarate buffer (pH 2.5 - 5.0) and citrate buffer (pH 5.0 and 7.5) were used to get adaptability to the enzyme. The Na-acetate buffer with pH range of 3.5 - 6 for (MnP, MIP and Laccase) while Na-tartarate buffer with pH range 2.5 - 5.0 for (LiP) was found to be more adaptive to the enzyme activity.

Effect of temperature

To determine the thermo-stability of the different enzymes**,** standard enzyme assay was carried out at different temperatures over the range 10-40 ºC. The effect of temperature on the enzyme activity was calculated in 0.1 M Na-acetate buffer pH 3.5-5.0 (MnP and MIP), 50 mM Na-acetate buffer at pH 3.5 and 4.0 (Laccase) and 50 mM Na-tartarate buffer pH 2.5- 3.0 (LiP) individually after 10 minutes of incubation period.

Effect of metal ions

Effect of metal ions on the enzyme activity was studied by the addition of 1mM, 0.1ml Fe^{+2}/ Cu^{+2}/ Ca^{+2}/Mg^{+2}/Na^{+2} to the reaction mixture. The activity was estimated quantitatively after 10 minutes of the incubation at room temperature.

Partial purification of crude extract

Ammonium sulphate precipitation and Dialysis

For the partial purification of enzyme, different percent saturations (20 to 80 %) of crude extract of enzyme were achieved by addition of ammonium sulphate according to ammonium sulphate precipitation table by **(**Dawson *et al.* 1969). Ammonium sulphate precipitation was performed in cooling centrifuge (Thermo Scientific, India; at DBT-ILSPARE central instrumentation center of M. S. University of Baroda) at 8000 rpm, 4 ºC for 10 minutes. Enzyme was dialysed in 12000-14000 Da membrane cut off value against acetate buffer ranging from pH 3.5-4.0. Dialysed enzyme was collected and stored for further characterization.

Molecular weight determination

Electrophoresis using SDS-PAGE

Molecular characterization of the enzyme was done using SDS-PAGE and activity staining using CBB (Coomasie brilliant blue R-250). Electrophoresis of the partially purified enzyme was performed by method as described by Laemmli (1970).

Decolourization and degradation Experiments

Dyes

The textile dyes used in the present study were selected on the basis of their frequency of use in textile industries and structural diversity. The textile dyes were provided by dyeing, printing and processing houses *viz*. Reactive Red HE8B, Reactive Orange 2R, Reactive Black B, Reactive Red ME4BL and Reactive Yellow FG.

Dye preparation and liquid decolourization assay

Each of the synthetic dyes was dissolved in distilled water to prepare stock solutions of different concentrations ranging from 1, 10, 50, 100, 250 and 500 mg/L. The liquid decolourization assay was carried out in 150 ml Erlenmeyer flasks containing 25 ml of 2 % Malt Extract Broth (MEB) supplemented with various concentrations of different dyes.

Respective dye concentration was added aseptically in the culture media after their separate sterilization by autoclaving them at 120 ˚C for 20 mins. Each flask containing the sterilized culture media was inoculated with three discs (10 mm diameter) of fungal inoculum from seven days old culture of pure isolates. Non-

inoculated flask containing MEB supplemented with dyes was considered as control while the flask containing only MEB (without dyes and fungal inoculums) was used as blank.

Harvesting and analytical assay

The inoculated flasks were assessed for decolourization of the dyes in the liquid medium by harvesting them after an interval of every 2nd day, i.e. on 3, 5, 7, 9, 11 and 13 days of inoculation. The content of the flask was filtered with Whatman filter paper No. 1 and decolourization was monitored spectrophotometrically, by subjecting the filtrate at the maximum visible wavelength of absorbance (λ_{max}) for individual dyes. The decline of dye concentrations was measured by monitoring the decrease in the absorbance in a UV–visible spectrophotometer (Shimadzu). All the experiments were performed in triplicates and the average values were considered in calculations.

