MATERIALS AND METHODS

2.1 Collection and Isolation of fungi:

Eighty five strains of wood rot fungi were collected from the different forests *viz.* Junagarh (Saurashtra region), Pavagarh (Central Gujarat) and Waghai (Dangs forest from South Gujarat) of Gujarat State. Samples of the rot fungi were collected in two different methods: i) collection of fruiting bodies and ii) isolation of fungi from the infected wood samples. In the former type, fungal fruiting bodies were either collected from the decaying wood or plant debris lying on the ground. These fruiting bodies were packed in the sterile poly ethylene bags. In the later type, wood samples were collected from the infected trees, in which half of the samples were packed in poly ethylene bags; whereas rest of the samples were fixed in Formaldehyde: Acetic acid: Alcohol (Berlyn and Miksche, 1976) for histological study. After coming back from the field, collected fruiting bodies and wood samples were surface sterilized by routine method of 0.1% $HgCl_2$ with an intermediate washing by sterile distilled water followed by a treatment of 70% ethanol. Finally, these samples were

treated with absolute alcohol and again washed with sterile distilled water before inoculating on different media.

2.2 Optimisation of growth media:

Initially, isolation of fungi was carried out by using various media to optimize the growth conditions (Table 2.1). To prevent the bacterial contamination, the media were supplemented with 0.1% streptomycin and chloramphenicol. Following the optimisation of different growth media, the pure cultures were maintained with suitable growth media at 4°C.

Sr. No.	Growth media used	pH
1.	Potato Dextrose Agar	5.0-5.5
2.	Malt Extract Agar	6.8-7.0
3.	Malt Extract Glucose Agar	5.8-6.0
4.	Yeast Extract Agar	5.5-6.0
5.	Sabouraud Agar	5.8-6.0
6.	Yeast Extract Glucose Agar	5.5-6.0
7.	Czapex Dox Agar	5.5-6.0

Table 2.1: Different growth media

2.3 Screening of white rot fungi:

For screening of white rot fungi, all the collected fungal strains were subjected to Bavendamm's test to distinguish between the white rot and brown rot fungi. Malt agar plates containing 0.1% tannic acid were exploited to inoculate 10mm fungal mycelial plugs of five days old cultures. These plates were incubated in B.O.D. incubator at 28°C and regularly checked for the browning of media as a confirmatory observation of being white rot. As all the species were isolated wild from the forest, the temperature required for the growth varied according to the species.

From all eighty five strains, only thirty six strains were found to be positive with Bavendamm's test (Bavendam, 1928); however, from these species only two giving promising results were considered for the further studies. Molecular identification of both the strains was carried out by the help of Chromous Biotech Pvt. Ltd., Bangalore (India). Pure cultures of both the strains *viz. Irpex lacteus* and *Phanerochaete chrysosporium* were revived regularly on freshly prepared media at the interval of every 15 days and maintained at 4°C.

2.4 Chemicals:

Malt Extract powder, Sudan Black B, and Safranin were obtained from LOBA Chem (Laboratory Reagents and Fine chemicals; India). Agar agar powder, Potassium di-hydrogen orthophosphate (KH_2PO_4), Di-potassium hydrogen orthophosphate (K_2HPO_4), Ferric chloride, Formaldehyde, Acetic acid, Potassium iodide and nitrogen sources (ammonium sulphate, sodium nitrate, sodium nitrite, urea and asparagine), were supplied by Qualigens Fine chemicals, India. All carbon sources (sugars) and Astra blue were procured from Himedia (India). DMAB (3-dimethyl amino benzoic acid), MBTH (3-methyl-2-benzothioazolinone hydrazone hydro chloride), H_2O_2 and Manganese Sulphate ($MnSO_4$) were provided by National chemicals Ltd., (India).

Electrophoresis chemicals such as Acrylamide, Bisacrylamide, Tris buffer, Ammonium per sulphate, Sodium dodecyl sulphate (SDS), TEMED (N, N, N', N'-Tetramethyl ethylenediamine), β-mercaptoethanol, Bromophenol blue, and Commassie Brilliant Blue R-250 (CBB) were acquired from Himedia (India). Sterilised double distilled water was used for all the experiments related with protein purifications and molecular determination. For partial purification of enzymes dialysis membrane having cut off value 12000-14000 Da was used. Medium range molecular marker was procured from New England Biolabs (NEB). All the other chemicals used were commercially available products of analytical grade.

