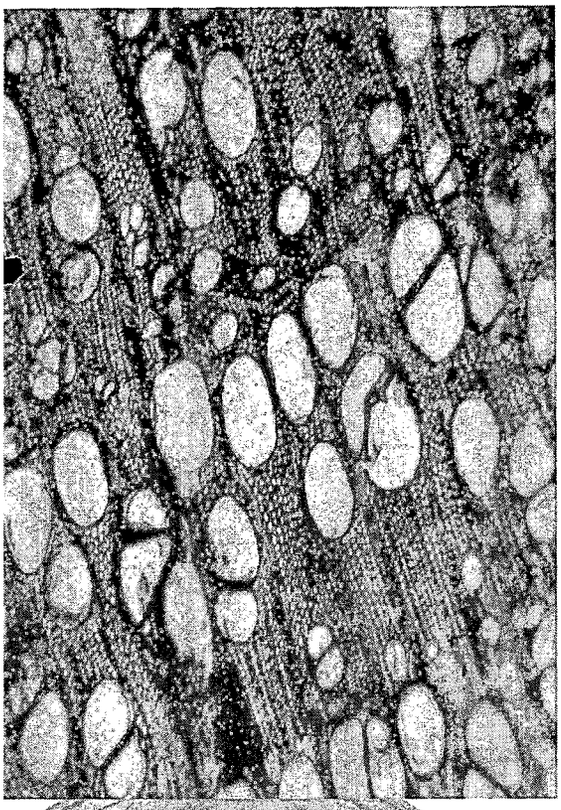
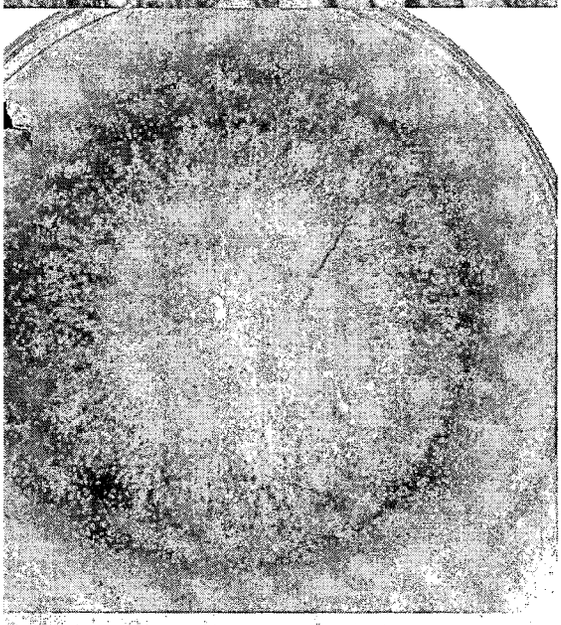
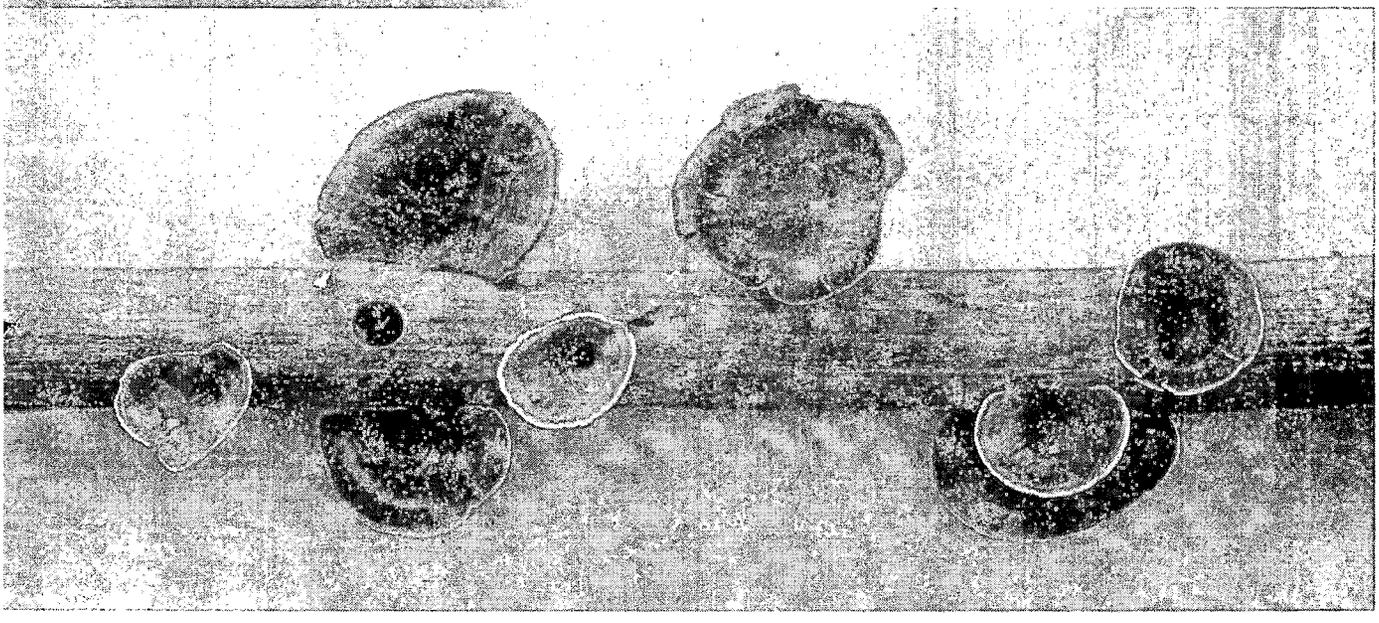




Materials and Methods



1) Survey of Timber Markets/ Saw Mills and Forest areas of Gujarat

A survey was undertaken in Ratanmahal Wildlife Sanctuary (RWLS) between Dec. 2005 to July 2009 and basidiocarps of different fungi were collected. RWLS is an area of 55.65 km^2 consisting of dry deciduous forest. The total existing sanctuary area lies between the river Panam and Orsang. The 11 villages of Ratanmahal forest are situated at the southernmost part of Limkheda taluka of Dahod district of Gujarat state. Ratanmahal lies nearly 35 km south-east from Devgadh Baria, the head quarter of Baria taluka. It is situated between 74° 37' to 74° 11' E Longitude and between 22° 32' to 22° 35' N Lat. The forest of the area was part of Kanjeta state. It is bounded by Jhabua district of Madhya Pradesh on its south-eastern side and Devgadh Baria on north-western side. The climate is sub-tropical arid, which turns damp and humid during monsoon. Rainfall ranges between 957 to 2101mm.

A survey was undertaken in sawmills of Vadodara, Ahmedabad, Jambusar, Bharuch, Rajkot and Jamnagar between January 2007 to July 2009 to find out the occurrence of timber degrading fungi and problems related to the wood decay in 5 districts. The infected wood along with sporophores were brought to the laboratory to identify the associated fungi.

