2. 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

2.1 Chemicals

All growth media *viz*. Yeast Extract Agar, Sabouraud Agar, Czapex Dox Agar, Potato Dextrose Agar, Rose Bengal Agar were procured from Himedia (India) while Malt Extract and Agar Powder was obtained from LOBA Chem (Laboratory Reagents and Fine Chemicals; India). The stains for histological study Safranin'O' was obtained from SRL Pvt. Ltd. (India) and Astra blue from Himedia (India). Other chemicals such as Formaldehyde, Acetic acid, Potassium iodide, Potassium di-hydrogen orthophosphate (KH_2PO_4), Di-potassium hydrogen orthophosphate (K_2HPO_4), Ferric

chloride and Agar powder were provided by Qualigens Fine Chemicals, (India). Manganese Sulphate ($MnSO_4$), H_2O_2, MBTH (3-methyl-2-benzothioazolinone hydrazone hydro chloride) and DMAB (3-dimethyl amino benzoic acid), were procured from SRL Pvt. Ltd. (India). All the chemicals used were commercially available products of analytical grade.

For the partial purification of enzymes, dialysis membrane with cut off value of 12000-14000 Da and other electrophoresis chemicals like Sodium dodecyl sulphate (SDS), TEMED (N, N, N', N'-tetramethyl ethylenediamine), β-Mercaptoethanol, Acrylamide, Bisacrylamide, Tris buffer, Ammonium per sulphate, Bromophenol Blue, and Coomasie brilliant blue R-250 (CBB) were obtained from Himedia (India). Medium range molecular marker was procured from GeNei Bangalore (India). All the experiments related to protein purification and molecular determinations were carried out using sterilized double distilled water.

2.2 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.

2.3 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 (Table 3).

Growth media	pH
Yeast Extract Agar	5.5-6.0
Sabouraud Agar	5.8-6.0
Czapex dox Agar	5.5-6.0
Potato Dextrose Agar	5.0-5.5
Rose Bengal Agar	5.5-6.0
Malt Extract Agar	6.8-7.0

Table 3: Various growth media used for optimization of fungal cultures.

2.4 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). Mycelia 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 (FRI), 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.

2.5 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/).

After molecular identification, characteristics of identified fungal species were compared with the literature of the standard references to confirm morpho-taxonomic characteristic of the species with our isolate.

2.6 Determination of enzyme activity

2.6.1 Production of 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.

2.6.2 Optimization of assays under SSF

i) Optimization of agro-industrial waste used as substrates

To determine the maximum production of ligninolytic activity by SSF, different agro-industrial waste listed in (Table 4) were used as solid substrates.

Agro-industrial waste (Substrates)	Availability
Rice Straw Wheat straw	Local Agricultural farms near Vadodara
Saw dust	Saw Mills at Harni Road, Vadodara
Sugarcane bagasse	Sugarcane Industries near Vadodara
Pigeon pea (*Cajanus cajun*) pod shells	As a waste from household use

Table 4: Substrates used for production of ligninolytic enzymes by SSF.

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.

ii) Optimization of particle size

The influence of substrate size on the growth of fungi is a necessary factor. Therefore, it is necessary to check the effect of particle size on enzyme activity. 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).

iii) Optimization of incubation time

The optimization of incubation period was carried out from 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 were 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.

iv) 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.

2.6.3 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). The reaction mixture of MnP contained 100 µl MBTH (1 mM), 200 µl DMAB (25 mM), 10 µl $MnSO_4$ (20 mM), 10 µl H_2O_2 (10 mM), 1000 µl Na-acetate buffer (0.1 M) at pH 4.5- 6.0, and 100 µl enzyme in a total volume of 2 ml. In case of MIP, the same reaction mixture was used as MnP except the addition of $MnSO_4$.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 $cm^{-1}M^{-1}$). The reaction mixture of laccase contained 100 µl ABTS (1 mM), 800 µl sodium acetate buffer (50 mM) ranging from pH 3.5-6.0 and 100 µl of enzyme in a total volume of 1 ml incubate. The rate of ABTS oxidation was determined spectrophotometrically at 420 nm. 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 reaction

mixture contained 0.2 ml Azure B (0.32 mM), 1 ml sodium tartarate buffer (50 mM) at pH 2.5- 5.0, 0.4 ml H_2O_2 (2 mM) and 0.4 ml of enzyme extract in a total volume of 2 ml. The reactions were monitored at 651 nm. 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. Enzyme activity was measured by using following equation:

