Materials and Methods

(1) Isolation of fungi from axenic culture

Fruiting bodies naturally growing on dead/living logs of different trees growing at Pavagadh forest located close to Vadodara in Panchmahal district and the Arboretum of The Maharaja Sayajirao University of Baroda, Gujarat, India were previously collected. The fruiting bodies were excised, packed in sterile poly ethylene bags and brought to the laboratory. Fruiting bodies were surface sterilized by 0.1% $HgCl_2$ for 60 seconds and washed thoroughly with sterile distilled water followed by 70% ethanol for few seconds and inoculated on Potato Dextrose Agar (PDA) Medium and Malt Extract Agar (MEA) Medium under asceptic conditions and incubated at $25(\pm1)^0$C with 70% relative humidity. Pure cultures were established by routine methods and after development of colony these were subcultured in slants of PDA/MEA medium and maintained for the experimental studies. Remaining portion of the basidiocarps of collected fungi were dried and a small piece of the fruiting body was packed in locked polythene bags, assigned identification number and sent to Forest Research Institute, Dehradun for confirmation and authentic identification. All the identified cultures were maintained on Potato dextrose agar (PDA) at 4 $(+1)^0$C in seed Anatomy laboratory of Department of Botany at The Maharaja Sayajirao University of Baroda, Gujarat, India.

For the present study *Daedaleopsis confragosa* and *Phellinus pectinatus* were subcultured from the identified pure culture maintained in the laboratory. Pure cultures of six *Basidiomycetes* fungi *Irpex lacteus*, *Pycnoporus sanguineus*, *Pleurotus eryngii*, *Pleurotus florida*, *Pleurotus ostreatus*, *Pleurotus sajorcaju* and two *Ascomycetes* fungi namely *Trichoderma viride* and *Trichoderma harzianum* were obtained from Forest Research Institute, Dehradun. Cultures of *Trichoderma reesei* belongs to (*Ascomycetes*) and *Phanerochaete*

chrysosporium belongs to (*Basidiomycetes*) were procured from MTCC (Microbial Type of Culture Collection) Chandigadh.

All the cultures were maintained on Potato dextrose agar (PDA) at 4 $(+1)^0$C in seed Anatomy laboratory of Department of Botany at The Maharaja Sayajirao University of Baroda, Gujarat, India.

For further studies petridish containing potato dextrose agar medium were inoculated with 0.5 cm diameter agar plug, cut from the growing edge of colonies of the isolates and incubated in incubator at 25($\pm$1)^{0}C in dark with 70% relative humidity.

(2) Preparation of Media:

PDA (Potato Dextrose Agar) Medium (Stevens 1981)

The PDA medium was prepared with the following composition (gm/lit): Potato, 200.0; Dextrose, 20.0; Agar agar, 20.0 and Distilled water 1 lit. Potato was peeled and cut into small cubes, weighed, and boiled in water until soft. Boiled potatoes were squeezed through a sieve. Agar Agar was added and boiled till it gets dissolved, then dextrose was added and stirred until dissolved, sterilized by autoclaving at 121˚ C temperature and 15 psi pressure for 20 minutes.

MEA (Malt Extract Agar) Medium (Stevens 1981)

The MEA medium containing Malt extract 30gm/Lit, Agar agar 30gm/L and Distilled water 1 Lit. Malt extract powder was boiled in the water until it dissolved, to this mixture agar agar powder was added and sterilized by autoclaving at 121˚ C temperature and 15 psi pressure for 20 minutes.

(3) Screening

(a) Screening of the fungal isolates for ligninolytic and cellulolytic enzyme activity

Screening was performed by Bavendam test, Fungal isolates were cultured on malt extract agar medium (MEA). For screening of cellulolytic and ligninolytic ability malt extract agar medium (3%) was substituted with respective enzyme substrates viz; tannic acid for ligninases and carboxy methyl cellulose for cellulases (Bains *et al* 2006). The petri plates were inoculated at 28 ± 2°C for 7days. Three sets of replicates were maintained for all the selected fungi. Ligninolytic enzyme activities were assessed by observing the dark brown colored zone around the fungal colony. The cellulolytic enzyme activities were evaluated by observing the zone of clearance if any, formed by flooding the plates with visualizing dye congored for 15min.