2) Studies on Asco and Basidiocarps leading to their identification

2.1 Identification of Fungi

During field survey the materials were collected in clean polythene bags from different locations and brought to the laboratory. Basidiomes were studied using macroscopic (e.g. size, colour, number of pores/mm, length of tubes) and microscopic (presence/absence of structures, dimensions, vegetative and reproductive characters (Ryvarden 1991). To observe basidia and setae, free hand sections were taken. For the clear observation of setae, trammel setae and setal hyphae, lacto phenol cotton blue was used as staining medium. Xanthochoric reaction was also tested using potassium hydroxide solution. The various details of specimens were compared with available literature i.e. *Hymenochaetaceae of India* (Sharma, 1995) *Indian Polyporaceae* (Bakshi 1971), CBS Aphyllophorales database, New Zealand Fungi database, and *Species Fungorum*. Certain specimens were sent to the Royal Botanical Gardens, Kew, U.K. for final confirmation. These fungi are kept in fungal collection of Botany Department of The M. S. University of Baroda, India.

2.2 Isolation of Fungi

The fungi associated with the samples were isolated. Wood chips measuring 5 mm × 5 mm × 2 mm were aseptically removed from the samples and transferred to petriplates containing 2 different cultural media: 2% malt extract agar and PDA medium amended with 250µg Streptomycin sulphate per ml. The first medium was intended to isolate Basidiomycetous fungi and the second medium to specifically isolate total fungi. Eight pieces of wood were cut from each sample and placed in 2 petriplates. These plates were incubated at 25±2°C for 7 days. Once fungal colonies were formed in the agar plates, each colony was transferred to a new agar slant to obtain a pure culture.

3) Cultural Characters of Certain Timber Degrading Basidiomycetous members

For cultural studies Petri dishes of (100mm outer diameter, containing 20 ml agar) were inoculated with a piece of mycelium at the edge and were kept in diffused daylight at room temperature (25±2°C) and examined at 7 days intervals. Separate slides were prepared from the marginal region, the aerial mycelium and the submerged mycelium; they were mounted in lactophenol with cotton blue, which gives a cyanophilic reaction with certain structures.

3.1. Diagnostic Characters

3.1.1. Chemical tests

A rapid plate assay method was used to determine the presence of laccase, peroxidase, and catalase producing isolates. One to two weeks old mycelial growth on MEA plates was flooded with 25mM guaiacol in 5 mM $KHPO_4$ pH 6, to determine the presence of laccase. If no reaction occurred, the plate was then flooded again with 2 mM $H_2O_2$to determine the presence of peroxidase (Garraway *et al.* 1989, Harkin and Obst, 1973). To determine the presence of catalase activity, approximately 0.5 ml of 3% hydrogen peroxide was placed on a 1-3 week old colony on a MEA plates. The evolution of continuous bubbling was examined immediately and then again after a few min for positive reaction (Smibert and Krieg, 1981).

One drop 4% KOH was placed on the aerial mycelium and any colour change was noted. Also a mycelial preparation was made in water; while observing the hyphae under the microscope, the water was gradually replaced by 10% KOH. The most common reaction is a colour change from yellowish to reddish or purplish and from brown to dark purple or blackish.

3.1.2. Growth rate

The colony radius was measured after one and two weeks. The growth rate used in the key is the colony radius after two weeks.

3.1.3 Characters of the Fungal mat

3.1.3.a. Advancing zone: At the very margin of the colony the medium can be submerged(Growing in the agar), appressed (Prostrate on the agar surface) or raised. The hyphae may grow so densely that the individual tips cannot be seen with the naked eye when the plate is held against the light or they may be so distant as to appear fimbriate. The outline of the margin may be even but is sometimes fringed or bayed.

3.1.3.b. Aerial mycelium: The texture of the mycelial mat is described by on or more of the following terms like absent, downy, fariaceous, granular, silky, cottony, wooly, floccose, plumose, pellicular or subfelty, velvety, crustose, and zonate (Stalpers, 1978).

3.1.3.c. Colony colour: Seven large groups have been distinguished. Colony of a fungus may be (a) uncoloured, (b) white, (c) cream, (d) yellowish or ochraceous, (e) brownish, (f) orange or reddish and (g) pink, pale lilac, blue, vinaceous or violaceous.

3.1.4. Other macroscopic characters

3.1.4.a. Odour: Little attention has been paid to odours, unless they were very distinct and definable.

3.1.4.b. Reverse: The term reverse is used here for colour change in the agar underneath mycelium, induced by the fungus. It may be unchanged, bleached or darkened.

3.2. Characters of hyphae

3.2.1. Clamps: The distribution of the clamps is generally considered as having major taxonomic importance in Basidiomcetes members. It is constant within a species and generally also within a genus unless the fungus returns to the haploid condition. Four groups can be recognized

1) Clamps present at all septa
2) Clamps absent or rare in the advancing zone, but present at nearly all other septa
3) Clamps of erratic or rare occurrence, when present usually only at the boarder hyphae. There may be more than one clamp at each septum
4) Clamps absent.

3.2.2. Hyphal width: Five intervals of hyphal width have been assessed: (a) hyphae $\leq$ 1.5 µm, (b)Hyphae 1.5-3 µm (c) hyphae 3-5 µm (d) hyphae 5-7.5 µm and (e) hyphae $\geq$ 7.5 µm.

3.2.3. Differentiation of the hyphae

a) Generative hyphae: septate, branched, thin to thick-walled hyphae. For the thickness of the wall three categories are distinguished (a) thin walled (0.2 μm or less), (b) firm walled (0.2-0.3 μm) and (c) thick-walled (0.3 μm or more)

b) Skeletal hyphae: non-septate, unbranched or rarely branched, thick walled, straight or slightly flexuous.

c) Much-branched binding hyphae: non-septate, much branched, thick walled, strongly interwoven, typically narrow.

d) Hyphae with irregularly thickened walls, with meandering lumen or with thick walled, refractive areas inside the wall.

e) Hyphae with encrusted and usually contorted hyphal tips, usually erect and arising directly from the agar, giving the colony a mealy appearance.

f) Encrusted hyphae, occurring in many species in the aerial or submerged mycelium.

g) Hyphae covered with resinous material or oil drops, often yellowish or brownish.

h) Hyphae covered with minute projections which may be spiny or blunt but consist of wall material.

i) Aerial hyphae containing oil drops or resinous material.

j) Much branched thin walled hyphae, often described as witches brooms can be observed in the marginal zone.

k) **Setae**: Thick walled, brown more or less pointed hyphae, typically rather short and with a basal median swelling.

l) **Setal hyphae**: Long thick walled brown, pointed hyphae with or without some inconspicuous swellings.

m) **Asterohyphidia:** Thick walled, brown hyphae with radiating branches.

n) **Acanthohyphidia:** Clavate or cylindrical structures with pin like out growths.

o) **Cystidia:** Terminal or rarely lateral, cylindrical, clavate or ovoid structures which are thin to thick walled hyaline or brownish in colour.

p) **Gyoeocystidia:** Cylindrical, clavate or ovoid structures, terminal or more rarely, lateral, thin to firm walled, filled with refractive or resinous material, hyaline or yellowish.

q) **Cuticular cells**: Terminal, lateral or intercalary swelling or complexes of swelling which form a pseudo-paranchymatous crust.

