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3. MATERIALS AND METHODS

MATERIALS

3.1 ORGANISMS

Four standard strains of *Phanerochaete chrysosporium* were used in this study.

(i) *Phanerochaete chrysosporium* BKM-1-1767 (ATCC NO 24725) was procured from American Type Culture Collection. This wild strain was isolated by T.I. Novabranova from *Vitis vinifera* wood in Alma Ata, Kazachstan, USSR, and classified as *Sporotrichum pulverulentum* Novobranova.

(ii) *Sporotrichum pulverulentum* was obtained from Swedish Forest Products Laboratory, Sweden.

(iii) *Phanerochaete chrysosporium* K-3 is a heterokaryon grown from a single condium of wild type strain P-127-1 (ATCC No.32629). The wild type was originally isolated by O.Berghem and T.Nilsson from spruce wood chips in Sweden. It was procured from Swedish Forest Products Laboratory, Sweden.

(iv) *Sporotrichrum pulverulentum* NRRL 6360 was obtained from Northern regional research laboratory, U.S.A.

Besides standard strains, lignin degrading fungi were isolated from soil samples. All the organisms were propagated and maintained on malt agar containing 2 % each of malt extract and agar at 25°C.
3.2 PULPS

Unbleached mixed wood kraft pulp and mixed grass kraft pulp were obtained from Ballarpur Industries Limited (Shree Gopal Unit) a commercial Paper Mill, Yamuna Nagar. These pulps were prepared from a mixture of different woods and grasses in a specified ratio under standard cooking conditions (Appendix I). Unbleached kraft pulps made of single raw material were prepared in laboratory scale with defined cooking cycles at mill site only (Appendix II). Eight different raw materials used were bamboo, eucalyptus, softwood, khar grass, kai grass, sabai grass, sugarcane bagasse and wheat straw.

3.3 EFFLUENT

Effluent from first alkaline extraction stage following chlorination of eucalyptus kraft pulp in bleach plant was used. Eucalyptus kraft pulp of kappa number in the range 25 to 26 was treated with 5% chlorine for 45 minutes and 1% alkali for 45 minutes. The effluent from alkali extraction stage was collected.

3.4 CHEMICALS AND REAGENTS

Dimethyl succinate, paranitro phenyl β-D-glucopyranoside (pNPG) and carboxymethyl cellulose were purchased from Sigma Chemical Company whereas veratryl alcohol and veratraldehyde were from Aldrich Chemical Company. Indulin AT- a lignin preparation was procured from Westvaco Company, USA. All other chemicals used in growth media as well as in analytical methods were from E.Merck/BDH.
METHODS

3.5 ISOLATION AND SCREENING OF LIGNIN DEGRADING FUNGI

3.5.1 BASAL MEDIUM

A chemically defined nitrogen limiting basal medium was used for isolation of lignin degrading organisms and the growth of *P.chrysosporium* for production of cellulolytic enzymes and bleaching studies (Kirk et al., 1978) with following composition per litre.

\[
\begin{align*}
\text{KH}_2\text{PO}_4 & \quad 0.2 \text{ g} \\
\text{MgSO}_4\cdot 7\text{H}_2\text{O} & \quad 0.05 \text{ g} \\
\text{CaCl}_2\cdot 2\text{H}_2\text{O} & \quad 0.03 \text{ g} \\
\text{Thiamine-HCl} & \quad 0.0025 \text{ mg} \\
\text{Mineral salt solution} & \quad 7 \text{ ml}
\end{align*}
\]

\[\text{NH}_4\text{NO}_3 \text{ and Asparagine were used as nitrogen source in equimolar concentration (both 1.2mM = 2.4mM N). One percent glucose served as the carbon source and the medium was buffered with 10mM phthalate buffer, pH 4.5. Oleic acid (0.03%) emulsified with 0.01% Tween 80 (sorbitan polyoxyethylene monooleate) was also added to the growth medium.}

Mineral salt solution (per litre of distilled water) :

\[
\begin{align*}
\text{Nitrilotriacetate} & \quad - \quad 1.5\text{g} \\
\text{MgSO}_4\cdot 7\text{H}_2\text{O} & \quad - \quad 3.0\text{g} \\
\text{MnSO}_4\cdot \text{H}_2\text{O} & \quad - \quad 0.5\text{g} \\
\text{NaCl} & \quad - \quad 1.0\text{g} \\
\text{FeSO}_4\cdot 7\text{H}_2\text{O} & \quad - \quad 100\text{mg} \\
\text{CoCl}_2\cdot 6\text{H}_2\text{O} & \quad - \quad 100\text{mg} \\
\text{CaCl}_2 & \quad - \quad 82\text{mg} \\
\text{ZnSO}_4\cdot 7\text{H}_2\text{O} & \quad - \quad 100\text{mg} \\
\text{CuSO}_4\cdot 5\text{H}_2\text{O} & \quad - \quad 10\text{mg} \\
\text{Al}_2\text{K}_3\text{SO}_4 & \quad - \quad 10\text{mg} \\
\text{H}_3\text{BO}_3 & \quad - \quad 10\text{mg} \\
\text{NaMnO}_4\cdot 2\text{H}_2\text{O} & \quad - \quad 10\text{mg}
\end{align*}
\]

3.5.2 ISOLATION OF LIGNIN DEGRADING FUNGI

Soil samples with decayed matter were collected from
the premises of a paper mill. One ml of 10% soil suspension was inoculated in 50ml growth medium enriched with 1% cellulose and 0.5% indulin AT (separately sterilised at 10 psi for 30 minutes). One ml of sample was withdrawn after 7 days incubation on a rotary shaker (200 rpm) at 30 ± 2°C and inoculated on agar plates containing 1% cellulose, 0.5% indulin AT and 2% agar in basal medium. The growing organisms were further subcultured on basal medium containing 0.5% indulin AT as sole carbon source. Only those isolates which managed to grow on this medium were picked up, purified and preserved on malt agar slants.