(b) Screening of fungal isolates for xylanolytic enzyme activity

Fungal isolates were screened for their abilities to produce extracellular xylanase during their growth on enriched malt extract agar medium (MEA) containing xylan as the sole carbon source (Nakamura *et al* 1993).The composition of the medium used was (g/l): birch wood xylan 1.0, peptone 5.0, yeast extract 5.0, K_2HPO_4 0.2 and agar 20.0. The inoculated plates were incubated for 5 days at 28 ± 2°C. Three sets of replicates were maintained for all the selected fungi. Positive xylanolytic isolates were detected based on the clear zones of hydrolysis after flooding the plates with 0.1% aqueous Congo red followed by repeated washing with 1 M NaCl (Teather and Wood 1982).

(4) Antagonistic activity

(a) Paired interaction test to detect antagonistic activity of the different fungi:

In vitro antagonistic interactions of the fungi were evaluated through dual culture technique. For the antagonistic effect the agar disc method was carried out on 3% malt extract agar (MEA). Five mm mycelial discs by the help of borer was taken from the margin of young vigorously growing 7 days old culture of fungi was inoculated at the margin of the petridish (90mm) containing 20ml sterilized MEA medium at opposite sides of the each other and then

incubated in dark at 25±2˚c with 70% relative humidity for 4 weeks. Petri dishes inoculated with individual fungi were used as controls. Three replicates were used for each experiment.

All measurements were done from day 2 to 30 days while the fungi were actively growing. The isolates were screened for their compatibility/antagonistic potential against the other fungal isolates by observing the relative growth of both fungi till one fungus inhibits the growth of other or one kills the other or they both overgrow each other and form overlapping zone. Outcomes of the interactions were also recorded and photographs were taken on digital Sony Cybershot model no. DSC-H2O.

Interactions between the fungal cultures placed opposite to each other were also assessed visually using a key based on the observations of Porter (1924) cited by Skidmore and Dickinson (1976), and Stahl and Christensen (1992). Porter recognized five separate modes of interactions while Stahl and Christensen recognised three, which are as follows: Porter (1924):

1. Mutually intermingling growth where both fungi grew into one another without any macroscopic signs of interactions.

2 (i) Intermingling growth where the fungus being observed is growing into the opposed fungus either above or below or above and below its colony, and its corollary.

(ii) Intermingling growth where the fungus under observation has ceased growth and is being overgrowth by another colony.