3.2.4 Propagative structures

Chlamydospores, Blastoconidia, Arthroconidia, Conidiophores and Basidiospores were seen (Stalpers 1978).

4) Molecular Characters of Certain Timber Degrading Fungi

4.1. Genomic DNA Extraction

Genomic DNA was extracted from freshly grown culture of fungal mycelium on MEA plates and extraction was carried out in two phases as described below:

4.1.1. Solutions for extraction:

Tris saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1), 70% and 80% ethanol, 5 M NaCl, 3 M sodium acetate (pH 5.2) and TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0). Solutions and buffers prepared were autoclaved at 121 °C temperature and 15 psi pressure (Tommy autoclave, Japan). The stock solution of RNase 10 mg/ml was prepared freshly as per the user manual (Sigma, USA).

4.1.2. Extraction phase:

- 0.1 g of mycelial mat was grinded in a pre-cooled mortar with pestle to a fine powder using liquid nitrogen along with 10 mg (2% of extraction buffer) of PVP (Sigma, USA). The powdered tissue was scraped into a 2.0 mL microcentrifuge tube containing pre-heated (65 °C) extraction buffer in 1:5 ratio (0.5 mL). β-mercaptoethanol was added to the final concentration of 0.2 M and mixed well. The mixture was incubated in water bath at 65 °C for 90 min and cooled for 5 min.
- An equal volume of chloroform:isoamyl alcohol mixture (24:1) was added to the extract and mixed by gentle inversion for 5 to 10 min to form a uniform emulsion. The mixture was centrifuged at 8000 rpm for 8 min at RT. Chloroform: isoamyl alcohol extraction step was done twice. The aqueous phase was pipetted out gently, avoiding the interface.
- To the above solution, 5 M NaCl (to final concentration 2 M) and 0.6 volumes (V/V) isopropanol was added and incubated at RT for 1 h.
- Two volumes of 80% ethanol was added to the above solution and incubated again for 10 min at RT for DNA precipitation. After incubation the mixture was centrifuged at 10,000 rpm for 15 min at RT. The white/translucent pellet was washed with 70% ethanol, dried and resuspended in 200 µl of TE buffer.

4.1.3 Purification phase:

- The sample was treated with RNase (10 µL of 10 mg/mL of RNase) and incubated at 37 °C for 60 min. After incubation with RNase, one volume of Tris saturated phenol (pH 8.0) was added and mixed gently by inverting the micro-centrifuge tube till it formed a milky white emulsion. The emulsion was then centrifuged at 10,000 rpm for 5 min at RT. The supernatant was pipetted out into a fresh tube.
- The sample was then extracted with equal volume of chloroform: isoamyl alcohol (24:1) twice. The DNA was reprecipitated with 0.6 volumes of isopropanol, 2.0 M NaCl (final concentration) and incubated for 10 min.
- To the above solution, 20 µl of sodium acetate and 1 volume of 80% ethanol was added, incubated at RT for 30 min, and centrifuged at 10,000 rpm for 15 min to pelletout the DNA. The pellet was then washed with 70% ethanol twice; air dried and finally suspended in 40-50 µl of TE buffer.

4.2. Quantification of Genomic DNA

After extraction of genomic DNA, quantification was done according to Sambrook *et al.,* (1982). 15 µl of extracted DNA was dissolved in 735 µl of TE buffer and O.D. was taken at 260 and 280 nm (using CARY 500 scan UV visible spectrophotometer).

Quantity of DNA was calculated by using following formula:

Amount of DNA (ng/µl) = O.D. at 260 X dilution factor X 50 (extension coefficient)

Quality was assessed by taking the (O.D. at 260)/(O.D. at 280).

Samples which gave the O.D. between 1.6-1.8 were used in further work.

4.3. Restriction Digestion

The extracted genomic DNA was digested by incubating with *EcoR*I, *Mse*I restriction endonucleases along with control (without adding enzyme) in the corresponding buffers at 37 °C for 3 h according to the users manual. Digested DNA along with control was analyzed by running the samples in 1.2% agarose gel at 50 V and stained with ethidium bromide.

4.4. Polymerase Chain Reaction (PCR)

PCR was carried out as per the required volume with stock concentrations of reaction buffer (10X), MgCl2 (25 mM), dNTPs, Taq DNA Polymerase (5 U/µl) (Bioenzyme, USA) and, template DNA (250 ng/µl). The reaction was carried out in Thermal cycler (Eppendorf ep gradient S)

Final concentration of PCR reagents in reaction mixture (100 µl)

Taq polymerase 5 U

1X reaction buffer

3.5 mM MgCl2

800 µM dNTPs

0.4-1 µM of each Primer

50-100 ng Template DNA

Reaction was carried out as per the program given bellow:

Step	**Temperature**	**Time**
Initial denaturation	94 °C	3 min
Denaturation	94 °C	30 sec
Annealing	----	30 sec
Extension	72 °C	----
Final extension	72 °C	5 min

4.5. Agarose Gel Electrophoresis

Plate preparation and casting the gels: Cleaned agarose gel casting cassette and comb were wiped with methanol. The open sides of the tray were sealed with gel sealing tape. The comb was placed in the given slits of the plate. Calculated amount of agarose in TBE buffer was mixed to prepare 1.5% solution. The agarose was dissolved completely in the buffer by heating the mixture at 80-85 °C in microwave oven and was cooled to 50 °C. Liquid was gently poured into the casting tray before it gets solidified. The combs and sealed tape were removed slowly after complete solidification of the agarose gel.

4.6. Preparation of Samples and Scanning of Gels

The amplified DNA samples having approximately 15 µl volume were mixed with 4 µl gel loading dye and were carefully loaded in the wells using gel loading tips. Electrophoresis was carried out at 100-50V. The gel was stained by ethidium bromide solution having of 0.1mg/ml concentration for 15-20 min. The gel images were recorded in JPEG or TIF formats using gel documentation system (Syngene, USA). The gels were analyzed by using the software Gene Tool (Syngene, USA).