3.5.3 COMPARISON OF ZONES OF BLEACHING

The isolates and standard strains were tested for production of zones of bleaching on the sheets made from kraft pulps of mixed woods and grasses. Pulp sheets (1g, 10cm diameter) were soaked with 10ml basal medium in petri dishes and autoclaved. A loopful of spores were inoculated in the centre of the sheet and bleached zone was measured after 5 days incubation at 25°C and 39°C.

3.5.4 ENZYMATIC PROFILES

3.5.4.1 INOCULUM PREPARATION

Spore suspension was prepared by suspending fresh conidial spores (obtained from 1-2 weeks old malt agar slants of culture) in sterilised water to give absorption of 0.4 at 550 nm.

3.5.4.2 PRODUCTION OF CELLULASES

50ml basal medium containing 1% mixed wood pulp, mixed grass pulp or cellulose powder as carbon source was
inoculated with one ml spore suspension and incubated for 5 days on a rotary shaker (200 rpm) at 39°C. The cultural filtrate was assayed for cellulolytic enzymes i.e. carboxymethyl cellulase (CMCase), filter paper assay (FPA) and β-glucosidase.

3.5.4.3 CMCase ASSAY (Reese and Mandels, 1963)

The procedure essentially consisted of estimating reducing sugars formed by the action of cellulase on carboxymethyl cellulose (CMC). The reaction mixture containing 0.5ml of 1.1% CMC in citrate buffer (0.05M, pH 5.0) and 0.5 ml of enzyme filtrate was incubated at 50°C for one hour. The reducing sugars formed were measured as glucose equivalent by the dinitrosalicylic acid method (Miller et al., 1959). One CMCase unit was defined as one μmole of reducing sugars liberated per ml of enzyme per hour.

3.5.4.4 FILTER PAPER ASSAY (Mandels et al., 1976)

The culture supernatant (0.5ml) was incubated with one ml of 0.05M citrate buffer (pH 4.8) and 50 mg of Whatman no.1 filter paper (1x6Cm strip) for one hour at 50°C. The reducing sugars released were quantitated by the dinitrosalicylic acid method (Miller et al., 1959). One FPA unit was defined as one μmole of reducing sugars liberated per ml per hour of enzyme filtrate.

3.5.4.5 REDUCING SUGARS ESTIMATION

Reducing sugars after enzyme hydrolysis were estimated by a colorimetric method of Miller (1959). The test sample
upto 1ml was mixed with 3ml Dinitrosalicylic acid (DNSA) reagent. The standard curve was prepared by taking 0.1 to 1.0mg glucose (in a total volume of 1ml) in test tubes containing 3ml DNSA reagent. The tubes were immersed in boiling water and removed after 15 minutes and allowed to equilibriate to room temperature. The volume was made upto 25ml in each tube and absorbance measured at 550nm. The DNSA reagent had the following composition.

Dinitrosalicylic acid = 1.0%
Phenol = 0.2%
Sodium sulfite = 0.05%
Sodium hydroxide = 1.0%
Sodium potassium tartarate = 20.0%

The reagent was prepared by taking the solid components in the container and dissolving them in the required volume of sodium hydroxide and phenol with constant stirring. The solution was filtered and stored in a coloured bottle in the refrigerator.

3.5.4.6 β-GLUCOSIDASE ASSAY (Berghem and Petterson, 1976)

The reaction mixture containing 0.5ml of cultural filtrate and 2ml of 10mM para-nitrophenyl-β-D-glucopyranoside (pNPG) in 0.05M citrate phosphate buffer, pH 7.0 and incubated for 30 minutes at 50° C. The reaction was stopped by the addition of 3ml of 1M sodium carbonate and absorbance was read at 420nm. Quantitation was done from a calibration curve obtained using 0.1-1.0mM p-nitrophenol (pNP) as a standard. One β-glucosidase unit was defined as one μmole of pNP released per ml enzyme per minute.
3.5.4.7 PRODUCTION AND ASSAY OF LIGNINASE (Tien and Kirk, 1989)

Shallow stationary cultures for ligninase production were grown in 100ml Erlenmeyer flasks. The growth medium for ligninase production had the same composition as previously described except that 2,2 dimethyl succinate and ammonium tartarate served as buffering agent and nitrogen source respectively. Flask cultures containing 10ml medium were inoculated with 1ml spore suspension and kept under stationary conditions at 39°C. Veratryl alcohol (0.04M, final concentration) as an inducer was added at the time of inoculation. The extracellular fluid (ligninase) was obtained by centrifugation at 10000 rpm for 5 minutes at 4°C after 5 or 6 days of incubation.