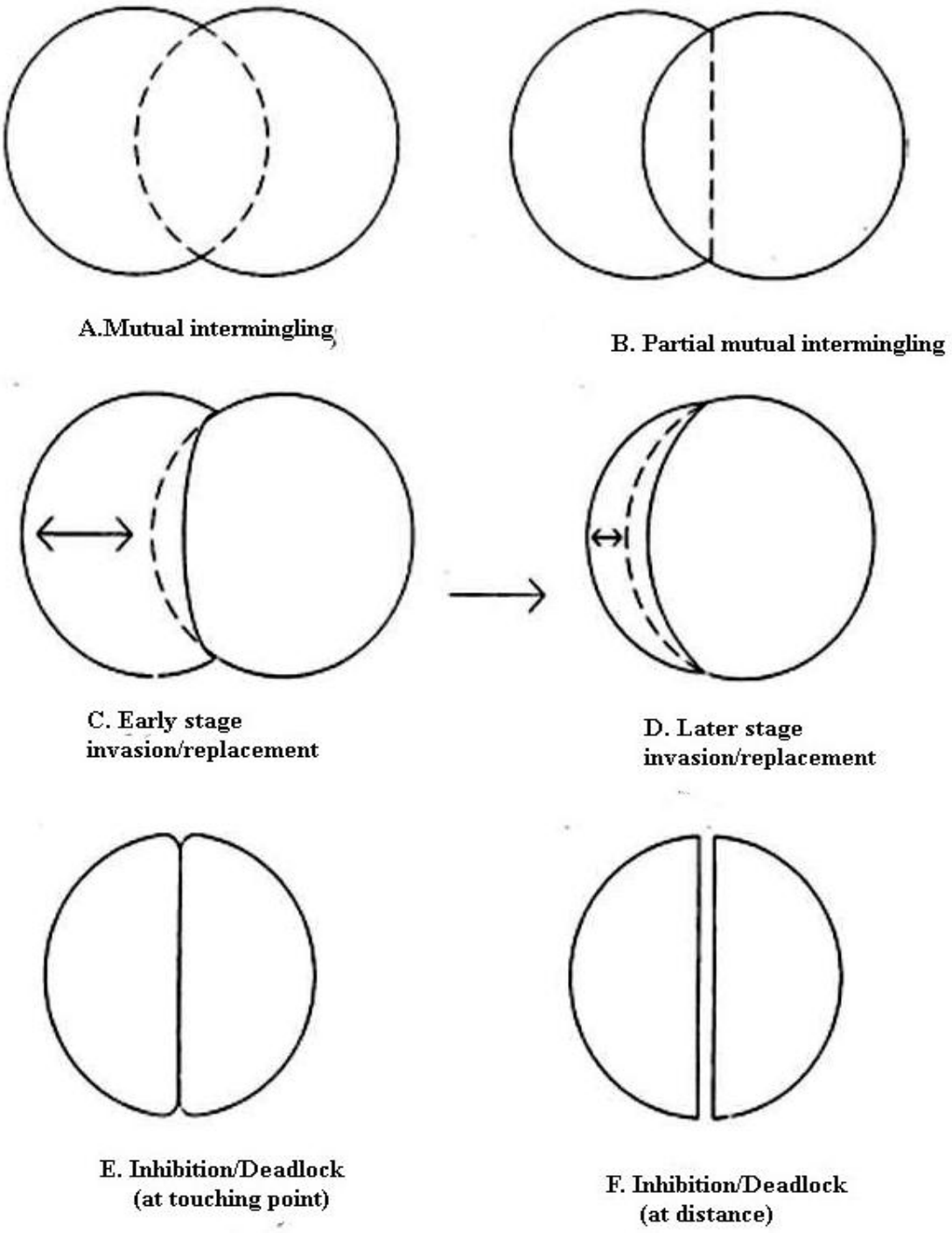


Fig. 18 Schematic diagram representing interactions between two different fungal isolates grown adjacent (4Cm apart) on MEA culture media observed after one week incubation period (Based

on the observations of Porter 1924 and cited by Skiddmore and Dickinson 1976 and Stahl and Christensen 1992)

3. Slight inhibition where the fungi approached each other until almost in contact and a narrow demarcation line, 1–2 mm, between the two colonies was clearly visible.

4. Mutual inhibition at a distance of >2 mm.

Stahl and Christensen (1992):

1. Neutral intermingling – one or both colonies grow into the other with no apparent adverse effect on the mycelium of either.

2. Replacement – one mycelium grows into the other and begins to consume and replace it.

3. Deadlock – neither mycelium can enter territory occupied by the other.

Schematic diagram of interactions between two different fungal strains, which were grown adjacently (4 cm apart) on MEA culture media (based on the observations of Porter (1924) cited by Skidmore & Dickinson (1976), and Stahl and Christensen (1992)) is as shown in figure 18.

(5) Selection of fungi

Monocultures and cocultures of fungi were selected for further analysis of enzyme activity on the basis of above experiment (Screening and antagonistic activity).

(6) Evaluation of enzyme activities

(a) Preparation of fungal enzyme

For the evaluation of enzymatic assay 100ml of Malt extract broth medium was prepared and autoclaved at 121˚C temperature and 15psi pressure for 20 minutes. After cooling, the medium was inoculated with 9mm disc of 10 days old culture under aseptic condition. For monoculture single disc and for coculture single disc of two different fungal isolates were inoculated in 100ml medium and incubated at 25±1˚C for the desired incubation period. After completion of

incubation period fungal mycelium in the flask were homogenized by laboratory hand blander and filtered through preweighed whatman paper no. 1 to separate the fungal mycelia and culture filtrate (Bettin *et al* 2009). The filtrated obtained were used as enzyme solution to determine various lignocellulolytic enzyme activity.