4.7. Random Amplified Polymorphic DNA (RAPD) Analysis

Amplification of RAPD fragments was performed according to Williams *et al.* (1990) using decamer arbitrary primers (Operon technologies Inc, USA; IDT, USA). The

reaction was carried out in a volume of 25 µl of reaction mixture containing final concentration of 10 mM Tris-HCl (pH 9.0), 50mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 3.0 mM MgCl2, 0.4µM primer, 25 ng template, 1unit Taq DNA polymerase (Sigma, USA). Amplification was performed in programmed thermal cycler with a program of initial denaturation at 94 °C for 3 min, 42 cycles of denaturation at 94 °C for 30 sec, primer annealing at 32 °C for 1min, extension at 72 °C for 2.5 min, and final extension at 72 °C for 4 min. amplification products were electrophoresed in 1.5% TBE. The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, UK). Experiment with each primer was done three times those primers gave reproducible fingerprints were considered for data analysis.

4.8. Amplified Fragment Length Polymorphism (AFLP) Analysis

4.8.1. Reagents for AFLP:

*Eco*RI/*Mse*I [1.25 units/µl each in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1mM DTT, 0.1 mg/mL BSA, 50% (v/v) glycerol, 0.1% Triton X- 100]. 100 µl

- 5X reaction buffer [50 mM Tris-HCl (pH 7.5), 50mM Mg-acetate, 250mM Kacetate] 250 µl
- Distilled water 1.25 ml
- Adapter/ligation solution [*Eco*RI/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl (pH 7.5), 10mM Mg-acetate, and 50 mM K-acetate] 1.2 ml
- T4 DNA ligase [1 unit/µL in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 50% glycerol (v/v)] 50 µl
- TE buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA] 4.5 ml
- Pre-amp primer mix 2 ml
- Selective primers (6.7 ng/µl, dNTPs)

EcoR I primers (27.8 ng/µl)

Primer E-AAC 46 µl

Primer E-AAG 46 µl

Primer E-ACA 46 µl

Primer E-ACT 46 µl

Primer E-ACC 46 µl

Primer E-ACG 46 µl

Primer E-AGC 46 µl

Primer E-AGG	46 µl
Mse I primers	(6.7 ng/µl, dNTPs):
Primer M-CAA	900 µl
Primer M-CAC	900 µl
Primer M-CAG	900 µl
Primer M-CAT	900 µl
Primer M-CTA	900 µl
Primer M-CTC	900 µl
Primer M-CTG	900 µl
Primer M-CTT	900 µl

4.8.2. Steps involved in AFLP technique:

(1) Restriction endonuclease digestion of genomic DNA

(2) Ligation of adapters

(3) Pre-amplification reaction

(4) Selective amplification reaction

4.8.2.a Endonuclease digestion:

The purified DNA sample showing sutable OD of 260/280 was taken and digested with *Eco*RI and *Mse*I which generated the template for next step. *Eco*RI has 6 bps recognition sites and *Mse*I has 4 bps recognition sites. Using these two enzymes together, small DNA fragments of optimal size range (<1 kb) are generated to obtain good fingerprints. The reaction was carried out as follows:

Component	**Control**	**Sample**
5X reaction buffer	5 µl	5 µl
Sample DNA (250 ng in ≤18 µl)	—	≤18 µl
EcoR I/Mse I	2 µl	2 µl
Distilled water 1	8 µL	5.5 µL-25 µL

Tubes containing reaction mixture were mixed gently and total reaction mixture was collected to bottom of the tube by centrifugation. Tubes were incubated at 37 °C for 120 min followed by heat inactivation of endonuclease at 70 °C for 15 min. The reaction mixture was stored at -20 °C till further step.

4.8.2.b. Ligation of adapters:

Following the heat inactivation of the restriction endonuclease, the genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters to generate template DNA for preamplification.

The common adapter sequences flanking variable genomic DNA sequences serves as primer binding sites on these DNA fragments. Using this strategy, it was possible to amplify many DNA fragments without having prior sequence knowledge.

Ligation reaction was carried out as follows:

Component	Volume
Adapter ligation solution	24 µl
T4 DNA Ligase	1 µl

Reaction mixture was gently mixed at room temperature, centrifuged briefly to collect contents, and incubated at 20 °C ± 2 °C for 120 min.

1:10 dilution of the ligation mixture was done (10 µl of reaction mixture was taken and transferred to a 1.5 mL microcentrifuge tube. 90 µl of TE buffer was added to it and mixed well). The unused portion of the reaction mixture was stored at –20 °C.

4.8.2.c. Pre-amplification:

In this step genomic DNA template obtained from step 3.12.2.1 was amplified with AFLP primers each having one selective nucleotide. The PCR products of the preamplification reaction were diluted and used as a template for the selective amplification.

The pre-amplification was carried out as follows:

Component	Volume
Diluted template DNA (from Ligation mix)	5 µl
Pre-amp. primer mix	40 µl
10X PCR buffer plus Mg+	5 µl
Taq DNA polymerase (5 unit/µL)	1 µl
Total volume	51 µl

Gentle mixing of contents was done and mixture was centrifuged briefly to collect the Reaction mixture. 20 cycles were performed at:

94 °C for 30 sec.

56 °C for 60 sec.

72 °C for 60 sec.

Final incubation temperature was kept at 4 °C.

15 µl of pre-amplified product was transferred to a 1.5 ml microcentrifuge tube containing 135 µl TE buffer. This was sufficient to carryout 30 selective AFLP amplifications. If necessary, new dilutions can be made from the pre-amplification

reactions to give additional template for the selective AFLP amplifications. Both unused diluted and undiluted reactions were stored at -20 °C.

4.8.2.d. Selective amplification:

In the selective amplification reaction, genomic DNA was amplified with two AFLP selective primers, each containing three selective nucleotides. For each primer pair, following components were added to a 1.5 ml microcentrifuge tube and labelled as "Mix 1".

Component	**Volume**
Diluted *Eco* RI primer	5 µl

(For non radioactive detection, primers were diluted as follows: 18 µl of *Eco*RI primer with 32µl of distilled water for use with the AFLP Non-Radioactive Probe)

*Mse*I primer (contains dNTPs)	45 µl
Total volume (sufficient for 10 reactions)	50 µl

Following components were added to another 1.5 mL microcentrifuge tube and were labelled as "Mix2".

Component	**Volume**
Distilled water	79 µl
10X PCR buffer plus Mg+	20 µl
Taq DNA polymerase (5 units/µL)	1 µl
Total volume (sufficient for 10 reactions)	100 µl

Each AFLP amplification was conducted by combining the following in a 0.2- or 0.5-mL thin-walled PCR tube:

Component	**Volume**
Diluted template DNA (from preamplification)	5 µl
Mix 1 Primers/dNTPs	5 µl
Mix 2 (Taq DNA polymerase/buffer)	10 µl

- One cycle was performed at 94 °C for 30 sec; 65 °C for 30 sec; and 72 °C for 60 sec.
- Annealing temperature was lowered at each cycle by 0.7 °C for 12 cycles. This gives a touch down phase of 13 cycles.
- 23 cycles were performed at:

  94 °C for 30 sec,

  56 °C for 30 sec, and

  72 °C for 60 sec.