Ligninase activity in the extracellular fluid was measured by determining the rate of oxidation of veratryl alcohol to veratraldehyde. Reaction mixtures contained 1ml of extracellular fluid, 2mM veratryl alcohol, 0.4mM H₂O₂ and 50mM tartaric acid, pH 2.5. The reaction was started by H₂O₂ addition immediately after buffer was added and the linear increase in absorbance at 310 nm was monitored over 3 to 5 minutes at room temperature. Optical measurements were performed with a Beckman DU7 spectrophotometer.

3.6 BIOBLEACHING OF KRAFT PULPS

3.6.1 INOCULUM PREPARATION

For preparation of mycelial inoculum, 1ml spore suspension (0.4 O.D. at 550 nm) was inoculated into 250ml erlenmeyer flasks containing 50ml basal medium with 1%
glucose. The flasks were incubated at 39°C under stationary conditions for 5 days. The fungal growth so obtained was harvested, washed with dist. water and broken with glass beads. The final mycelial concentration in dist. water was adjusted to 10mg/ml (W/V, on dry weight basis).

3.6.2 CULTURAL CONDITIONS

Biobleaching of mixed wood and grass pulps was studied under three cultural conditions with four standard strains i.e. P.chrysosporium ATCC 24725, S. pulverulentum, P.chrysosporium K-3 and S.pulverulentum NRRL 6360 and isolate VJ1.

3.6.2.1 SURFACE CULTURE

Pulp sheets (1g, 10cm diameter), with 10ml basal medium placed in petri dishes were autoclaved at 10 psi for 30 minutes and inoculated with 1ml of mycelial inoculum. The fungal treated pulp (seven replicates) was collected after 3 days incubation at 39°C, air dried and used for further analysis.

3.6.2.2 SUSPENDED CULTURE

Fifty ml of pulp slurry (1% W/V) prepared in basal medium was autoclaved at 10 psi for 30 minutes and inoculated with 1ml vegetative mycelium. The contents of the flasks were filtered after 3 days incubation at 39°C under stationary conditions, air dried and used for further analysis.

3.6.2.3 SHAKE CULTURE

The cultural conditions were same as for suspended culture except that the flasks were incubated on a rotary
shaker (200 rpm) for 3 days at 39°C.

3.6.3 PULP ANALYSIS

Three pulp characteristics i.e. kappa number, viscosity and copper number were measured after the fungal growth of the pulps.

3.6.3.1 KAPPA NUMBER ESTIMATIONS

This estimation directly relates to the lignin content of the pulp (Tappi Test method T236). It is adapted to the determination of the relative hardness, bleachability or degree of delignification of the pulp.

Appropriate quantity of pulp (3 to 8 gm) depending on its source was disintegrated in 500 ml of distilled water, until free from fibre clots using an electric stirrer at moderate speed to avoid cutting of fibres. The disintegrated sample was transferred to a 2 litre beaker, using 200 ml distilled water to rinse out the apparatus. The beaker was placed in a constant water bath set at 25°C ± 0.2°C. The stirrer speed was now adjusted to obtain a vortex of approximately 25mm in the solution. Hundred ml each of 0.1N KMnO$_4$ solution and 4.0N $\text{H}_2\text{SO}_4$ were taken in a 250ml beaker and tempered to 25°C. This mixture was quickly added to the disintegrated sample and a stop watch was switched on. Hundred ml distilled water was used to rinse out the beaker and the washings added to the reaction mixture. At the end of exactly ten minutes, the reaction was stopped by adding 20 ml of 15% (W/V) potassium iodide solution. Immediately after mixing, the free iodine was titrated with 0.02N sodium
thiosulphate solution. A few drops of the 0.2% starch indicator solution were added towards the end of the reaction. Blank estimation was carried out using distilled water in place of pulp slurry. Kappa number (K) was then calculated as follows:

\[ K = \frac{axd}{m} \]  
where \( a = \frac{(b-c)N}{0.1} \) and
\( a \) = volume in ml of potassium permangate solution used.
\( b \) = volume in ml of sodium thiosulphate consumed in blank determination.
\( c \) = volume in ml of sodium thiosulphate consumed in test.
\( N \) = normality of sodium thiosulphate solution.
\( d \) = factor for correction to 50\% KMnO₄ consumption dependent on the value of \( a \).
\( m \) = mass in grams of the test specimen (based on oven dry pulp).

3.6.3.2 VISCOSITY MEASUREMENTS

Viscosity of the pulp is determined by dissolving the pulp to 0.5\% solution using 0.5M cupriethylenediamine as the solvent and by using a capillary viscometer (Tappi test method T230). The solution viscosity of the pulp measures the average degree of polymerisation of the cellulose. This test, therefore, gives a relative indication of the degradation (decrease in cellulose molecular weight) resulting from the pulping and/or bleaching process. The reagents used during estimation were 50\% glycerine and cupriethylenediamine solution (1.0M cupric ion and 2.0M ethylenediamine concentration). The solution was stored under nitrogen pressure.

The pulp samples were formed into thin sheets on a buchner funnel. The sheets were air dried and average moisture content was determined. An amount of the sample that is equivalent to 0.125 ± 0.0005g of oven dry pulp was
taken in a 100ml beaker. To it, 12.5ml distilled water was added and mixed for 30 seconds followed by 12.5ml cupriethylenediamine solution and the mixture stirred for 15 minutes on a magnetic stirrer.