The textile dyes used in the present study were kindly provided by the dyeing and printing, as well as processing houses. Dyes were selected on the basis of their structural diversity and frequency of use in the textile industries. The dyes used for the investigations are presented in Table 2.2.

Sr. No.	Dyes	λ_{max} (nm)
1.	Reactive Yellow FG	422.0
2.	Reactive Orange 2R	481.5
3.	Reactive Red ME4BL	542.0
4.	Reactive Red HE8B	522.0
5.	Reactive Black B	581.0
6.	Reactive Golden Yellow HR	422.0
7.	Reactive Golden Yellow HRNL	408.0
8.	Reactive Violet 5R	558.0
9.	Reactive Magenta HB	558.6
10.	Reactive Yellow MERL	421.5

Table 2.2: Different reactive textile dyes used

2.5 Experimental methods:

2.5.1 Measuring ligninolytic activity of fungi using dye decolourisation method:

2.5.1.1 Solid plate decolourisation:

The evaluation of on plate decolourisation of 10 different dyes was carried out on the Malt Extract Agar (MEA)-dye plate containing 2% malt and 2.5% agar in the presence of individual dye. The medium and dyes were separately autoclaved for 20 minutes at 120°C for sterilization purpose. Malt Extract Agar plates supplemented with different dye

concentrations (10, 50, 100, 250 and 500mg/l) were prepared with 25ml medium/plate. These petri plates were inoculated centrally with 10mm diameter agar disc removed from the actively growing fungi on Malt Extract Agar medium.

The plates were periodically observed for the growth of colony and decolourisation of growth medium up to 18 days. The decolourisation efficiency was assessed by visual disappearance of colours of the dyes on the plates from 3rd to 18th day of inoculation. At the interval of every 2 days, the growth zone and zone of decolourisation were measured.

Effect of carbon and nitrogen sources on solid plate decolourisation:

Influence of different carbon and nitrogen sources on the solid plate decolourisation was checked by the addition of 1% of carbon sources (dextrose, sucrose, fructose, maltose and lactose) and nitrogen sources (asparagine, urea, sodium nitrate, sodium nitrite and ammonium sulphate) into MEA media. Growth medium, dyes and carbon/nitrogen sources, all of them were sterilized separately in autoclave at 120°C for 20 minutes and mixed before plating into petri dishes (90mm diameter). A disc (10mm diameter) of fungal mycelium was centrally inoculated and incubated at 30 to 35°C. The diameters (cm) of the decolourisation zone and growth zone were determined in two perpendicular directions of the plates. Un-inoculated plates containing dyes and carbon/nitrogen sources were treated as control. Regular observations followed by measurement of growth zone and decolourisation zone were carried out at the interval of every 2 days. All experiments were carried out in triplicate and the results were expressed as the mean values.

Influence of inoculum size on solid plate decolourisation:

Effect of inoculum size on solid plate decolourisation was assessed by inoculating the MEA-dye plates with four different inoculum sizes (1 to 4, and 10mm agar plugs). These plates were observed regularly prior to

assess the decolourisation efficiency of fungi. Decolourisation zone and growth zone were measured periodically from the 3rd to 9th day of inoculation.

2.5.1.2 Decolourisation in liquid growth medium:

Dye decolourisation experiments in the liquid media were carried out in the 150ml Erlenmeyer flasks containing 25ml of 2% Malt Extract Broth (MEB) supplemented with various concentrations of different dyes. Test solutions of synthetic dyes were prepared by dissolving the dyes in the distilled water at five different concentrations of 10, 50, 100, 250 and 500mg/l. Prior to inoculation of fungal plugs, dyes and culture media were sterilized separately by autoclaving at 120°C for 20 minutes and thereafter respective concentrations of dyes were added in the culture media. Three discs (of 10mm diameter) of fungal inoculum taken from seven days old cultures of pure isolates were inoculated in each flask containing sterilized media.