  Total Time: 82 min.

4.9. RAPD and AFLP Data Analysis

Acquired RAPD finger prints were statistically analyzed with following assumptions.

1) The populations are in hardy Weinberg equilibrium.

2) Each band represents the phenotype at a single biallelic distinct locus.

3) Comigrating band represent homologous loci.

4) Polymorphic loci are inherited in a Mendelian fashion.

Fragment sizes were designated as loci, and were considered as Biallelic (present = 1, absent = 0) and made the binary matrix. Only those loci amplified strongly in each instance with reproducibility were scored and included in the analyses ignoring the intensity of the bands.

Genetic similarity was calculated according to the following formula:

$$F = \frac{2Nxy}{(Nx+Ny)}$$

Nxy = the number of bands shared by two species.

Nx and Ny = are the number of fragments in each sample.

Genetic disparity was calculated by formula.

$$P=1-F$$

Percentage of polymorphism was calculated by using following formula:

$$\text{Percentage of Polymorphism} = \frac{\text{No. of Polymorphic Bands}}{\text{Total number of Bands}} \times 100$$

Phylogenetic trees were constructed according to Jaccard (1908) using binary data generated by RAPD and AFLP excluding the intraspecific polymorphic markers, followed by bootstrapping analysis across the loci (Felsenstein, 1985) with the help of statistical analysis software NTSYS-pc version 2.11f.

5) Physiological studies on certain Timber degrading fungi

5.1. Selection of suitable culture media

To select a suitable culture medium for better growth of *L. sterioides, T. pini, H. apiaria* and *N. floccosa*, the following media were used. For physiological studies 25 ml of the liquid basal medium was taken in 150ml (Borosil) conical flasks. Unless otherwise stated the culture media were autoclaved at 15 lbs. p.s.i for 20 min. With the help of agar disc method (Garrett 1936) ten to twelve days old cultures were used for inoculating the flasks containing different media.

Malt Extract Agar Medium:

Malt extract	– 20g
Dextrose	– 20g
Peptone	– 1g
Agar	– 20g
Distilled water	– 1000ml

Modified Asthana and Hawkers Medium 'A'

D – glucose	-10g
KNO_3	– 3.5g
KH_2PO_4	– 1.75g
Mg SO_4 $7H_2O$	– 0.75g
Distilled water	– 1000ml

Czapek Dox's Medium

Sucrose	– 30g
Na NO_3	– 2g
K_2HPO_4	– 1g
Mg SO_4 $7H_2O$	– 0.5g
KCl	– 0.5g
$FeSO_4$ $7H_2O$	– 0.01g
Distilled water	– 1000ml

Basal Medium (Hacskaylo *et al.* 1954)

D- Glucose	25 g
KNO_3	0.425 g
KH_2PO_4	2 g
$MgSO_4$ 7 H_2O	0.5 g
$FeSO_4$ $7H_2O$	0.2 mg
Zn SO_4 $7H_2O$	0.2 mg
$MnSO_4$ $4H_2O$	0.1 mg
Thiamine HCl	100 µg
Biotin	5 µg
Distilled water	1000ml

1 % Malt Extract Agar Medium

Malt extract	– 10g
Agar	– 20g
Distilled water	– 1000ml

Inoculated flasks were incubated at 25± 2°C for 5,10 and 15 days. At the end of incubation period, change in pH of the medium was determined. In order to assess the growth of organisms, their fungal mats were harvested at the end of incubation period on previously dried and weighed Whatman filter paper No. 1. the filter papers were again dried in an electric oven at 60°C for 72h and then they were cooled in a desiccator at least for 2 h.

The difference between the initial and final weights of the filter paper indicated the dry weight of fungal mat. Each set of the treatment was run in triplicate and only the average dry weight was always taken as standard value for comparison of growth. The dry weight results were statistically analyzed by using the MS office Excel software and the significant values were taken for study.

Dry weight of mycelial mat was graded into three categories good, moderate and poor. The general mean (G.M) of the experiment ± SD with anova has been considered moderate. The dry weights higher or lower than the moderate have been designate as good or poor respectively.

5.2. Selection of suitable Hydrogen Ion Concentration

To select a suitable hydrogen ion concentrations for better growth of timber degrading fungi, the following hydrogen ion concentrations i.e. 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 8, 9, and 10 were used. The pH of the Czepek Dox's medium was adjusted by using 1N HCl and 1N NaOH solutions. After adjustment of suitable hydrogen ion concentration 25 ml of the medium was transferred to 150 ml conical flasks (Borosil grade). Other method was same as described above.

5.3. Selection of suitable temperature

To select a suitable temperature for the growth of timber decaying fungi, the temperatures like 5,10,15,20,25,30,35, and 40°C were used. The same procedure was followed as above mentioned in suitable growth and hydrogen ion concentration tests.

5.4. Effect of carbon sources on timber degrading fungi

For the study of the effect of carbon the amount of carbon present in individual substance in the basal medium was calculated, and a quantity equivalent to that was singly substituted in the basal medium by replacing the original corresponding substance viz, Sucrose. The amount of polysaccharides was similar to the amount of sucrose present in the basal medium. The medium devoid of sucrose served as control for carbon. To study the effect of carbon sources on growth of timber degrading fungi, the Xylose,

Arabinose, Maltose sugars were used. These sugars will acts as carbon sources which are supplemented in Czapak Dox medium. Wood degrading fungi like *L. sterioides, T. pini, H. apiaria* and *N. floccosa* were grown on basal medium containing 1% Malt extract as carbon source. After inoculation with test fungi the flasks were incubated in dark for 21 days. After completion of incubation period the fungi were filtered with Whatman filter paper No.1 and dried for 72 h at 60 °C in oven. The dried filter papers were weighed to calculate the growth of each test fungi. The filtrate was used to determine the final pH.