Ten ml of this reaction mixture was pipetted into the small diameter leg of the viscometer. The viscometer was wiped clean from the outside and placed in a vertical position inside the constant water bath maintained at 25°C ± 0.1°C. Then by applying suction to the other end, the solution was drawn up to a level above the second etch mark in the measuring leg of the viscometer. The efflux time was determined by measuring the time required for the meniscus to pass between the two marks. 50% glycerine solution was used to calibrate the viscometer and finding viscometer constant. The viscosity (V) measurement calculations were done as follows:

\[ V = C t d \]

\( V \) = Viscosity of pulp in cupriethylenediamine solution
\( C \) = Viscometer constant found by calibration using 50% glycerine.
\( t \) = average efflux time in seconds
\( d \) = density of the pulp solution (1.052).

3.6.3.3 COPPER NUMBER DETERMINATIONS

This estimation measures the free reducing groups of the cellulose fibres, thus indirectly indicating the extent of damage caused to the length of cellulose fibres, during the bleaching process (Tappi test method T215). Reagents used for this estimation are as follows:

Solution A: 60g of CuSO\(_4\) \(5\)H\(_2\)O dissolved in one litre distilled water.
Solution B: 200g of Sodium potassium tartarate and 100 g of NaOH dissolved in one litre distilled water.

Solution C: To 50g of anhydrous ferric sulphate, 200 ml of distilled water and 100ml of concentrated H₂SO₄ was added. The mixture was warmed till it became clear and then the volume was made to 1 litre with distilled water.

Solution D: 0.1N KMnO₄ solution.

Properly disintegrated pulp samples, equivalent to 1g of oven dry pulp was taken for estimation. Twenty ml each of solution A and B were taken in a 200ml beaker and heated. Just as it started boiling the pulp sample was added to the solution. The contents were boiled for 3 minutes and diluted with hot distilled water. The reaction mixture was filtered through whatman no.1 filter paper and given washings with about one litre of hot followed by cold distilled water. The washings were discarded and 50ml of solution C were added to the residue. The sample was washed with 500ml distilled water. The dissolved copper (earlier deposited on the pulp sample) in the filtrate was then titrated with 0.1N KMnO₄ till a faint pink colour appeared.

Copper number was then calculated as follows:

\[
\text{Copper number} = 0.636 \times V \times N/W,
\]

where

- \( V \) = ml of KMnO₄ required for titration
- \( N \) = normality of KMnO₄ solution
- \( W \) = weight of oven dry sample of pulp.

3.6.4 BIOBLEACHING OF DIFFERENT PULPS

Biobleaching of kraft pulps prepared from eight different raw materials individually i.e. bamboo, eucalyptus, soft woods, khar grass, kai grass, sabai grass,
sugarcane bagasse and wheat straw was carried out with P. chrysosporium K-3 and the soil isolate J1 in surface culture. The pulps were mixed after 3 days incubation at 39°C, dried and analysed for kappa number, viscosity and copper number.

3.6.5 EFFECT OF NUTRITIONAL FACTORS IN BIOBLEACHING

The effect of some nutritional factors in biobleaching of eucalyptus and khar grass pulp by P. chrysosporium K-3 was studied in surface culture as described in section 3.6.2.1. Some of the constituents of nutrient medium like carbon source (sugars), nitrogen source buffering agents and surfactant were varied but their concentrations were kept constant. Besides effect of addition of chelators and glycols were also studied.

3.6.5.1 SUGARS AND GLYCEROL

Various monosaccharides (Glucose, Xylose), disaccharides (Sucrose, maltose, cellobiose, lactose) and glycerol were added to the basal medium at a concentration of 1% as carbon source. The other constituents of basal medium were kept constant.

3.6.5.2 NITROGEN SOURCE

Different salts containing nitrogen were substituted in place of an equimolar mixture of asparagine and ammonium nitrate so as to give a final concentration of 2.4mM nitrogen in the medium. These were ammonium nitrate, ammonium chloride, ammonium sulphate, ammonium tartarate, ammonium oxalate, sodium nitrate, potassium nitrate and urea.
3.6.5.3 BUFFERING AGENTS

Seven buffering agents i.e sodium hydrogen phthalate, 2,2 dimethyl succinate, sodium tartarate, sodium succinate, sodium maleate, sodium citrate and sodium acetate were tried in basal medium at 10mM concentration, pH 4.5.

3.6.5.4 SURFACTANTS

Tween 80, Tween 60, Tween 40, Tween 20 and Triton X 100 (0.01%), oleic acid (0.03%) and an emulsified mixture of Tween 80 (0.01%) and oleic acid (0.03%) were used as surfactants in the growth medium. Two ionic surfactants i.e. sodium deoxycholate and sodium lauryl sulphate were also tried.

3.6.5.5 CHELATORS

Effect of some chelators like EDTA, EGTA, sodium citrate and ammonium oxalate was also studied at 1mM concentration.

3.6.5.6 GLYCOLS

Ethylene glycol, propylene glycol and polyethylene glycol at 10mM concentration were also tested in the nutrient medium.

3.7 STUDY OF PAPER MAKING CHARACTERISTICS OF BIOBLEACHED PULPS

Biobleaching of eucalyptus kraft pulp was carried out using optimised nutritional medium with P.chrysosporium K-3 on a relatively larger scale (sheet size, 3g, 16cm diameter) in surface culture and its various paper making characteristics were examined according to Tappi test methods (Appendix III). The optimised nutritional medium had
1% glucose as carbon source, ammonium tartarate as nitrogen source (2.4mM N, final conc.), dimethyl succinate as buffering agent (10mM, pH 4.6) and 0.1% Tween 80 as surfactant.