(b) Enzyme assays

(i)Lignin peroxidase (LiP) and Manganese-dependent peroxidase (MnP)

Lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP) activities were measured according to the procedure of Castillo *et al.* (1994) using as substrate 0.167 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) which interact with 2.37 mM 3-(dimethylamino) benzoic acid (DMAB) producing a purple coloured reaction. Substrates were prepared on 0.1 M succinic-lactic acid buffer at pH 4.5. Cuvette (1.5 ml) contained 417.5 µl MBTH, 417.5 µl DMAB, 100 µl $MnSO_4$, 50 µl supernatant (enzyme) and 15 µl H_2O_2 to measure the enzyme activity. To distinguish between manganese dependent and independent peroxidases reaction was performed in the presence and absence of 3mM $MnSO_4$. 4mM H_2O_2 was used to initialise the reaction. Reactions were carried out at 37°C and monitored spectrophotometrically at 590 nm during 1 min. The enzyme activity was calculated using as extinction coefficient ε = 53000 $M^{-1}cm^{-1}$.

(ii)Laccase

Laccase activity was determined by the oxidation of 2, 2'-azino-bis (3-ethylbenziazoline-6-sulphonic acid) (ABTS) by laccase causes by blue discoloration of the substrate (Niku-paavola *et al.* 1988). Enzyme assay were performed with 500 µM ABTS in 50 mM Sodium acetate buffer at pH 4.5. A 20 µl aliquot of enzyme solution was added to 580 µl of the ABTS. Change in the absorbance at 420 nm were observe (ε = 36, 000 $M^{-1}cm^{-1}$).

(iii)Aryl alcohol oxidase (AAO)

Aryl alcohol oxidase (AAO) activity was estimated according to the procedure of Guillen *et al* 1990 following the increase in absorbance due to the vertraldehyde formation from veratryl Aryl alcohol oxidase alcohol (3, 4-dimethoxybenzyl alcohol). The reaction mixture contained 5mM veratryl alcohol in a 0.1 mM sodium phosphate buffer pH 6 and 50 µl supernatant (enzyme solution). Reaction was performed at 30°C and visualized spectrophotometrically at 310 nm during 2.5 min and activity expressed in µkat/g dw using ε = 9300 $M^{-1}cm^{-1}$as the extinction coefficient of vertraldehyde.

(iv)Xylanase

The amount of xylanase produced was measured by using 1% birch wood xylan as the substrate (Bailey *et al* 1992). Xylanase activity was assayed in 3.0 ml of a reaction mixture containing 0.1 ml of crude extracellular enzyme sample, 0.5 ml of 1% birch wood xylan (prepared in 0.05 M citrate buffer, pH 5.3). The mixture was incubated at 55°C for 30 min. The reaction was stopped by the addition of 0.6 ml of 3, 5- dinitrosalicylic acid (DNSA) and 1.8 ml of distilled water was added to the tubes to make the reaction mixture 3 ml. The contents were boiled for 15 min (Miller 1959). After cooling, the color developed was read at 540 nm. The amount of reducing sugars liberated was quantified using xylose as standard. One unit of enzyme activity is defined as the amount of enzyme which releases 1 µmol of xylose in 1 min under assay conditions (Khan *et al* 1986).

(v)Cellulase

Cellulase (CMCase) activity was determined by mixing 0.5 ml of 1% (w/v) CMC (prepared in 0.05 M acetate buffer pH 5.3) with 0.1 ml of crude extracellular enzyme solution incubating at 50°C for 30 min (Casimir *et al* 1996). The reaction was stopped by the addition of

0.6 ml of 3, 5-dinitrosalicylic acid (DNSA) and 1.8 ml of distilled water was added to the tubes to make the reaction mixture 3 ml. The contents were boiled for 15 min. The colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1 µmol of glucose equivalents per minute under the assay conditions (Mandels *et al* 1981).

(7) Parameters affecting enzyme activity

(a) Effect of incubation period

Enzyme filtrates were collected after 3, 5, 10, 15, 20, 25, 30 and 35 days of incubation and all the enzyme assays were carried out.

(b)Effect of Temperature

Enzyme containing supernatants were incubated at 25, 35, 45, 55, 65, 75 ˚ C in water baths for four hours to allow ample time for denaturation. Then all enzyme assays were carried out.