FTIR (Fourier Transform Infrared Spectroscopy) Analysis

In the present study, the dye biodegradation of decolourization values achieved through the spectrophotometric measurements was characterized by FTIR analysis. The samples containing the mixture of 10 ml of dye (10 mg/L concentration) were treated with 500 µl of partially purified enzyme. Subsequently, untreated and the treated dye solution was evaporated till complete drying at room temperature where untreated dye solution was considered as control. Powder obtained after drying was further processed for the FTIR analysis by KBr pellet method (Shah *et al.* 2013). The samples were analysed by using Shimadzu 8400 (Department of Applied Chemistry, M. S. University of Baroda) at 10^{-4} resolution and 30 scan.

II. HISTOLOGICAL STUDY

Wood blocks/material and *in vitro* laboratory decay test

To study *in vitro* decay, healthy wood disks of *Eucalyptus globulus* Labill., *Azadirachta indica* A. Juss, *Tectona grandis* L.f. and *Leucaena leucocephala* (Lam.) de Wit., were obtained from the main stems of 12-15 years old trees from the forest depots, Sawmills and the M.S. University Arboretum. Cubic wood blocks measuring 2x2x2 cm were prepared from the stem disc free from knots. Some of the blocks were marked for weighing, after weighing these blocks were soaked in water for 24 hours and hydrated. Next day, after autoclaving these blocks at 120°C for 30 minutes, they were surface sterilised with 70% alcohol and inoculated with 15 days old pure

cultures of *T. versicolor and T. hirsuta*. These samples were incubated for 30, 60, 90 and 120 days at 27+1°C and 70% relative humidity. After each incubation period, test blocks were removed and cleaned to take out mycelia. The marked blocks were weighed after oven drying, to determine percent weight loss while the rest of the blocks were fixed in FAA (Berlyn and Miksche, 1976). After 12 hours of fixation, these samples were transferred in 70% alcohol. The experiment was performed in triplicates and percent weight loss was determined.

Light microscopy

Suitably trimmed samples were dehydrated with a tertiary butyl alcohol series and embedded in paraffin. Transverse, radial and longitudinal sections of 10–12 μm thickness were cut with a rotary microtome (Leica RM 2035, Leica Microsystems, Germany). Sections were dewaxed in a xylene–ethanol series and stained with safranin–astra blue (Sigma, Germany) combinations (Srebotnik and Messner 1994). After dehydration in an ethanol–xylene series, the sections were mounted in dibutyl phthalate xylene. Sections were micro-photographed using a Leica DM 2000 trinocular research microscope with a digital camera (Canon S70D).

***Confocal Laser Scanning Microscopy (CLSM*)**

Samples were washed in water, followed by 0.01M phosphate buffer (pH 9.0). Hand sections (approximately 40-80μm thickness) were taken from the wood block and mounted in buffered glycerol (pH 8-9). Slides were examined with Zeiss confocal laser scanning microscope using a Krypton/argon laser emitting at a wavelength of 488 and 568nm (Donaldson and Lausber, 1998).

RESULTS AND DISCUSSION

I. ENZYME AND BIODEGRADATION STUDY

Isolation and Purification of Fungi

Isolation and purification was carried out from sterilized fruiting bodies of fungi and wood samples to check their adaptation potential on different growth media. Among different media used, Malt Extract Agar (MEA) media was found to be more suitable for the growth of the most of fungal isolates. Pure cultures were established by serial

culture technique and pure cultures of all these strains were maintained at 4 ^{o}C in refrigerator to study further parameters.

Screening of white rot fungi for extracellular ligninolytic enzyme study and dye degradation

All the purified strains were subjected to Bavendamm's test to distinguish between the white rot and brown rot fungi by using the technique proposed by Bavendam (1928). Among them, *Trametes hirsuta* and *Trametes versicolor* were selected for further study due to higher production of ligninolytic enzymes during primary screening. Compared to other strains *Trametes hirsuta* and *Trametes versicolor* grew much adaptively on Malt Extract Agar media. We also procured above mentioned both strains are also procured from Forest Research Institute, Dehra Dun. When compared with our isolates, pure cultures of *Trametes hirsuta* (Acc No NTCC 729/C) and *Trametes versicolor* (Acc No NTCC 165/S) obtained from Forest Research Institute, Dehra Dun were found to be more potent in the production of ligninolytic enzymes. Therefore, for subsequent study FRI strains were used. Therefore, for subsequent study was carried out on strains obtained from FRI.