3.7.1 FREENESS DETERMINATIONS

Freeness test depends mainly upon the quantity of debris present in the pulp and is an arbitrary measure of the rate at which a suspension of 3g of pulp in 1 litre of water may be drained (Tappi T227 OS-58). The pulp sample is completely defibred and gently poured in the freeness tester, previously cleaned and wetted. After an interval of 5 seconds from the time that the addition of sample is completed, the air cock is opened to start the flow. The volume (ml) discharged from the side orifice is recorded as freeness.

3.7.2 HANDSHEETS PREPARATION FOR BRIGHTNESS TEST OF PULP

Six grams of moisture free pulp is diluted to 2000 ml with water at room temperature and disintegrated for 15000 revolutions (5 minutes) (Tappi T218 om-83). The contents are divided equally into two parts and poured rapidly over a buchner funnel with 150mm filter paper placed on it. The suction is applied and continued until excess of water has been removed and the vacuum is broken immediately. The sheets are then transferred to the press and the drier sequentially. The filter paper is detached and sheets stored in the conditioned room to be tested after at least 2h but not more than 24h, as their optical properties may change with time.
3.7.3 BRIGHTNESS TESTING

Brightness is the numerical value of the reflectance factor of a sample with respect to blue light of specific spectral and geometric characteristics (Tappi T452 om-87). Representative portion of the sample sheet is cut into seven or more tabs at least 30mm longer and 20mm wider than the specimen aperture. The tabs are assembled in a pad with the top sides up using the top tab as a cover only. Without touching the test areas with the fingers, the pad is placed over the specimen aperture of the brightness tester and the reflectance reading is recorded. The average brightness of the sample is reported to one decimel place in percent photo volt.

3.7.4 HANDSHEET PREPARATION FOR PHYSICAL TESTS OF PULP

This method provides a precise standard procedure for making test sheets from pulp that ensures a high degree of reproducibility (Tappi T205 om-81). Representative specimen of pulp is weighed equivalent to 24 grams of moisture free fibre. The sample is diluted to 2000 ml (1.2% consistency) with water at 20 ± 2°C and disintegrated in the standard disintegrator. The stock is further diluted to 8.0 litres (0.3% consistency) with water alongwith stirring to ensure proper mixing. 400ml of this stock is measured out for each handsheet to be formed into separate containers.

The measured sample is poured into the standard sheet machine and water is added. The perforated stirrer is inserted, rapidly moved up and down and gently withdrawn.
After a pause, the drain cock of the machine is fully opened with a rapid movement and all the water is allowed to drain under suction from the sheet. The sheet is then couched off the wire of the machine by using standard blotting paper and the couch plate. Two pressings are given to the sheets followed by drying and conditioning under standard conditions.

3.7.5 PHYSICAL TESTING OF PULP HANDSHEETS

3.7.5.1 BASIS WEIGHT

The average basis weight (g/m²) is measured by weighing five conditioned sheets together on a balance sensitive to 0.01 gms. The area of each sheet is approximately taken to be 200 cm² (Tappi T220 om-83).

3.7.5.2 THICKNESS

The thickness of the sheet (in microns) is measured using a motor driven micrometer and the reading recorded to nearest 2 μm (Tappi T411 om-84).

3.7.5.3 BULK

Bulk of handsheets is expressed as cm³/gm and calculated as thickness divided by basis weight.

3.7.5.4 BURST FACTOR

Burst factor is widely used as a measure of resistance to rupture in many kinds of paper (Tappi T403 om-85). A specimen strip (62x62mm) is clamped securely in position in bursting tester. The hydrostatic pressure is applied as specified until the specimen ruptures and the maximum pressure registered is recorded. Ten tests are made and bursting strength is reported as the arithmetic mean to
three significant figures. Burst factor \((X)\) is calculated as
\[
X = \frac{P}{W}
\]
where
\[
P = \text{bursting strength, g/cm}^2.
W = \text{weight per unit area, g/m}^2.
\]

3.7.5.5 TEAR FACTOR

This method determines the average force perpendicular to the plane of the paper required to tear a single sheet of paper through a specified distance after the tear has been started using a tearing tester (Tappi T414 om-82). The test specimen at least 53mm long by 63mm wide is centered in the clamps of tearing tester using approximately same pressure on both clamps. The initial slit is made and the pendulum stop is depressed quickly as far as it will go to release the pendulum. The stop is held down until after the tear is completed and pendulum is caught on the return swing without disturbing the position of the pointer. The scale readings are recorded to the nearest half division to give average tearing force. The tear factor is calculated as
\[
\text{Tear factor} = \frac{\text{tearing force}}{\text{basis weight}}.
\]

3.7.5.6 TENSILE STRENGTH

It is the maximum tensile stress developed in a test specimen before rupture on a tensile tester under prescribed conditions (Tappi T494 om-81). The test specimen is cut 15mm wide and long enough to be clamped in the jaws of tensile testing machine when they are 180 ± 25mm apart. The clamps are set to an initial test span i.e. 180 ± 25mm. The
specimen is aligned and clamped first in the upper jaw and then in the lower jaw after removing any noticeable slack in the lower jaw. A clamping pressure is used which is satisfactory so that neither slippage nor damage to the specimen occurs. The breaking force is read and recorded to 0.5% of the full scale. Tensile strength is reported as lbs/15mm width.