(c) Effect of pH:

To estimate the effect of different pH on enzyme activity, all enzyme assays were carried out at different pH in 0.1 M of HCl-KCl buffer, pH 1 and 2; citrate buffer, pH 3 and 4; acetate buffer pH 5 and 6 and phosphate buffer, pH 7 and 8. Then all enzyme assays were carried out spectrophotometrically (Trivedi 2002).

(8) Selection of cellulase free monocultures and coculture

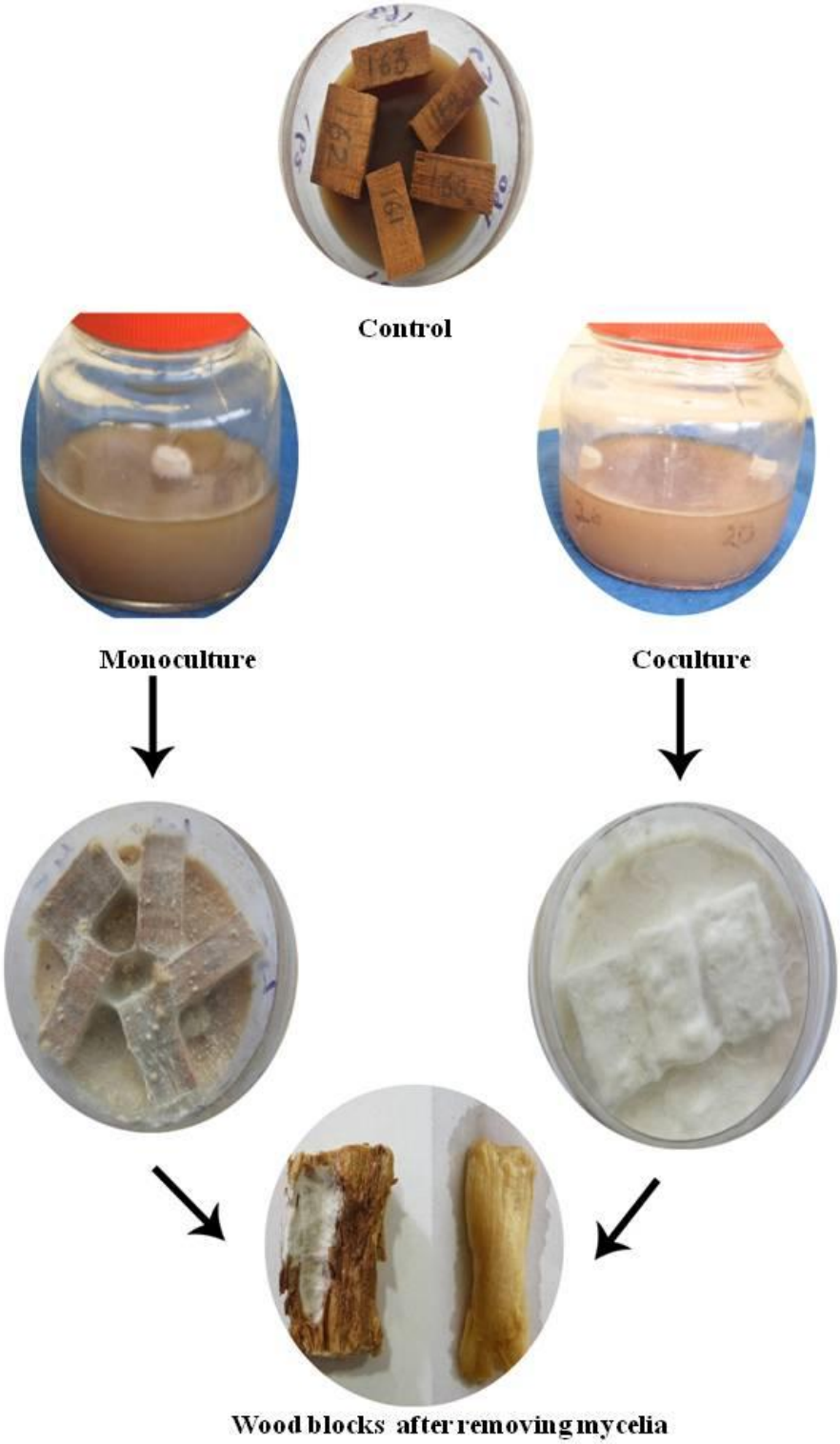
On the basis of results obtained in the evaluation of enzyme activity monocultures and coculture giving best production of all the enzymes were selected for further experiment of enhancement.