Determination of enzyme activity

Solid State Fermentation (SSF)

Solid Substrate Fermentation (SSF) is an important mode of fermentation occurring in the absence of free water, employing an inert or a natural substrate used as solid support (Pandey *et al.* 2000; Couto and Sanroman 2005) which also mimics the natural condition for the organism. In the present work, efforts have been made for the production of ligninolytic enzymes using different agro-industrial waste by SSF.

Optimization assays under SSF

Optimization of agro-industrial waste used as substrates

Different agro-industrial waste, i.e. wheat straw, rice straw, sawdust, pigeon pea (*Cajanus cajan*) pod shells and sugar cane bagasse as a sole source of substrate without any mineral supplementation using *Trametes hirsuta* and *Trametes versicolor*.

Trametes hirsuta showed maximum enzyme productivity of manganese peroxidase and manganese independent peroxidase with sawdust, while laccase and lignin peroxidase with wheat straw. Though *T. hirsuta* showed sufficient growth on

sugarcane bagasse, it was found less efficient for enzyme production. In case of *T. versicolor* all the substrates showed efficient enzyme activity, but comparatively pigeon pea pod shells gave the maximum production of manganese peroxidase, manganese independent peroxidase, laccase except production of lignin peroxidase which was recorded highest in sugarcane bagasse. Our results indicate that differences in lignocellulolytic enzyme yields depend upon variation of the growth substrates, which is in agreement of earlier researches (Lorenzo *et al.* 2002; Silva *et al.* 2005; Songulashvili *et al.* 2007; Elisashvili *et al*. 2008; Elisashvili *et al.* 2009).

Optimization of particle size

Evaluation of different particle size of substrate may result in variation in the enhanced production of enzymes; this variation may be associated with the surface area available to the fungi (Sanghvi *et al*. 2010). Therefore, appropriate particle size of wheat straw i.e.1, 1.40, 2.0, 2.80 and 4.0 mm was assessed to obtain maximum enzyme production. Compared to other substrates used, *T. hirsuta* produced highest activity of MnP, MnIP, laccase and LiP at a 1mm particle size of wheat straw. The smaller particle size may ensure an increase in the ratio of accessible surface area to volume to the microorganism facilitating its growth (Woiciechowski *et al.* 2014).

Optimization of incubation time

In the present study, to conclude the optimum incubation period for maximum enzyme production; the enzyme activity was determined from the cultures harvested at the interval of every three days of incubation for 24 days. *T. hirsuta* started production of MnP, MnIP on sawdust as a substrate of SSF media while Lac and LiP on wheat straw as a substrate. Similarly, *T, versicolor* showed initiation of MnP, MnIP and Lac production on pigeon pea pod shells and LiP on sugarcane bagasse as a substrate.

It demonstrated an increasing trend of enzyme activity of all four enzymes, i.e. MnP, MnIP, Lac and LiP from the 6^{th} day and reached to its peak on 12^{th} day of incubation. After that, a gradual decrease in the enzyme activity of all four enzymes was observed. In contrast, *T. versicolor* showed peak of activity of laccase on the 9^{th} day, whereas MnP, MnIP and LiP attained their highest peak on the 12^{th} day of incubation. Subsequently, enzyme production declined after the 9^{th} day in laccase and after the 12^{th} day in MnP, MnIP and LiP. Hence, in the present investigation peak

enzyme activity by both the species occurs on the 12th day of incubation except for laccase of *Trametes versicolor* that peaked highest on the 9th day under SSF.

Optimization of reaction time

In the present investigation, 5 to 45 minutes of reaction time was checked to find out the maximum enzyme activity by *Trametes hirsuta* and *Trametes versicolor*. Both fungal strains showed increased activity up to 10 minutes of reaction time after that a gradual decrease in activity of all the four enzymes was noticed.

Production of extracellular ligninolytic enzymes by Trametes hirsuta and Trametes versicolor

White rot fungi differ from other group of fungi with respect to their enzyme production patterns and produce various combinations of enzymes (Vares and Hatakka 1997). In the present study, both the fungal strains *T. hirsuta* and *T. versicolor* produced all ligninolytic enzymes *viz*. Manganese Peroxidase (MP), Manganese Independent Peroxidae (MnIP), Laccase (Lac) and Lignin Peroxidase (LiP).