5.5. Effect of Nitrogen on growth of wood decay fungi

The basal medium supplemented with five nitrogen sources was used for growth of test fungi. The amount of Nitrogen present in 2g NaNO3/L was calculated and was replaced with same amount of nitrogen in other nitrogen sources

5.6. Utilization of the sugars by wood decay fungi

Utilization of different mono-oligo, and poly sachharides as well as the hydrolytic products of di- and tri sachharides was studies. Paper chromatography was used for this purpose. The quantity of various sugars was similar to that used in experiment dealing with carbon requirements. Dry weight of mycelial mat and pH of the medium was recorded after incubation period of 5, 10 and 15 days and filtrates were analyzed daily to detect the presence of various sugars. Drops of known volume (0.05 ml) were taken from the filtrates every days and were placed on the chromatogram by micropipette at a position located for this purpose. The running solvent was n-butonol-acetic acid- water (4:1:5) v/v. A mixture of 5 vol of 4% aniline, 5 vol of 4% diphenylamine and 1 vol of orthophosphoric acid (Buchan and Savage, 1952) was used as spraying reagent for the detection of sugars. Chromatograms were developed after drying at room temperature by heating in an electric oven at 100°C for 90 sec. the Rf values were calculated by the following formula:

$$Rf = \frac{\text{Distance traveled by the solute}}{\text{Distance traveled by the solvent}}$$

6) Biochemical changes of certain Timber Rotting fungi

6.1 Tests for different Enzymes

The screening of all species of fungi for their lignolytic and cellulolytic ability was done by substituting the malt extract agar medium (2%) with tannic acid for ligninase (0.5%) as suggested by Nobles (1965) and carboxymethyl cellulose for test of cellulase (0.5%) (Bains *et al.*, 2006). Streptomycin sulphate was added prior to

sterilization to avoid bacterial contamination except in case of lignolytic activity where tannic acid itself act as bactericidal agent. After autoclaving, media was cooled and poured to sterile Petriplates aseptically. On solidification, the plates were inoculated at the center with 1cm^2 mycelial disc of different fungal cultures under study and incubated at 28±1°C for a week. The replicates were maintained for each set of observations. The respective enzyme activities were evaluated by observing and measuring the zone of clearance if any, found by flooding the plates with visualizing reagent or dye (0.25% Congo red) for 15min (Teather and Wood 1982) for detection of cellulolytic activity, while the lignolytic activity was assessed by observing brown colored zone around respective fungal colonies.

6.2. Enzyme Essay

Wood decay fungi like *S. commune, L. sterioides, G. lucidum, N. floccosa, F. flavus, C. versicolor, H. apiaria,* and *T. pini* were tested for production of lignin degrading enzymes. Estimation of Laccase, Peroxidase, Aryl alcohol oxidase, and Lignin peroxidase was done by using Guaiacol and veratryl alcohol as substrates. The basal medium described above supplemented with KNO_3, Na NO_3, NH_4NO_3, and $Ca(NO_3)_2$ was used for growth of these fungi. Same procedure was followed as described in effect of nitrogen sources on growth with some modifications i.e. the filtrate was centrifuged at 10000 rpm for 15 min to get the clear supernatant. The supernatant was used as crude enzyme source for all ligninolytic enzyme assays.

In another experiment, white rot fungi i.e. *S. commune, L. sterioides, G. lucidum, N. floccosa, F. flavus* were grown in malt extract broth to test lignin degrading capacity by secreting enzymes. 1% malt extract supplemented in Basal medium was prepared as method described above. After the completion of incubation period the flasks were filtered with Whatman filter paper no1 and then the filtrate was centrifuged at 10000rpm for 15 min to get the clear supernatant. The supernatant was used as crude enzyme for all lignolytic enzyme assays.

6.2.1. Preparation of Sawdust Basal Medium

Sawdust of Teak was reduced to particle size measuring 152 µm using a grinding machine. About 6g sawdust was transferred into 500 ml flask and made to the level with distilled water. The pH of the solution was adjusted to 6.8 using 0.1 M NaOH. The solution was sterilized at 121°C for 20 min and inoculated with white rot fungi like *S. commune, L. sterioides, G. lucidum, N. floccosa* and *F. flavus.* Lignin degrading

enzymes were estimated described as below after an incubation period of 2 -10d. same procedure was followed described as above for the extraction of crude enzyme from flasks.

6.2.2. Extraction of crude enzyme from wooden blocks

The artificially inoculated wooden blocks were grinded to pass through 56µm sieve. The enzyme was extracted with water by mixing 1g of decayed sawdust. The solution was filtered and centrifuged at 6000 rpm for 10 min, The clear solution of sawdust was used as crude enzyme fraction and ligninolytic enzyme activity was estimated by the methods as detailed below.

6.2.3. Laccase Activity: It was assayed according to the procedure suggested by Coll *et al.*, (1993). For measuring laccase activity, source enzyme was added to 50 mM Na-acetate buffer (pH 4.5) containing 1mM guaiacol (Sigma grade.) as substrate, to make a final volume of 5ml. The tubes were incubated at 37°C for 15 min. The blank contained substrate and the source enzyme that was inactivated by boiling. The optical density of the reaction tubes was measured against reagent blank in a Jenway 6105 spectrophotometer at 465 nm wavelength. One unit relative enzyme activity was described as the amount of enzyme causing a 0.1 unit increase in the optical density of the reaction mixture under the experimental conditions.

6.2.4. Aryl-alcohol oxidase activity (AAO): It was assayed to the procedure suggested by Mansur *et al.,* (2003). The AAO activity was assayed spectrophotometrically, as the oxidation of veratryl (3,4dimethoxybenzyl) alcohol to veratraldehyde, monitored at 465 nm (ε = 9300 *5.1*-'cm-I). The reaction mixtures contained 10 mM veratryl alcohol in 100 mM sodium phosphate, (pH 6.0). One U of enzyme activity is defined as the arnount of enzyme releasing 1 µmol/min-' oxidized product at 25°C in enzymatic determination.

6.2.5. Peroxidase activity: The enzyme activity was determined using guaiacol as substrate (Lewis *et al.* 1987). The assay mixture consisted of 0.1 M potassium phosphate buffer (pH 7.0 at 25°C), 50 µl of 20.1 mM guaiacol (Fluka) solution, and the enzyme. The reaction was initiated by the addition of 30 µl of 12.3 mM H_2O_2. The total volume of the reaction mixture was 3.18 ml. Activity was monitored using a spectrophotometer at 436 nm. The enzyme activity was calculated by using an extinction coefficient (436 nm) of 6.39 cm^2/pmol for guaiacol. One unit of enzyme activity is defined as 1pmol of guaiacol consumed (oxidized) per min.

6.2.6. Lignin peroxidase (LiP) Activity: it was measured using the method of Tien and Kirk (1984). In this method the increase of absorbance at 310 nm, due to the oxidation of

the veratryl alcohol to veratryl aldehyde, was measured. The reaction mixture contained: 2.2 ml of sodium tartrate buffer (50 mM, pH 4,5 at 25°C), 40 µl of veratryl alcohol (2 mM) and 240 µl of culture supernatant. The reaction was initiated by the addition of 20 µl of H_2O_2 (0.2 mM). The absorbance was measured immediately (ε 310 = 9333 M-1cm-1). One unit enzymatic activity was defined as the quantity of enzyme that produced 1 µm of oxidized product.