(9) In vitro decay experiment

The fungi for wood decay were selected on the basis of the results from the paired interaction test. Two monocultures viz. *D. confragosa*, *I. lacteus* and their co-cultures viz. *D. confragosa* along with *I. lacteus* were taken for the experiment. Test cultures for mono cultures were prepared by inoculating pieces of agar from pure culture plates in 250 ml of decay chambers containing 100 ml of Malt Extract Agar (MEA) medium and in case of co-culture/dual culture agar piece of both the selected fungal mycelium were placed opposite to each other in the decay chamber and incubated for 2 weeks under controlled conditions (25°C, 70% Relative humidity and dark) prior to inoculation of *Eucalyptus* wood blocks in to decay chambers. Decay chambers for wood decay were prepared according to modified method of Blanchette 1986. *Eucalyptus* wood blocks were used for the in vitro decay experiments as this wood is traditionally used as raw material in the pulp and paper industries. Logs of *Eucalyptus* were purchased/collected from Jalaram timber mart situated at Harni in Vadodara and cut into planks of 3 inches thickness. These wooden planks were oven dried and after the bark portion of 0.5-1.5 cm was removed it was cut into wood blocks of (1cm × 3cm size) by Jackshow machine and dried at 72 °C for 24-48 hrs until constant weight, marked and dry weight was recorded for each block. The blocks were then soaked in distilled water for 24hrs to get enough humidity and autoclaved for 1hr at 121°C.

Five sterile blocks were then inoculated aseptically on the distributed actively growing two week old cultures of a single species and for a mixed culture in each decay chamber three wood blocks were placed in the middle of the chamber where the overlapping zone of both the fungi has occurred. Five sterile wood blocks inoculated in a decay chamber without fungus culture was kept as control for each incubation period. Five replicates were prepared for each isolate of monoculture and eight replicates were kept for co culture and kept for four different incubation periods i.e, 45days, 3 months, 6 months and 12 months at 25±1 °C and 70% relative humidity.

The plate below depicts the methodology followed to obtain treated wood blocks.



Decayed experimental wood block samples obtained after the completion of the prescribed incubation period were analysed/evaluated for weight loss, anatomical alterations and biochemical alterations.

(a) Weight loss

After each incubation period the wood blocks were removed from decay chambers and cleaned properly to remove mycelia. The marked blocks were weighed after oven drying to determine final volume. To monitor the progress of the wood degradation, the percentage weight loss due to degradation was calculated as per the ASTM standard 1978.

(b) Anatomical

After desired incubation period completed wood blocks were removed from the decay chambers, mycelium was removed and five blocks were fixed in FAA (Berlyn and Miksche 1976) and further analysis was conducted using light microscopy.

Light microscopy

After completion of the different incubation periods wooden blocks were taken out, mycelium was removed from the surface and preserved in FAA (Formaldehyde: acetic acid: alcohol 5:5:90) for the anatomical studies (Blanchette 1986). 15-20 µm thick transverse(TS), radial (RLS)and tangential sections(TLS) of the block were cut on sliding microtome (Ernst Leits Wetzlar) and firmly tied with cotton thread onto the slide and stained with Astrablue-saffranin, toluidene blue and saffranin (Johansen 1940) then after the slides were passed through alcohol-xylene series for dehydration and made permanent by mounting in DPX. The sections were observed under Leica DME 750 and important results were photographed at different magnifications.

(c) Bio chemical analysis

Prior to biochemical analysis of the wood, both control and decayed wood blocks were ground and passed through a 40-mesh-sized screen and biochemical estimation was done by Tappi standard 1995. For analysis of lignin sample must be free from extractives so extractive content was removed by following method.

Determination of Extractive content in the wood sample was done by modified method of Anon 1985. 95% ethanol-toluene (1:2 by volume) were added to the flask containing 5 gm of oven dried sample for 6 hours followed by addition of 95% ethanol for 4 hours and then by distilled water for 2 hours and washed with acetone.