Enzyme activities of MnP, MnIP, laccase and LiP in crude extracts of *T. hirsuta* and *T. versicolor* varied depending on the selected agricultural wastes used. Under SSF, *T. hirsuta* showed the highest activity for MnP (168.69IU/ml) and MnIP (177.64 IU/ml) when sawdust was used as substrate while laccase (221 IU/ml) and LiP (58.44IU/ml) showed the highest activity in wheat straw as a substrate. In case of *T. versicolor* highest MnP (171.91 IU/ml), MnIP (168.69 IU/ml) and Lac (203.61 IU/ml) were revealed in pigeon pea pod shells, whereas LiP (56.39 IU/ml) was the highest in sugarcane bagasse. Among all the four enzymes, both species showed highest activity of laccase subsequently followed by MnIP, MnP and LiP by *T. hirsuta* and MnP, MnIP and LiP in *T. versicolor*. Thus, with present experiments, laccase activity is highly expressed indicating that it is the predominating enzyme, whereas LiP activities in both the strains were significantly lower compared to the other three enzymes.

Earlier, Cilerdzic *et al*. (2011) noted that *T. hirsuta* is the best producer of laccase among all the *Trametes* species studied so far. However, Remeshaiah and

Reddy *et al*. (2015) reported *T. versicolor* as one of the best- species that secretes various enzymes such as the phenol oxidase, laccase and perioxidase, which take part in the transformation of aromatic compounds.

Effect of physiochemical parameters

Effect of pH

Effect of pH on ligninolytic enzyme was investigated at the room temperature in the present study. The Na- acetate buffer (0.1 M) with pH range of 3.5 -6 for MnP and MnIP, while Na- acetate buffer (50 Mm) with pH range of 3.5-6 for laccase and Na-tartarate buffer (5 mM) with pH range 2.5-5.0 for LiP was found to be more adaptive to the enzyme activity with their specific substrate. In case of *Trametes hirsuta* highest activities of MnP, MnIP, Lac and LiP were recorded at pH 5, 3.5, 3.5 and 2.5 respectively. While in *Trametes versicolor* highest activities of MnP, MnIP, Lac and LiP was recorded at pH 3.5, 3.5, 4 and 2.5 respectively.

Most constructive growth pH of ligninolytic fungi is around pH 3-5 (Fu and Viraraghavan 2001). According to previous studies, the optimum activities of MnP of various white rot fungi vary from 4-7, laccase vary from pH range of 2-10 and LiPs vary between pH 2-5 (Bermek *et al.* 2004; Ürek and Pazarlioglu 2004; Yang *et al.* 2004; Baborová *et al.* 2006; Hakala *et al.* 2006; Asgher *et al.* 2007, 2008; Snajdr and Baldrian 2007).

Effect of temperature

Temperature affects the fungal growth, production, activity and stability of ligninolytic enzyme in white rot fungi (Snajdr and Baldrian 2007). In the present study, the enzyme activity of all four enzymes was estimated at varying temperature from 5-45 ºC. Both strains *viz*. *T. hirsuta* and *T. versicolor* showed fluctuations upon varying incubation temperatures. All enzymes showed an increase in titres of activities up to 30 ºC, and enzyme activity gradually declined upon increasing temperature. Therefore, temperature optima for maximum ligninolytic enzymes production were found to be 30-35 ºC for MnP, MnIP, Lac and LiP by *T. hirsuta* and *T. versicolor*. It was also noticed that enzyme production in both fungal strains increased substantially with temperature up to the optimal value. The temperature ranging from 25 to 37 ºC found to be optimum for ligninolytic enzyme production by

different white rot fungus (Zadrazil *et al.* 1999; Arora and Gill 2001; Tekere *et al.* 2001; Tripathi *et al.* 2008).