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

or

µmoles/ml/minute

ΔD : O.D. at λ_{max}

V : Volume of Total Assay

ΔE : Volume of Enzyme Used

E : Extinction Coefficient

Δt : Incubation Time (minutes)

2.6.4 Effect of physicochemical parameters

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

i) 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.

ii) 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 a-tartarate buffer pH 2.5-3.0 (LiP) individually after 10 minutes of incubation period.

iii) Effect of metal ions

Effect of metal ions on the enzyme activity was studied by addition of 1 mM, 0.1 ml 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.

2.7 Partial purification of crude extract

2.7.1 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 (Table 5).

%	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 5: Concentrations of ammonium sulphate used for the precipitation of manganese peroxidase, manganese independent peroxidase, laccase and lignin peroxidase during partial purification (Dawson *et al.* 1969).

Ammonium sulphate precipitation was performed in cooling centrifuge (Thermo Scientific, India; at DBT-ILSPARE central instrumentation center of The M. S. University of Baroda) at 8000 rpm, 4 ºC for 10 minutes. Pellets obtained after centrifugation were dissolved in minimum amount of buffer required for respective samples. 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 acetate buffer ranging from pH 3.5-4.0. Dialysed enzyme was collected and stored for further characterization.

2.7.2 Molecular weight determination

i) 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). Gel electrophoresis was performed by using GeNei electrophoresis unit (Bangalore GeNei, India, at Department of Biochemistry, The M. S. University of Baroda). 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.

The enzyme was treated with treatment buffer (Table 6a) for 3 to 5 minutes in boiling water bath. Approximately 15 to 20 µl of treated enzyme were loaded in gel (Table 6b and 6c). Glycine SDS buffer (Table 6d) 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.

a. Composition of Treatment buffer (4X)

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

b. Composition of 10 % Resolving gel (5 ml)

Chemicals	Per 5 ml
Distilled water	1.9
30 % Polyacrylamide	1.7
1.5 M Tris Buffer (pH 8.8)	1.3
10 % SDS	0.05

10 % APS(Ammonium per sulphate)	0.05
TEMED	0.003

c. Composition of Stacking Gel (4ml)

Chemicals	**Per 4 ml**
Distilled water	2.7
30 % Polyacrylamide	0.67
1.5M Tris Buffer (pH 6.8)	0.5
10 % SDS	0.04
10 % APS (Ammonium per sulphate)	0.04
TEMED	0.004

d. Composition of Glycine SDS buffer (5X)

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

Table 6: Composition of buffers and gels used for SD-PAGE electrophoresis.

ii) Staining

For the detection of protein separated by polyacrylamide gel electrophoresis, gel was stained with CBB (Coomasie brilliant blue R-250) dye solution (Table 7). Gel was stained overnight followed by de-staining with the same solution except CBB with 7 to 8 washes. After staining bands were observed and photographed by using Sony digital camera DSC T10- 8 megapixel. Gel was preserved in 0.1 % acetic acid solution.

Growth media	**gm/100ml**
Coomasie brilliant blue R-250	0.005
Methanol	30
Acetic acid	10
Distilled water	60

Table 7: Compositions of CBB dye solution.

2.8 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 are listed below in (Table 8).

Dyes	**λ_{max} (nm)**
Reactive Red HE8B	522.0
Reactive Orange 2R	481.5
Reactive Black B	581.0
Reactive Red ME4BL	542.0
Reactive Yellow FG	422.0

Table 8: Textile dyes used in the present study.