Wood of *Eucalyptus* was reported containing polyphenols known as kinos which show resistance to the extraction procedure described above (Anon 1985). To avoid complications in determination of lignin by Klason method, alkali extraction was carried out by Appita standard method. From the obtained residues of extractive free content 1.5 gm residues were taken in the flask and 75 ml of 0.1M sodium hydroxide was added and boiled for one hour. After boiling the residues were filtered from preweighed glass filtering crucible and washed with hot distilled water, 10% acetic acid solution and again by hot distilled water.

(a) Lignin (Klason method)

From the alkali extracted sample 1gm was weighed and taken in the beaker. Beaker was soaked at 20°C in water bath and then 20 ml of 72% sulfuric acid was added and stirred for 4 hours and 765 ml of distilled water was added and after four hours reaction the residues were filtered through preweighed glass filtering crucible and washed with hot distilled water. Residues were dried under vacuum and weighed to determine the lignin content.

(b) Cellulose

Hollocellulose was first extracted from which cellulose was determined. Hollocellulose content was measured by taking 3 gm of extractive-free sample and placed in flask. Flask was soaked in water bath at 70°C. To this flask 180 ml of 0.2% sodium acetate (pH 3.5) was added which is followed by addition of 1.2 gm of sodium chlorite and stirred. After 30 minutes again 1.2 gm of sodium chlorite was added. After 45 minutes the content was filtered through preweighed glass crucible and washed with cold distilled water and acetone. The residues were dried under vacuum and weighed to determine the holocellulose content.

Cellulose content was determined by taking 1 gm of holocellulose sample in beaker. To this beaker 25ml of 17.5% sodium hydroxide was added and stirred. After 4 minutes the residues were smashed for 1 minute and again stirred for 16 minutes. After 16 minutes 25 ml of distilled water was added and stirred for 1 minute and After 5 minutes the residues were filtered through preweighed glass crucible and washed with distilled water till it becomes neutral. To these residues 40 ml 0f 10% acetic acid was added and kept for 5 minutes. The residues were dried under vacuum and weighed to determine the α-cellulose content.

(c) Hemicellulose

Hemicellulose content was obtained by subtracting cellulose from holocellulose.

(10) Study on the enhancement of enzyme activity

(a) Effect of different chemical enhancers on enzyme activity

3 % MEB medium was prepared, autoclaved and supplemented with different concentration of chemicals (Ethanol, Veratryl alcohol, Xylidine, Yeast Extract, Peptone) and inoculated with a 9 mm disc of 10 days old culture. After completion of incubation period enzyme assays were carried out.

Ethanol

Effect of different concentration of ethanol on biomass yield and enzyme production was evaluated in the MEB medium. The ethanol was added to media after the media was autoclaved in the concentration of 1%, 2%, 3%, 4%, 5% (Lee *et al* 1999). A control treatment was included in the experiment that does not contain ethanol.

Veratryl Alcohol

In the experiment to determine the optimal concentration of veratryl alcohol for induction of biomass yield and enzyme production MEB medium was used. Veratryl alcohol added to MEB media to obtain final concentration of 4 mM, 8 mM, 12 mM, 16 mM, 24 mM. (Barbosa *et al* 1996) A control treatment was included in the experiment that contained no veratryl alcohol.

Xylidine

To determine the effect of xylidine 3 % MEB medium was utilized. Xylidine was added to the MEA media to obtain final concentrations 4 µM, 7 µM, 10 µM, 20 µM, 30 µM. The control treatment did not include xylidine in the MEB medium.

Yeast Extract

To determine the effect of Yeast Extract it is substituted in the MEB medium. Yeast extract is added in MEB medium in the concentration of 0.1 g/l, 0.2 g/l, 0.4 g/l, 0.6 g/l, and 0.8 g/l. In the control the yeast extract is not added.

Peptone

In the experiment to determine the optimal concentration of peptone for induction of biomass yield and enzyme production MEB medium was used. Peptone was added to MEB

media to obtain concentration of 0.1 g/l, 0.2 g/l, 0.4 g/l, 0.6 g/l, and 0.8 g/l (Kanwal and Reddy 2011). A control treatment was included in the experiment that contained no peptone.