Effect of metal ions

In the present study, five different metal ions like Mn^{+2}, Zn^{+2}, Cu^{+2}, Ca^{+2}, and Mg^{+2} were examined for their ability to enhance the enzyme activity. The activity of MnP, MnIP, Lac and LiP are not altered by the presence of metal ions in enzyme reaction mixture. The strong negative effect of heavy metals on the growth of wood-rotting basidiomycetes is already well documented *in vitro* (Baldrian and Gabriel 1997; Mandal *et al*. 1998, Baldrian *et al*. 2000). LacII was strongly inhibited by Cd^{+2} followed by Cu^{+2}, Mn^{+2} and Zn^{+2} did not affect the enzyme activity at the concentrations tested in *Trametes versicolor* (Lorenzo *et al*. 2005). According to Johnsy and Kaviyarasan (2014), in *Neolentinus kauffmanii* compared to the control conditions, laccase activity remain stable for Fe^{+2} and decreased in $ZnSO_4$ and $CaCl_2$; while MnP activity was decreased on addition of aluminium oxide and calcium chloride.

Partial purification of crude extracts

Ammonium sulphate precipitation

Considering the high amounts of laccase produced by *T. hirsuta* and *T. versicolor*, ammonium sulphate precipitation was carried out at different percent saturations (20 to 80 %) described by Dawson *et al.* (1969) and the fraction with highest laccase activity was subjected to molecular weight determination. Crude extract of laccase obtained from *T. hirsuta* and *T. versicolor* exhibited high laccase activity at 60 % and 20 % saturated fraction respectively. These fractions were further dialyzed with dialysis membrane having 12000- 14000 Da cut off value.

Molecular weight determination

T. hirsuta produce laccase of molecular mass 68 kDa and laccase of *T. versicolor* produced molecular mass of 29 kDa. Laccases from the same or related species of *Trametes* or other fungi, range from 61 ~ 81 kDa (Yaver *et al.* 1996; Shin and Lee 2000; Min *et al.* 2001; Iyer and Chattoo 2003; Han *et al.* 2005). Shleev *et al*. (2004) reported the molecular mass of laccases from *Trametes hirsuta, Trametes ochracea, Coriolopsis fulvocinerea,* and *Cerrena máxima* within a range of 64 to 70 kDa. Laccase of *Trametes versicolor* of a molecular weight as low as 29 kDa as reported

in this work have never been found. In contrast in previous research, with some basidiomycetes such as *Trametes versicolor* and *Fomitella fraxinea*, the molecular weight of the laccases ranged from 97 to 80 kDa (Park and Park 2008; Zapata-Castillo *et al*. 2012). Han *et al*. (2005), also reported 97 kDa molecular mass of denatured laccase from *T. versicolor* 951022. Zhu *et al*. (2011) revealed 60 kDa molecular mass of laccase from *Trametes versicolor* sdu-4. According to Zapata-Castillo *et al*. (2012), these variations in laccase size may be attributed to the glycosylation degree of the protein.

Decolourization and degradation experiments

Dye decolourization in liquid medium by Trametes hirsuta and Trametes versicolor

The present study investigated decolourization of five textile dyes (Reactive Red HE8B, Reactive Orange 2R, Reactive Black B, Reactive Red ME4BL and Reactive Yellow FG) by *Trametes hirsuta* and *Trametes versicolor*. Initially a series of experiments were performed by using the liquid culture method to evaluate the rate of dye decolourization; wherein different concentrations from 1, 10, 50, 100, 250 and 500mg/L dye were checked. Decolourization was measured at the wavelength of maximum absorbance (λmax, nm) of respective dyes.

The colour change in all the five dyes was observed for the 3rd day of incubation in both the fungal strains. Pronounced decolourization by both *T. hirsuta* and *T. versicolor* was found in 10 mg/L concentration wherein all five dyes showed complete decolourization at the end of the 13 days of incubation. *T. hirsuta* decolourized all the five dyes on the 13th day of incubation. Similar to former species, *T. versicolor* also showed complete decolourization on the 13th day of Reactive Red HE8B, Reactive Orange 2R, Reactive Black B but Reactive Red ME4BL and Reactive Yellow FG were decolourized up to 97.9 % and 92.3 % respectively at the end of 13 days and complete decolourization was observed on the 15th day of fungal inoculation. This indicates that Reactive Red ME4BL and Reactive Yellow FG were more difficult to be decolourized as compared to all other three dyes. The decolourization efficiency of both species differed which showed that rate of decolourization by *Trametes hirsuta* was relatively faster than that of *Trametes versicolor.*