3.7.5.7 BREAKING LENGTH

Breaking length (in meters) is the calculated limiting length of a strip of uniform width, beyond which, if such a strip was suspended by one end, it would break of its own weight (Tappi T494 om-81). It is calculated as

$$\text{Breaking length} = \frac{\text{Tensile strength in lb/15 mm width}}{\text{Basis weight in g/m}^2} \times 30240$$

3.7.5.8 ZERO SPAN TENSILE STRENGTH

This method is used to determine an index of the average ultimate strength of the longitudinal structure of individual fibres in a pulp test handsheet (Tappi T231 Su-70). Test specimens are paper strips cut 15mm wide and of any convenient length over 15mm. An end of the specimen test strip is inserted between the opened jaws of the zero span jaw attachment. The pendulum of the tensile tester is locked at zero and its jaws are set just over 180mm apart. The attachment is placed with the specimen, centrally in the grips and clamped firmly in position. The load is applied at such a rate that the specimen will break in 2.5 ± 0.5 seconds. The breaking load is recorded to the nearest 0.1Kg. Zero span tensile strength is reported in Kg.
3.7.5.9 FIBRE STRENGTH FACTOR

It signifies the intrinsic fibre strength of the paper (Casey, 1980) and is derived from zero span tensile strength. It is calculated as

\[
\text{zero span tensile strength in Kgx60 g/m}^2 \div \text{g/m}^2
\]

3.7.5.10 FOLD

Fold or folding endurance tests have been used for the estimation of the suitability of paper in use to withstand repeated bonding folding and creasing (Tappi T423 om-84). The test specimen having a width of 15.0 ± 0.1 mm and a length of 100 mm is placed in the vertical slot of the folding tester, without touching its center and the ends are fastened firmly in the jaws. The tension is applied and the specimen folded until it breaks. The number of double folds made before fracture are recorded. The average test result is reported as double folds (folding number).

3.7.5.11 GURLEY POROSITY

It is evaluated by obtaining the time for a given volume of air to flow through a specimen of given dimensions under standard conditions of pressure, pressure difference, temperature and relative humidity (Tappi T460 om-83). The specimen having dimensions of 50mm width and 125mm length is clamped in the air resistance apparatus. The number of seconds are determined to the nearest 0.25, required for the first two consecutive 50ml intervals to pass the rim of the outer cylinder of the apparatus, starting at zero point. The results are reported as seconds/100ml/6.4 cm².
3.7.6 LABORATORY REFINING OF PULP (PFI MILL METHOD)

Laboratory refining or beating of the pulp is a widely accepted method of simulating commercial refining practices. Physical testing of laboratory refining pulps provides significant data that aid in determining the ultimate performance of pulp when converted to paper (Tappi T248 pm-74).

A pulp specimen weighing 30.0 ± 0.5g (oven) dry basis is obtained for each run. The pulp is thoroughly softened by soaking to ensure that preliminary disintegration results in the least possible beating effect. The pulp is completely disintegrated at 1.5% consistency following which the pulp suspension is drained on a buchner funnel using a coarse filter paper to approximately 20% consistency. The pulp is peeled from the filter and weighed. Appropriate amount of distilled water is added to a total mass of 300 ± 5g corresponding to a 10% stock consistency.

The 10% pulp suspension is transferred to the beater housing and distributed as evenly as possible over the wall. The beater roll is inserted in the housing and the cover pressed into position. The beater housing is set rotating so that the pulp is slung against the wall and the roll is started. When both elements have attained full speed, the required beating pressure is gradually applied over a 4 second interval and the revolution counter is started simultaneously. The beating is discontinued by removing pressure from the roll after the required number of
revolutions of the roll. The motors are shut off and the roll is centered. Then both the roll and housing cover are lifted. All the pulp is transferred to a 200 cm$^3$ graduated cylinder. The stock is diluted with distilled water to 2000 cm$^3$ and cleared in the disintegrator for 10000 revolutions. The pulp is then ready for freeness determinations and making and testing of handsheets.

3.8 PROCESS OPTIMISATION FOR BIOBLEACHING

Effects of varying a few important parameters viz. incubation time, PFI refining, concentration of mycelial inoculum, concentration of carbon source, initial Kappa number of pulp and prerefining of pulp were studied on biobleaching of eucalyptus kraft pulp by $P. chrysosporium$ K-3. Large pulp sheets (3g, 16cm diameter) were prepared from eucalyptus pulp and inoculated with 5% fungal mycelium after addition of optimised nutritional medium. After 3 days incubation at 39°C in surface culture, various replicates in each case were mixed and washed with distilled water. The pulp samples were then analysed for pulp characteristics as well as handsheet properties without and with PFI refining.

3.8.1 INCUBATION TIME

Unbleached eucalyptus kraft pulp inoculated with vegetative mycelium of $P. chrysosporium$ K-3 was incubated for different time intervals i.e., 0, 1, 2 and 3 days at 39°C in surface culture and analysed.

3.8.2 REFINING

Biobleached pulps obtained after fungal growth for all incubation intervals were subjected to refining in the PFI
mill to freeness levels of 400, 350 and 300ml and analysed for handsheet properties.