Decolourisation of the dyes in the liquid medium was investigated by harvesting the inoculated flasks after 3, 5, 7, 9, 11 and 13 days of inoculation. Dye decolourisation was monitored spectrophotometrically, by subjecting the filtrate after removing mycelia at the maximum visible wavelength of absorbance (λmax) for individual dyes (Table 2.2). Un-inoculated sterile media containing only nutrient broth medium was used as blank while medium with dye but without inoculum was considered as control.

Analytical methodology:

Flasks inoculated with the fungal strains were harvested at the interval of every 3 days up to 15 days of inoculation. Before the spectrophotometric analysis, the liquid cultures were filtered with the Whatman filter paper No. 1 to separate out the mycelia and decolourised extract. The reduction of dye concentrations were measured by monitoring the decrease in the absorbance against the initial absorbance of control with a UV–visible spectrophotometer (Perkin-Elmer, USA).

The decolourisation efficiency was expressed as per the following equation.

$$P\% = \frac{A0 - A1}{A0} \times 100$$

Where, **P%** is the percentage decolourisation,

A0 and **A1** represent the initial and final concentrations of azo dyes, respectively.

All the experiments were performed in triplicates and the average values were considered in calculations.

***Effect of carbon and nitrogen sources on liquid growth medium decolourisation*:**

Various sources of carbon (such as dextrose, sucrose, fructose, and lactose) and nitrogen (such as ammonium sulphate, sodium nitrite, asparagine, and urea), at the concentration of 10g/l were used as co-substrates to investigate their effects on decolourisation. Three plugs of fungal mycelia (10mm diameter) were inoculated in the flask containing MEB (Malt Extract Broth) supplemented with different dyes and carbon/nitrogen sources (1% concentration). Before inoculation, the medium, dyes and carbon/nitrogen sources were autoclaved separately and thereafter they were mixed together.

Medium without any of the supplement was used as blank, whereas media with dyes and carbon/nitrogen sources but without inoculum were used as control. Decolourisation efficiency of the fungal isolates was measured at the interval of every 3 days using UV-visible spectrophotometer (Perkin-Elmer, USA) and percent decolourisation was calculated as per above mentioned equation.

***Influence of inoculum size on decolourisation of the dyes in the liquid medium*:**

Six flasks containing MEA-dye media were inoculated with different inoculum sizes (3, 6, 9, 12, and15 of 10mm diameter agar plugs).

After the 6th day of fungal inoculation, the contents of all six flasks were filtered and subjected to spectrophotometric analysis. Medium with dye but without inoculum was considered as control and medium containing only sterile media without any supplement was used as blank.

2.5.2 Determination of enzymatic activity:

2.5.2.1 Production of ligninolytic enzyme activities by solid state fermentation:

***Optimisation of fermentation process under SSF*:**

Optimisation of different Solid substrates:

Different substrates such as wheat straw, rice bran, saw dust, ground nut shells, sugarcane bagasse, and banana stems etc. were screened for determining the maximum production of ligninolytic enzymes in Solid State Fermentation (SSF). Some of these agro-industrial wastes were obtained from the local agricultural farms, while saw dust was obtained from saw mills situated near Vadodara and sugarcane bagasse procured from the sugarcane industry near Vadodara. All of the agro-industrial wastes were used individually as a substrate, in which wheat straw was found to be the best substrate for the production of enzymes under solid state fermentation. All the substrates were inoculated with the pure cultures of the fungal strains and crude extract of these substrates was used for enzyme assay. Each and every analysis was performed in triplicates.

Optimisation of particle size:

The process parameter was also assorted to optimize the enzyme production. Exposure of the surface of the substrate directly affects the growth of fungi. As the wheat straw was found to be the best substrate for the production of the enzymes, different particle sizes of the wheat straw (<1, 1, 1.5, 2, 2.5, 3, 3.5, 4 and >4 mm) was evaluated in connection to get the high efficient enzyme activity.