Previous studies mentioned 95% removal of HRB 8 dyes by *T. versicolor* in four days (Heinfling *et al.* 1997) and 92.17 % removal of Blue CA by *Trametes hirsuta* in 10 days of incubation (Sathiya-Moorthi *et al*. 2007). At lower concentration of 25 mg/L *Pleurotus florida* showed 93.54 % and 83.70 % decolourization of Blue CA and Corazol Violet SR respectively on the 10th day of incubation (Sathiya-Moorthi *et al*. 2007). *Phanerochaetae chrysosporium* removed 85 % of azo dye Orange II in 7 days (Sharma *et al*. 2009) while *Irpex lacteus* decolourized 100 % of Reactive Yellow FG, Reactive Violet 5R, Reactive Magenta HB, and Reactive Yellow MERL by after the 11th day of inoculation at a concentration of 10 mg/ml (Koyani 2011). Similar to our result, *T. versicolor* G-99 was slightly less effective, decolourizing Reactive Black B less than 60 % on the 8^{th} day (Mohorcic *et al*. 2006). However, a higher percentage of decolourizartion was reported for indigo carmine (96 %) and phenol red (69 %) in 24hr indicating its suitability for synthetic dye decolourization by using *Trametes hirsuta* (Dominguiz *et al*. 2005).

FTIR (Fourier Transform Infrared Spectroscopy) Analysis

FTIR analysis was carried out to confirm the biodegradation of these compounds by ligninolytic enzymes (Parshetti *et al.* 2006; Ghodake *et al.* 2009, Koyani *et al*. 2014). The FTIR spectra of all the tested control dyes and its treatment with fungi *Trametes hirsuta* and *Trametes versicolor* showed the specific peaks in specific region of stretching (4000 to 500 cm^{-1}), which clarifies the degradation of the dyes with fungal enzymes over decolourization. In the present study, the disappearance of a peak for an azo stretch clearly indicates the breaking of azo bond, by *Trametes hirsuta* and *Trametes versicolor* that would be an essential and foremost step for the color removal in all the five dyes. Broadband for-N-H stretching was the evidence for the aromatic amine group present in the parent dye compound; whereas peaks for C-H bend, and for C-N stretching of Ar-NH-R suggests the formation of aromatic amine. Comparison between the spectrum of control and treated dyes exhibited the changes in the positions of these peaks. The shifting of peaks from their original positions clearly indicates the degradation of the original molecular structure of the dye. Similar changes in the peak of different dyes has already been reported by earlier workers (Field *et al.* 1993; Parshetti *et al.* 2006; Ghodake *et al.* 2009; Gomare *et al.* 2009; Koyani *et al.* 2013).

II. HISTOLOGICAL STUDY

Trametes hirsuta and *Tramestes versicolor* are known to possess strong ligninolytic activity such as manganese peroxidase, laccase and lignin peroxidase. In the present study, wood decay pattern caused by *Trametes hirsuta* and *Trametes versicolor* is investigated on four different commercially important timber species *viz*. 1) *Eucalyptus globulus* Labill. 2) *Azadirachta indica* A. Juss 3) *Tectona grandis* L.f. and 4) *Leucaena leucocephala* (Lam.) de Wit.

Our results show variation in the percentage of weight loss depending upon different wood and the fungal species. Even though, both the strains completely ramify the wood blocks within the first fifteen days, no appreciable weight loss was observed by *T. hirsuta* and *T. versicolor* in all four woods at the end of the first month. According to Koyani and Rajput (2015), presence of several low molecular weight compounds in wood cells might be acting as a source of carbon during this stage, which consequently results in no weight loss. No appreciable weight loss at the end of one month indicates that the fungus either requires unusual conditions or the decay may develop slowly (Worrall *et al*. 1997). However, after 120 days of incubation, the highest percent of the weight loss occurred in *Eucalyptus* wood 51.9 % by *T. hirsuta* compared to samples of other wood samples. Similar to former fungal species, the percent of weight loss was highest in *Eucalyptus* 44.8 % incubated with *T. versicolor* whereas it was least in Teak wood. Thus, the percent weight loss was relatively more by *T. hirsuta* than *T. versicolor.*