3.8.3 MYCELIAL INOCULUM

The concentration of vegetative mycelium in the pulp, used as the inoculum for biobleaching, was varied to 2.5, 5.0, 7.5 and 10.0% (W/W) on dry weight basis.

3.8.4 GLUCOSE CONCENTRATION

Effect of different glucose concentrations (0.5, 1.0, 2.0 and 4.0%) used as carbon source was studied.

3.8.5 INITIAL KAPPA NUMBER

Unbleached eucalyptus pulps of three different kappa numbers (26.5, 22.3 and 18.1) were prepared by extending cooking cycles during kraft pulping. These were biobleached with *P. chrysosporium* K-3 and analysed to study the effect of initial lignin content of the pulp.

3.8.6 PREREFINING OF THE PULP

Unbleached eucalyptus pulp was refined to freeness levels of 550, 500 and 450ml in a PFI mill and then biobleached. Handsheet properties of the prerefined biobleached pulps were studied without and with further refining to 400 ml as in other cases.

3.9 COMBINATIONS OF BIOBLEACHING AND CHEMICAL BLEACHING PROCESSES

Biobleached pulp was obtained by growing *P. chrysosporium* K-3 on unbleached eucalyptus kraft pulp of 25.2 kappa number for 3 days at 39°C using 5% mycelial inoculum and 1% glucose. Chlorine bleached pulp was obtained by treating the same pulp in the bleach plant of a
commercial paper mill with 5% chlorine for 45 minutes. Both
the pulps were subjected to further bleaching by various
chemical agents viz. Sodium hydroxide, calcium hypochlorite,
chlorine dioxide and hydrogen peroxide individually as well
as in succession. Following each treatment, pulp
characteristics (kappa number, brightness and viscosity) and
physical handsheet properties were measured. The pulps were
refined in the PFI mill to freeness level of 400ml and
physical handsheet properties were again measured.

3.9.1 ALKALI EXTRACTION

Both biobleached and chlorine bleached pulps were
treated with 2% sodium hydroxide (on pulp basis) at 70°C for
90 minutes.

3.9.2 HYPOCHLORITE BLEACHING

The pulps were treated with 2% calcium hypochlorite
(based on available chlorine), 1% sodium hydroxide and 0.1%
sulphamic acid at 6% pulp consistency at 40°C for 90
minutes. Initial pH of the reaction mixture was 9.1 to 9.2.

3.9.3 CHLORINE DIOXIDE BLEACHING

Chlorine dioxide was added at a concentration of 1.5%
and 10% pulp consistency and initial pH of the reaction
mixture was brought to 6.0 with glacial acetic acid. The
reaction was carried out for 3 hours at 70°C.

3.9.4 PEROXIDE BLEACHING

Pulps were treated with 3% hydrogen peroxide, 4% sodium
silicate, 0.1% MgSO₄.7H₂O and 2.5% NaOH at 10% pulp
consistency and 70°C for 90 minutes.
3.9.5 SECOND STAGE HYPOCHOLORITE TREATMENT

Conditions for second hypochlorite bleaching were 6% pulp consistency, 1.5% calcium hypochlorite, 1% NaOH, 0.1% sulphamic acid, initial pH 9.3-9.4, temperature 40°C for 90 minutes.

3.9.6 SECOND STAGE ALKALI TREATMENT

The chlorine dioxide bleached pulps were treated with 1% sodium hydroxide at 10% pulp consistency at 40°C for 90 minutes.

3.9.7 THIRD STAGE CHLORINE DIOXIDE BLEACHING

Pulps treated with chlorine dioxide and then alkali extracted were further subjected to chlorine dioxide bleaching with 1.5% chlorine dioxide at 10% pulp consistency. Acetic acid was added to adjust pH of the reaction mixture to 6.0. The bleaching was carried out at 70°C for 30 minutes.

3.10 LIGNINASE EXPERIMENTS

Five days old shallow stationary cultures of *P.chrysosporium* K-3 were used as source of ligninase enzyme (see section 3.5.4.4.). The yellow supernatant obtained by centrifugation at 10000 g for 5 minutes at 4°C, was concentrated by ultra filtration (Millipore Minitan unit) using a 10 K.Da cut off membrane to about one tenth volume. The preparation was then filtered to remove precipitated mycelial slime and used as crude ligninase (Tien and Kirk, 1989).
3.10.1 STABILITY TESTING OF LIGNINASE

3.10.1.1 pH STABILITY

One ml enzyme preparation was incubated with 0.5 ml sodium tartarate buffer of different pH values ranging from 2.5 to 5.5 at 25°C. Enzyme assay was performed after 1 hour incubation as well as under normal assay conditions.

3.10.1.2 TEMPERATURE STABILITY

Effect of incubation temperature on enzyme activity was studied by incubating the enzyme with 100 mM tartarate buffer, pH 3.5 at different temperatures (25 to 45°C) for one hour followed by enzyme assay at room temperature.

3.10.2 LIGNINASE TREATMENT OF UNBLEACHED KRAFT PULP

Unbleached eucalyptus kraft pulp was treated with crude ligninase in the presence of hydrogen peroxide and tartarate buffer. The reaction mixture included 50mg pulp, 2ml concentrated enzyme, 1ml tartarate buffer (100 mM, pH 3.5) and 1 ml 0.4mM H₂O₂. The reaction mixture was centrifuged at 15000 rpm for 20 minutes after incubation at 25°C for 2, 8, 16 and 24 hours. The optical densities of the supernatants were read at three wavelengths i.e. 205, 260, and 280nm in Beckman DU7 spectrophotometer. UV spectra of the supernatants were also obtained in the range of 200 to 320 nm.