Optimisation of incubation time:

Time required for the growth of the fungi and production of the enzyme is another essential factor to be evaluated. In the present study, the optimisation of the incubation period was carried out till the 18 days of the inoculation. At the interval of every 3 days i.e. 3, 6, 9, 12, 15, 18, the flasks containing solid substrate covered with the fungal mycelia was harvested and checked for the maximum production of the enzyme.

***Enzyme production and harvestation*:**

The enzyme production was performed into 250ml Erlenmeyer flasks containing 5gm of agro-industrial wastes (wheat straw, rice bran, saw dust, groundnut shells, sugarcane bagasse, and banana stems) moistened with 50ml distilled water. This production media was sterilized by autoclaving it at 121°C, 45 minutes. Five plugs (10mm diameter) of fungal inoculum taken from seven days old cultures of pure isolates were inoculated in each flask containing sterilized production media and incubated at 35°C for 18 days. The flasks were harvested at every three days of the inoculation of fungal isolates and the enzyme activity was assessed for the maximum enzyme production. Crude extract of extracellular enzymes was prepared by addition of 50ml phosphate buffer to the harvested flasks. The contents in the flasks were gently beaten and incubated on the rotary shaker for 30 minutes. Liquor obtained was filtered by using Whatman filter paper No. 1 and the filtrate was used as a source of crude enzyme.

***2.5.2.2 Enzyme assay*:**

Crude culture filtrates obtained by Solid State Fermentation (SSF) were used for the estimation of extracellular MnP (Manganese Peroxidase), MIP (Manganese Independent Peroxidase) and Laccase activities.

MnP, MIP and laccase activities were determined by spectrophotometric measurement of DMAB (3-dimethyl amino benzoic acid) and MBTH (3-methyl-2-benzothioazolinone hydrazone hydro chloride) as substrates (Vyas *et al.*, 1994). The reaction mixture of MnP contained 100µl MBTH (1mM), 200µl DMAB (25mM), 10µl $MnSO_4$ (20mM), 10µl H_2O_2 (10mM), 1000µl buffer (0.1M), and 100µl enzyme in a total volume of 2ml. In case of MIP, the same reaction mixture was used as MnP except the addition of $MnSO_4$. On the other hand, in the reaction mixture of laccase, addition of $MnSO_4$ and H_2O_2 were excluded. Oxidation of DMAB and MBTH as chromogen was followed spectrophotometrically at 590 nm. The enzyme activity was calculated using the molecular extinction coefficient of MnP, MIP and laccase, and expressed in µmol/min. All measurements were run in quadruplicates. 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.

Enzyme activity was measured by using following equation:

$$\textbf{Enzyme Activity (IU/ml)} = \frac{\Delta D \times V}{\Delta E \times \varepsilon \times \Delta t} \times 10^6 \quad \text{units/ml}$$

or

µmoles/ml/minute

ΔD : O.D. at λ_{nm}

V : Volume of Total Assay (1ml)

ΔE : Volume of Enzyme Used (100ml)

ε : Extinction Coefficient

Δt : Incubation Time (minutes)

2.5.3 Partial purification:

Crude extract of enzyme was subjected to ammonium sulphate precipitation for the partial purification of enzyme. Different percent saturations (20 to 80%) were achieved by addition of ammonium sulphate according to ammonium sulphate precipitation table (Table 2.3).

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
10		28	57	86	118	137	190	183	216	251	288	326	365	406	449	494	540	592	640	694
15			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
20				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
25					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
30						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
33							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39

Table 2.3: Showing different concentrations of ammonium sulphate used for precipitation of Manganese Peroxidase, Manganese Independent peroxidase and Laccase during partial purification (As suggested by Dawson R.M.C., Elliott D.C., and Jones K.M. 1969. Data from Biochemical Research 2nd Ed. Oxford Press, London).

Ammonium sulphate precipitation was performed in cooling centrifuge (REMI, Bangalore, India) at 8000 rpm, 4°C for 10 minutes. Pellets obtained after centrifugation were dissolved in minimum amount of phosphate buffer (pH 5, 5.5). All saturated fractions were assayed for enzyme activity and the fractions having the maximum activity were subjected to dialysis. Enzyme was dialysed in 12000-14000 Da membrane cut off value against phosphate buffer ranging from pH 5 to 5.5. Dialysed enzyme was collected and stored for further characterization.