(b) Effect of different lignocellulosic material on enzyme activity

The different lignocellulosic materials used to study the effect on enzyme activities are Apple peels, Banana peels, Mandarin peels and Ash gourd pulp.

To evaluate the effect of different lignocellulosic materials on the enzymatic activity, four different lignocellulosic substrates in various concentrations 1%, 2%, 3%, 4%, 5%, 6%, 8%, 10% (Elisashvili and Kachlishvili 2008) and their effect on the enzyme activity were evaluated. In order to carry out the experiment 3 % MEB medium was supplemented with different concentration of substrates (Apple peels, Banana peels, Mandarin peels, Ash gourd pulp) and autoclaved. All the culture bottles were inoculated with a 9 mm disc of 10 days old culture. After the completion of incubation period enzyme assays were carried out.

(11) Fungal pretreatment under optimized condition for biopulping

From the above experiments the best incubation period, experimental conditions (pH and Temperature), concentration of chemical enhancers and concentration of lignocellulosic materials were selected for the biopulping experiment. 20 days of incubation period for coculture, pH 5 and temperature 25˚C were selected from the result of valuation of enzyme activity and parameters affecting enzyme activity. On the basis of the enhancement of enzyme activity 1 % of Ethanol, 16 mM of Veratryl alcohol, 30 µM of Xylidine, 0.6 g/l of Yeast Extract, 0.4 g/l of Peptone as chemical enhancers and 5% apple peels, 4% banana peels, 6% mandarin peels and 5% ash gourd pulp as lignocellulosic materials were selected for biopulping experiment. All these chemical enhancers and lignocellulosic materials were added in 3 % MEB medium with both substrates (*Eucalyptus* wood blocks and pulp) in 5 % concentration were

added. Then this was sterilized by autoclaving at 121˚ C temperature and 15 psi pressure for 20 minutes. After cooling, it was inoculated with 9 mm disc of 10 days old fungal culture under aseptic condition. Then culture was incubated at 25 ± 1 ˚ C for the desired incubation period. After completion of incubation period the cultures were removed, mycelium were homogenized with laboratory hand blander and filtered through whatman paper No. 1 disc. These filtrates were used as enzyme solution. The residual sample was used to analyze for lignin and cellulose content.

(12) Fiber Properties

From the wood blocks treated with the best enhancing substrate small slivers were obtained and macerated with Jeffery's solution (Sass 1958). (1:1 mix of 10% aqueous chromic acid + 10% aqueous nitric acid, chromic acid-potassium dichromate 10% in sulfuric acid). The slivers were washed, kept in 50 ml of distilled water and fiber bundles were separated in to individual fibers using small mixture. Macerated fibers were stained with Saffranin and the fiber suspension was placed on the slide using dropper.

(a) Fiber morphology

Morphological properties of the fibers from sound wood of *Eucalyptus globulus* were compared with the fibers obtained from the wood blocks treated with the best enzyme activity enhancing substrate. The parameters studied were shape of fibers, shape of lumen, shape of fiber ends, surface markings if any present.

(b) Fiber dimensions

For fiber diameter, lumen diameter and cell wall thickness determination, cross sections were used. All the measurements were made with the help of Leica
Qwin Pro image analyser.

(c) Derived values

With the help of fiber dimensions five derivative values were also calculated. The following five equations were used to find the derived values which are important criteria for determining fiber qualities in paper making (Saikia *et al* 1997, Ogbonnaya *et al* 1997, Kirchi 2006).

Felting rate/Slenderness ratio: Fiber length÷ Fiber diameter

Elasticity coefficient (%)/Flexibility coefficient: (Fiber lumen diameter÷ Fiber diameter) × 100

Rigidity coefficient (%): (Cell wall thickness ÷ Fiber diameter) × 100

Runkel index/Runkel ratio: (Fiber cell wall thickness × 2) ÷ Lumen diameter

F ratio (%): Fiber length ÷ Cell wall thickness × 100

(13) Statistical analysis

Data presented throughout the whole thesis were analyzed using Microsoft excel. Results were significant when subjected to ANOVA (Analysis of Variance) test at p=0.05 level. Standard deviations of mean (SD) were calculated.