6.4. Estimation of Protein in Sawdust Medium and Artificially inoculated woods

Protein content was determined using Biuret reagent. Standard albumin solution containing 10mg albumin per ml-solution was prepared and fractions of 0.2, 0.4, 0.7 and 1.0 ml were measured out into four different test tubes. Distilled water was added to the first three test tubes to make up the volume 1.0 ml solution. 4 ml of Biuret reagent was added to each of the test tubes and cautiously shaken to homogenize and left for 30 min at 29^{0}C. The average of three absorbance's measured using a spectrophotometer at 540 nm, each of the test tubes, were recorded from which the standard curve was obtained. The procedure was repeated using 1.0 ml of the test solution and corresponding concentration of protein was determined from standard curve.

7) Wood decay test

7.1. Wooden Chips Method

Fungi were grown on 2% malt extract agar in petriplates for 7d prior to inoculation in decay chambers. Wooden chips (0.5g) were added to decay chamber containing Modified Asthana and Hawker's medium 'A'. The decay chambers were sterilized at 121°C for 1 h. Three decay chambers were used for each isolate and the fungus inoculated in decay chamber without wooden chips served as control. Assembled decay chambers were incubated in the dark for 20 and 40 d at 27±1 °C. The wooden chips were filtered, oven dried, and weighed. Percent weight loss was determined as follows:

$$\textbf{Percent weight loss} = \frac{\textbf{Weight loss of oven-dried wood after incubation for 20d/40d}}{\textbf{Weight of oven-dried original wood}} \times 100$$

7.2. Wooden Block Method

Test fungi were grown on 2% malt extract agar for 7 d prior to inoculation. The PDA plates were prepared by inoculating each isolate of test fungi and incubated for 7-10 d. Wooden blocks (1x1x1cm) were cut from the respective logs and soaked in

distilled water for 30 min. These wooden blocks were autoclaved. After completely spreading of the test fungus, four blocks of each wood were placed on the medium and incubated for 3, 6 and 12 months at 27±1 °C. To maintain the moisture in test plates two layers of Whatmen filter paper No 1 were placed on the surface of the blocks. The strile distilled water was added to it regularly. The uninoculated wooden blocks acted as control. After completion of the incubation period each block was cleaned (of the mycelium), oven dried and weighed. The percentage weight loses was calculated as described above.

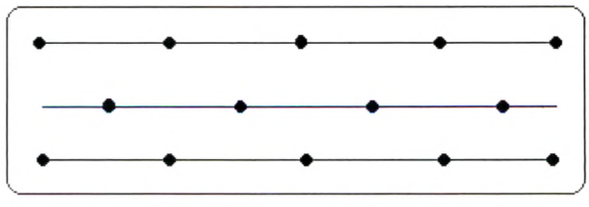
8) Biochemical analysis of certain artificially inoculated woods

8.1. Spawn Preparation

For the spawn preparation a media used for developing sporophores of wood rot fungi as suggested by Etter, (1929) was used. The spawn preparation medium consisted of 48g of corn-meal, 16g of corn-starch and 8g of powdered wood. The spawn was taken in a polypropylene bag and 2.5% malt extract was added. The bag was closed by putting the moist cotton swab to maintain moisture level inside the bag; such bags were sterilized at 121°C for 1 h and inoculated with four test fungi. The bags were incubated in dark for 15d at 27±1°C. The fully-grown spawn was used for artificial inoculation in wooden logs.

8.2. Wooden log preparation

The average size of wood plank used was 2 x 2 x 30 cm length. The bark was not removed. It helps to maintain moisture and keeps away the foreign fungi. A 5/16" drill bit was used to make holes in the logs for insertion of spawn as diamond drilling pattern shown below. After drilling the entire log, after 1 h autoclaving the spawn was inoculated into the holes. It was then sealed off with paraffin wax. Logs were covered with cheese cloth to maintain the moisture and packed in polythene bags and incubated in dark for 12 months.



Diamond drilling pattern

8.3. Physico-Chemical analysis of Decayed wood

The chemical composition of sound and decayed wood as determined by previously described technique (Dill and Kraepelin, 1986). Decayed wood was dried at 105°C and then ground to pass through a 60μ mesh screen. It was used for further analysis.

8.3.1 Water content: To obtain water present in the sample decayed wood (3g) was dried at 105 °C for about 48 h, cooled in a desiccator and weighed. The difference in two weights gave the water content in milligrams.

8.3.2. pH of samples: The pH was determined potentio-metrically after suspension of the samples in distilled water for about 30 to 45 min. analysis of decayed wood was made with a few grams of fresh material and that of the corresponding sound wood was made with 1 g of dry wood meal.

8.3.3. Solubility in hot water: One gram of dry wood meal was placed in a 250ml Erlenmeyer flask. After the addition of 100ml of distilled water, the mixture was slowly stirred at 80°C for 3 h. the samples was then filtered by using Whatman filter paper No 1, washed with hot water, dried at 105 °C for about 24 h, cooled in a desiccator and weighed.

8.3.4. Solubility in Ethanol- Benzene: About 1.5 g of dry wood meal was extracted with Ethanol – benzene (1:2 v/v) for 4 h in a Soxhlet extractor, keeping the liquid boiling briskly. Each extracted sample was washed with 50 to 100 ml of ethanol and dried at 105°C. After evaporation of solvent, each extract was dried at 105°C for 24 h, cooled in a desiccator and weighed.

8.3.5. Acid insoluble lignin: Flasks containing 1g of ethanol - benzene extracted wood meal and 20 ml of H_2SO_4 (72%) were gently shaken in a water bath at 30°C for 1 h. The acid was then diluted with H_2O to 4% (wt/vol), and the samples were autoclaved at 121°C for 30 min. The lignin that settled overnight was quantitatively collected by filtration through a Whatman filter paper No. 1, washed free of acid with hot water, and dried. The lignin content was calculated as a percentage of oven-dried, non-extracted wood meal.

8.3.6. Chlorite Holocellulose: Chlorite holocellulose was also determined as described by Seifert (1983). Extracted wood samples of approximately 400 mg were placed in 50 ml Erlenmyer flasks. Seven milliliters of buffer solution consisting of 60 ml glacial acetic acid and 1.3 g sodium hydroxide per 1000 ml distilled water was added to each flask. Three milliliters of 20% (w/w) aqueous solution of sodium chlorite was

immediately added and the flasks were sealed with parafilm and aluminum foil. The flasks were placed in an orbital shaker at 110 rpm at 45 °C for 36 to 40 h. after the incubation period; the flasks were placed in ice bath to stop the reaction. The contents were then transferred to pre weighed Whatman filter paper No. 1 using 100 ml of 1% acetic acid. The holocellulose was washed with 5 ml of acetone three times and oven dried at 105°C for 4 to 6 h before weighing.