Molecular characterization of enzyme was done using SDS-PAGE and activity staining using CBB (Commassie Brilliant Blue R-250).

2.3.4 Molecular weight determination:

The molecular weight and sample purity were evaluated by separating proteins through 10% SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) along with standard protein marker. Gel electrophoresis was performed by using Medox electrophoresis unit (Bioneeds Instruments, Ahmedabad, India). Electrophoresis of partially purified enzyme was performed by method as described by Laemmli (1970).

Enzyme was treated with treatment buffer (Table 2.4) for 3 to 5 minutes in boiling water bath. Approximately 15 to 20µl of treated enzyme was loaded in gel (Table 2.5 and 2.6). Glycine SDS buffer (Table 2.7) was used as running buffer for electrophoresis. The electrophoresis time was variable from 2 to 5 hours, depending on the gel size and the purpose of the experiment.

Chemicals	Per (10ml)
Bromophenol Blue	8.0 mg
100%Glycerol	4.0 ml
14.7M β-mercaptoethanol	0.4 ml
1M Tris Buffer (pH 6.8)	2.0 ml
SDS	0.8 gm
0.5M EDTA	1.0 ml

Table 2.4: Composition of Treatment buffer (4X)

Composition	ml
30% Polyacrylamide	2.25
1.5M Tris Buffer (pH 8.8)	1.75
10% SDS	0.07
10 % APS(Ammonium per sulphate)	0.07
TEMED	0.0028
Distilled water	2.75

Table 2.5: Composition of 10% resolving gel (7ml) used

Chemicals	ml
30% Polyacrylamide	0.68
1.5 M Tris Buffer(pH 6.8)	0.5
10% SDS	0.04
10% APS(Ammonium per sulphate)	0.04
TEMED	0.004
Distilled water	2.72

Table 2.6: Composition of stacking gel (4ml) used

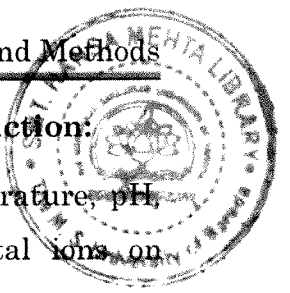
Chemicals	gm/l
Tris base	15.1
Glycine	94
SDS	10%
Distilled water	1000

Table 2.7: Composition of Glycine SDS buffer (5X)

After electrophoresis, gel was stained with CBB (Commassie Brilliant Blue R-250) dye solution (Table 2.8). Gel was stained overnight for the detection of protein bands followed by destaining with same solution except CBB. Nearly 7 to 8 wash were given to destain the gel. Bands were observed and photographed by using Sony digital camera DSC T10- 8 megapixel. Gel was preserved in 0.1% acetic acid solution.

Chemicals	gm/100ml
Commassie Brilliant Blue R-250	0.005
Methanol	30
Acetic acid	10
Distilled water	60

Table 2.8: Composition of CBB dye solution



2.5.5 Effect of physico-chemical factors on enzyme production:

Optimisation of enzyme activity with respect to its temperature, pH, substrate concentration, incubation time and effect of metal ions on enzyme activity was also studied.

Effect of reaction time:

Incubation period for enzyme assay reaction mixture directly affected the enzyme activity. During enzyme assay, the range of incubation period was extended from 5 to 45 minutes to get the maximum enzyme activity with appropriate reaction mixture.

Effect of pH:

Four different buffers viz. tartarate buffer (pH 2.5 and 3.0), succinate-lactate buffer (pH 3.0, 3.5, 4.0 and 4.5), citrate buffer (pH 5.0 and 7.5) and phosphate buffer (pH 4.5 to 6.5) were used to get adaptability to the enzyme. The range of pH 4.5 to 6.5 with phosphate buffer was found to be more adaptive to the enzyme activity. The pH profile was studied at the room temperature.