2.8.1 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/litre. 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.

2.8.2 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 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

(Table 8). The decline of dye concentrations were measured by monitoring the decrease in the absorbance in a UV–visible spectrophotometer (Shimadzu). The percentage of decolourization was expressed by following equation:

$$\textbf{Percent Decolourization (P \%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where: A_0 is the initial absorbance and A_1 final absorbance of dyes

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

2.8.3 FTIR (Fourier Transform Infrared Spectroscopy) Analysis

Enzymatic degradation leads to breaking of certain bonds of the dyes during treatment. This phenomenon can be confirmed by FTIR spectroscopy using transmittance mode. 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) was treated with 500 µl of partially purified enzyme. Subsequently, untreated and 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, The M. S. University of Baroda) at 10^{-4} resolution and 30 scan.

II. HISTOLOGICAL STUDY

2.9 Wood blocks/material and *invitro* 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. These wood disks obtained from all four species were further cut into small cubic blocks measuring 2x2x2 cm.

Wood blocks free from the knots separated and used for the *in vitro* laboratory decay test. From each of the four species, some wood blocks were marked for weighing and after weighing these wood blocks were soaked overnight in water to obtain optimum moisture level to facilitate the fungal growth. Subsequently, these test blocks were autoclaved at 120 ^{o}C for 30 min and surface sterilized with 70 % ethanol. Four test blocks from each species were kept in each autoclaved petri plate containing Malt Extract Agar (MEA) media and inoculated with 2-3 plugs (5 mm diameter disk obtained with cork borer) of mycelium taken from 15-days-old pure cultures of *T. hirsuta and T. versicolor* (one fungi/petri dish). These samples were incubated for 30, 60, 90 and 120 days at room temperature and 70 % relative humidity. After each incubation period, test blocks for each fungus were removed and cleaned with a brush to take out mycelia. The marked blocks were weighed after oven drying to determine percent weight loss while rests of the blocks were fixed in FAA (Berlyn and Miksche 1976). After 12 hours of fixation, these samples were transferred in 70 % ethanol. The experiment was performed in triplicates. Percent weight loss was determined as:

$$\textbf{Percent weight loss (\%)} = \frac{\text{Weight of dry wood block after fungal incubation}}{\text{Weight of dry original wood block}} \times 100$$

2.9.1 Sample processing for light microscopy studies

Test blocks were processed for paraffin embedding to obtain 10 to 12 µm thick sections from experimental wood blocks. Suitably trimmed samples were dehydrated with tertiary butyl alcohol (TBA) series and processed by routine method of paraffin embedding (Berlyn and Miksche 1976). Transverse, radial and longitudinal sections of 10-12 µm thickness were taken with a rotary microtome (Leica RM 2035, Germany). The sections were de-waxed in xylene-alcohol series and stained with safranin-astra blue (Sigma, Germany) combinations (Srebotnik and Messner 1994). After dehydration in ethanol-xylene series (Berlyn and Miksche 1976), the sections were mounted in DPX (Dibutyl Phathalate Xylene) and were micro-photographed on Leica DM 2000 microscope (Germany) with a digital camera (Cannon S70D).

2.9.2 Confocal Laser Scanning Microscopy (CLSM)

For Confocal Laser Scanning Microscopy (CLSM), samples fixed in FAA were washed thoroughly in water, followed by 0.01 M phosphate buffer (pH 9.0). Hand sections (approximately 40-80 µm thickness) were taken from these wood block and

stained with 0.001 % acridine orange for 2 hrs in dark and mounted in buffered glycerol pH 8-9 (Ma *et al.* 2011). Slides were examined with Zeiss Confocal Laser Scanning Microscope-LSM 710 (at DBT-ILSPARE central instrumentation center, The M. S. University of Baroda) using a Krypton/Argon laser with excitation at wavelength of 488 nm (excitation) and 568 nm (Donaldson and Lausberg 1998; Knebel and Schnepf 1991). Important results were micro-photographed using in-built camera fitted within the instruments.