9) Histological and Histo-chemical studies on certain artificially inoculated timbers

9.1. Anatomical studies

Anatomical studies were conducted to study the normal wood structure and pattern of decay caused by white rot fungi in selected timbers i.e. *Tectona grandis, Adina cordifolia, Terminalia crenulata* and *T. arjuna*. The wooden logs were infected with certain white rot fungi like *L. sterioides, C. versicolor, H. apiaria* and *T. pini*. For changes in morphology of infected wood and cell wall alteration due to initiation of decay, following methods were used. Histochemical localization was carried out to detect the histochemical changes taking place in infected wood components due to the initial and advanced stages of decay. The Ultra structural changes in the cell wall layers of different cells in infected teak wood samples were observed by using Scanning Electron Microscopy.

9.1.1 Light Microscopy

Samples of normal and infected wood blocks of approximately 2 x 1 x 1 cm were cut from the inoculated logs and fixed in FAA after the determination of weight loss. The fixed wood blocks were sectioned on the sliding microtome without embedding. However, very soft or heavily decayed wood required the support of an embedding matrix before sectioning could be performed satisfactorily. Wood blocks to be sectioned without embedding required softening and thorough hydration prior to sectioning. This was accomplished by boiling small wood blocks in distilled water until they sink. Very hard wood was softened by treating with concentrated hydrofluoric acid for 2 h. Sections of 15µm to 20 µm thickness were obtained on a sliding microtome. These were tied on to the slide with the help of nylon thread and stored in 50 % alcohol for further studies.

9.1.2 Toludine blue staining:

It was used for the general studies of wood structures in sound wood. The thin sections were transferred to the 30% alcohol. The wood sections were stained with 0.5% Toludine blue solution for 5 min. The stained sections were destained in a series of,

water: TBA, TBA and TBA: xylene in 1:1, 100%, 1:1 and 1:3 ratio respectively. The sections were mounted in DPX and air dried for 2 days. The anatomical details were studied with the help of trinocular research microscope Leica DME model with Canon digital camera attachment.

9.2. Differentiation of Wood Structures and Components

Various histological and histochemical methods have been devised for the examination of wood. Several of these common methods were also found useful in this study to know the changes in wood structure and composition resulting during decay.

9.2.1. Safranin and Fast Green

The decayed wood sections were studied by Double staining method (Wilcox, 1964a). By this method, hyphae were stained very lightly by the fast green and sometimes with a faint pink tinge. Although this method is very satisfactory for application to the observation of wood structure, hyphae of decay fungi stained by this method remained indistinct. The sections were first stained in a 1 % solution of safranin in 50 % ethyl alcohol for 5 min or more. They are washed in 50, 70 and 100% alcohol and stained for 2 sec. in a 0.5 % solution of fast green in a mixture of 9 parts 95 % alcohol and 1 part clove oil. They are then washed in 95 and 100 % alcohol, a series of Xylene: alcohol in the ratio of 1:3, 1:1 and 3:1 respectively, washed in xylene, and mounted in DPX and air dried for 2 days. Photomicrographs were taken with a Leica DME model research microscope, with Canon digital camera attachment.

9.3. Histo-chemical Studies of Decaying Wood

9.3.1. Potassium-iodide-iodine sulphuric acid Method:

It was used for the localization of cellulose. The sections fixed in 50% alcohol were used. These were bring down to the water. The sections were placed in Potassium iodide – iodine solution for 15 -60 min and then mounted. The cellulose stains yellow. For control, cellulose walls were dissolved in concentrated sulfuric acid, washed in water and then stained with IKI solution.

9.3.2. Phloroglucinol Method:

This method was used for localization of the lignin. The sections were flooded with 1% solution of Phloroglucinol in ethanol for 1-2 min. The Phloroglucinol was drained off. A few drops of hydrochloric acid was added and once the red colour was obtained, the acid was drain off. Mount in 50% glycerin and sections were observed under the trinocular microscope. The lignin containing cells appeared red.

10) Ultra-structural studies of teak wood samples associated with wood rotting fungi

For Scanning Electron Microscopy (SEM), blocks of six replicates were cut into 100- to 200-mm^3 pieces and placed in 4% formaldehyde–1% glutaraldehyde (Ted Pella Inc., Redding, Calif.) in 0.10 M Phosphate buffer (pH 7.2) for 72 h at 25^0C. Samples were then placed in an alcohol dehydration series of 20, 50, 80, 95, 95, 100, and 100% ethanol for 15 to 30 min per treatment and then either stored in alcohol or air dried. Dried wood samples were then sectioned with a razor blade to an approximate size of 5 x 5 x 3 mm, exposing one of three sections of wood: transverse, radial, or tangential. Sections were then mounted on SEM stubs, sputter coated to a thickness of 30 nm with Platinum, and observed in Philips model of JSM-5600 Scanning Electron Microscope. Xylem elements like vessels, fibers tracheids and ray parenchyma cells were compared for non-decayed and decayed wood.

11) Bio-control of certain Timber Degrading fungi

11.1. Bio-control studies

On the basis of field survey of forests and saw mills certain preventive methods are suggested for proper storage.. *In vitro* studies were undertaken to control certain wood rotting fungi by using different leaf explants. Fresh leaves of different plants were washed with running tap water and dried at 60°C in oven, and powdered. To prepare stock solution the 25 g of powdered plant material was soxhlet extracted with methanol for 8 h. After extraction the methanol was removed by distillation. The obtained plant extract was dissolved in 100 ml of 20% ethanol. In case of water extract the 25 g of powdered plant material was dissolved in 150 ml of distilled water and extracted in a water bath for 2 h and the solution was filtered with muslin cloth and then through the Whatman filter paper no.1. Extract thus obtained was utilized for the experiment.

Fresh *Aloe vera* leaves were taken to extract the gel from them. An incision was made on the leaves of *Aloe* to collected the gel released from them. After collecting the gel it was mixed in a mixer and whole content was made into different concentration like 5%, 10% and 25%.

11.2. Poisoned food technique

Effect of Methanolic and aqueous leaf extracts of 12 plants, 4 oils and 2 gels was tested on 5 different wood rotting fungi. The leaf extracts were taken and appropriate volume was mixed with medium (PDA) to obtain concentrations ranging from 2.0 to

10.0% in the final volume of 100 ml of medium. This 100 ml medium was dispensed into 10 cm petri plates. Fungal isolates of selected fungi were placed in the centre of each plate. Control sets were also prepared without plant extract. The plates were incubated at 25 °C ±2°C and growth of colony was measured after 7 days of inoculation. The radial growth of mycelium was measured at two points along the diameter of the plate and the mean of these two readings was taken as the diameter of the colony. The growth of the colony in control sets was compared with that of various treatments and the difference was converted into percent inhibition by following formula

$$\textbf{Percent inhibition} = \frac{\textbf{Diameter of control set- diameter of treated set}}{\textbf{Diameter of control set}} \textbf{ X 100}$$