Effect of metal ions:

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

Effect of temperature:

Optimum temperature for the activity of the enzyme was determined by carrying out the standard enzyme assay at selected constant temperatures ranging from 10 to 40°C. The temperature profile was calculated in 0.1M phosphate buffer at pH 5 and 5.5. The effect of temperature on the enzyme activity was determined individually after 10 minutes of incubation period.

2.5.6 FTIR (Fourier Transform Infrared Spectroscopy):

Decolourisation values achieved through the spectrophotometric measurements were checked by FTIR analysis confirming the degradation of the compound. FTIR analysis was carried out at Department of Applied Chemistry, Faculty of Technology and Engineering, The Maharaja Sayajirao University of Baroda, Vadodara. The samples containing the mixture of 10ml of dye (10mg/l concentration) treated with 500µl of partially purified enzyme were dried at room temperature and processed for the FTIR analysis by KBr pellet method. The samples were analysed by using Shimadzu 8400, at 10^{-4} resolution and 30 scan.

(Any reference for this method or have you developed this technique for your expts.)

2.6 Histological studies:

2.6.1 Naturally infected samples:

Wood samples of naturally infected *Ailanthus excelsa* Roxb., attacked by *Inonotus hispidus* were collected from trees growing in the M. S. University hostel campus as well as from the Arboretum, located near Boys Hostel Campus. Only those trees from which fruiting bodies of *Inonotus hispidus* were coming out from the stem and branches were sampled to study the extent of damage caused. Sample blocks measuring about 60x60x60 mm in length, width and depth were excised from the main stems and thick branches with the help of chisel and hacksaw. Samples were fixed immediately in FAA (Berlyn and Miksche, 1976), while some of the unfixed samples were inoculated in PDA (Potato Dextrose Agar) and MEA (Malt Extract Agar) media for isolation and identification of the fungal species.

Fungi: Fruiting bodies of both *Irpex lacteus* and *Phanerochaete chrysosporium* were collected from the wood logs of trees growing in Girnar Forest of Gujarat state (Western India). Wood samples along with fruiting bodies were excised from the wood logs with the help of chisel and hammer. These samples were immediately packed in sterile poly ethylene

bags and brought to the laboratory. Small portion of fruiting bodies as well as suitably trimmed wood blocks were surface sterilized by routine method of 0.1% $HgCl_2$ for 40 to 45 seconds with intermediate washing by sterile distilled water followed by a treatment of 70% ethanol for few seconds. Thereafter, these samples were inoculated on 4% Malt Extract Agar (MEA) media. Pure cultures were established by routine methods and these cultures were maintained at 4°C. Molecular identification of pure culture of *Phanerochaete chrysosporium* and *Irpex lacteus* was done from the Chromous Biotech Pvt. Ltd., Banglore. For *in vitro* studies, to prepare the inoculum both the strains were grown separately for 7 days in petri plates containing 4% MEA.

2.6.2 Wood samples and decay test:

Healthy wood of *Tectona grandis* L.f., and *Azadirachta indica* (L.) Del., obtained from the plants growing in University campus and from sawmill was used to study *in vitro* decay test. 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 for re-wetting. 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 *Irpex lacteus* and *Phanerochaete chrysosporium*. 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 rest of the blocks were fixed in FAA (Berlyn and Miksche, 1976). After 12 hours of fixation, these samples were transferred in 70% alcohol.

Transverse, radial and longitudinal sections of 12 to 15µm thickness were cut on sliding microtome and stained with Safranin-Astra blue combinations (Srebotnik and Messner, 1994). Some of the sections were also treated with potassium iodide, Coommasie Brilliant Blue (CBB),

Sudan Black B and ferric chloride for the localisation of starch, proteins, lipids and tannins respectively (Krishnamurthy, 1999).

Some of the samples (particularly sample exposed to 120 days of fungal incubation) were also processed for paraffin embedding to obtain 8 to 10μm thick sections. Suitably trimmed samples were dehydrated with tertiary butyl alcohol series (30%, 50%, 70%, 90% followed by 3X 100% pure TBA) and processed by routine method of paraffin embedding. After dehydration in ethanol-xylene series, the sections were mounted in DPX. Important results were micro-photographed with Leica DM 2000 trinocular research microscope.