Patterns of degradation may also depend on environmental factors which directly or indirectly contributes to the efficiency of fungi. Among them temperature, humidity and pH are important factors (Eaton and Halle 1993; Eriksson *et al*.1990; Koyani *et al*. 2010). However, white rot fungi show tremendous variability in patterns of wood degradation. Species of *Trametes* are considered to be most efficient degraders in which the ligninolytic enzyme system is comprised of laccase and Mn dependant peroxidase (MnP) as well as a series of cellulases and cellobiose dehydrogenase (Nakagame *et al*. 2006). In the present study, both *T. hirsuta* and *T. versicolor* showed decay pattern typical for white rot type. Based on the pattern of delignification of cell wall polymers by the fungus, white rot decay is categorized into two types 1) selective delignification and 2) simultaneous degradation of all cell wall

constituents (Schwarze and Fink, 1998; Schwarze 2007; Koyani *et al*. 2010; Pramod *et al*. 2015).

Both the species i.e. *T. hirsuta* and *T. versicolor* in *Eucalyptus*, *Azadirachta* and solely *T. hirsuta* in *Tectona* showed a combined pattern of degradation of selective and simultaneous rot. In contrast, in *Leucaena* both the species and exclusively *T. versicolor* in *Tectona*, showed typical simultaneous mode of decay during initial and advanced stage of decay.

In *Eucalyptus globulus, T. hirsuta* induced selective delignification that is evident from the typical anatomical features like cell separation, formation of oval shaped cavities that are rich in cellulosic polysaccharides and pit erosion while degradation of all wall constituents was noticed in advanced stages of decay resulting in transformation of oval cavities into large void areas. *T. versicolor* showed distinct simultaneous white rot decay pattern in the early stages of wood decay leading to formation of erosion channels across the wall and tunnels within the secondary wall. Although, *T. versicolor* showed simultaneous rot, its ability for selective delignification was evident on the basis of dissolution of middle lamellae and separation of cells, and this also forms a similar white rot feature exhibited by both the species of *Trametes* used in this study. Hence, our results suggest that both the species of *Trametes* possesses ability for selective and simultaneous modes of decay with a specific difference in its duration of former decay mode in the wood of *E. globulus*.

In case of *Azadirachta indica*, during initial stages of decay, *T. hirsuta* showed selective delignification while removal of carbohydrates during advanced stages of infection resulted in erosion of the cell wall. *T. versicolor* showed a typical simultaneous pattern of white rot with characteristic anatomical features such as erosion channels with U-notch appearance and separation of cells. Confocal microscopy revealed the removal of lignin from cell types including vessels and parenchyma which often resist degradation.

In case of *Tectona grandis, T. hirsuta* showed simultaneous degradation during the early stage of decay by formation of erosion troughs. On the other hand, further decay caused cell separation and dissolution of middle lamella, indicating a typical selective mode of delignification which was also confirmed in confocal

microscopy. In contrast, at the advanced stage formation of borehole, erosion channels and degradation of vessel and fibre wall depicted characteristic feature of simultaneous degradation. Also a typical L-bending pattern which is a distinct soft rot feature was also noticed. In contrast, *T.* v*ersicolor* showed majority of features characteristic to simultaneous rot with formation of erosion channels, erosion troughs and thinning of fibre wall leading to collapse of fibres.

In *Leucaena leucocephala*, present study revealed that both *T. hirsuta* and *T. versicolor* cause anatomical changes in the cell wall specific to simultaneous mode of decay. Many of the structural changes during advanced stage of decay such as thinning of fibre wall in two distinct patterns, formation of boreholes and pit erosion were similar for both the species of *Trametes*. However, cell separation without degrading the compound middle lamellae with preferential degradation of outer secondary wall layer during initial stages of decay was an unusual pattern showed by *T. hirsuta*. A detailed investigation on enzymatic activity with respect to the timing of structural alternation in the cell wall will be helpful to understand the physiology of the unusual degradation pattern shown by *T. hirsuta* in *Leucaena* wood.

Confocal microscopy also revealed the extensive degradation of lignin from the cell wall of all types of wood elements undergoing fungal degradation. During advanced stages of decay degradation of fibres and vessel wall delignification in cell wall components was evident in all wood species supporting the light microscopy observations. These results indicate the strong ligninolytic activity of *T. hirsuta* and *T. versicolor* as well as vulnerability of lignin to enzymatic delignification by the fungi against all the four wood species.

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