All the enzyme treated pulps (50 mg) were further subjected to alkali treatment with 2.5ml of 1% NaOH at 70°C for 90 minutes. The supernatants were obtained by centrifuging at 15000 rpm for 20 minutes and diluted three times followed by study of optical densities at three wave
lengths (205, 260, and 280nm) as well as UV spectra ranging from 200-320 nm.

After the optimisation of treatment time, 2g of unbleached eucalyptus kraft pulp was treated with ligninase and analysed for changes in kappa number and brightness.

3.11 BIOCOLOUR REMOVAL EXPERIMENTS

3.11.1 PRELIMINARY STUDY OF EFFLUENT

First alkaline extraction stage (AES) effluent following chlorination in the bleach plant was analysed for pH and colour measurements (APHA method, 1980).

3.11.2 OPTIMISATION OF BIOCOLOUR REMOVAL PROCESS

Alkaline extraction stage effluent was acidified to pH 4.5 with concentrated HCl and adjusted to a colour concentration of 3000 Pt/Co colour units. It was supplemented with basal salts KH₂PO₄ (14.7mM), MgSO₄ (2.1mM), CaCl₂ (0.9mM), 2mM NH₄Cl as nitrogen source and 1% glucose as the growth substrate. Colour removal experiments were carried out in 100ml flasks containing 10ml of the effluent with nutrients under stationary culture conditions. The contents of the flasks were autoclaved at 10 psi for 30 minutes and inoculated with the suspension of mycelial fragments (10mg/ml, dry weight basis). The supernatants were obtained by filtration through whatman no. filter paper every day over a period of 7 days incubation at 39°C under stationary conditions. Colour units, pH and reducing sugars (Nelson, 1944) were measured in each effluent sample. Various flask level colour removal experiments done were as follows:
3.11.2.1 SCREENING OF FUNGI

Four standard strains i.e. *P.chrysosporium* ATCC 24725, *P.chrysosporium* K-3, *S.pulverulentum* NRRL 6360, *S.pulverulentum* and soil isolate VJ1 were screened for their decolourisation efficiency.

3.11.2.2 EFFECT OF GLUCOSE CONCENTRATION

Rate of decolourisation of alkaline extraction stage effluent by *P.chrysosporium* K-3 was compared by addition of different glucose concentration ranging between 0 to 1% using effluent having 3000 Pt/Co colour units.

3.11.2.3 EFFECT OF ALTERNATE CARBON SOURCES

Effect of cellulose, pith and primary sludge at a concentration of 1% as alternate carbon sources to replace glucose was also investigated. These materials were added to effluents medium prior to sterilisation. The inoculation with mycelial was done as above.

3.11.2.4 EFFECT OF INITIAL COLOUR CONCENTRATION OF EFFLUENT

The initial colour concentration of the effluent was varied between 1000 and 6000 Pt/Co units to study its effect on bio colour removal by *P.chrysosporium* K-3 using 1% glucose.

3.11.2.5 EFFECT OF TEMPERATURE

*P.chrysosporium* K-3 inoculated effluent flasks of 3000 Pt/Co units colour were incubated at 3 different temperatures i.e. 25°C, 30°C and 39°C under stationary conditions to compare the rate of colour removal. Samples were withdrawn every day over a period of 7 days incubation.
3.11.2.6 EFFECT OF SUCCESSIVE SCALE UP AT FLASK LEVEL

Colour removal rate was compared in different flasks by taking effluent volume in a fixed ratio (10\% of the flask capacity) i.e. 10ml in 100ml flask, 25ml in 250ml flask, 50ml in 500ml flask and 100ml in 1000ml flask. The effluent of 3000 Pt/Co units colour concentration was inoculated with \textit{P.chrysosporium} K-3 using 1\% glucose and incubated for 7 days under stationary conditions at 39\(^{\circ}\)C.

3.11.3 BIOCOLOUR REMOVAL IN A STIRRED FERMENTER

Biocolour removal of alkaline extraction stage effluent (3000 Pt/Co colour units) with \textit{P.chrysosporium} K-3 was studied in a 6.0 litre stirred tank fermenter (LKB fermenter) with a working volume of 4.5 litres. Media constituents added to the effluent were similar to that in flask cultures with 1\% glucose as carbon source. The reactor was operated at 39\(^{\circ}\)C with an agitation rate of 200 rpm. Agitation was provided by a stainless steel agitator with four impeller blades mounted on a thick disk at the bottom of the reaction vessel which is powered by a magnetic stirrer. The fermenter was continuously sparged with humified, sterile air (0.3 vvm) via a gas dispersion tube with a fine glass frit located directly above the agitator.

\textit{P.chrysosporium} K-3 was inoculated with mycelial inoculum (10mg/ml) and the fermenter was run for 96 hours. Effluent sample was withdrawn after every 12 hours and analysed for pH, colour units and reducing sugars.
3.11.4 COLOUR MEASUREMENT

The colour of the effluent was determined essentially according to American Public Health Association (APHA) standard method (1980). The pH of the sample was measured and then adjusted to 7.6 by addition of up to 200ul of 2M NaOH. The effluent then filtered through 0.2-0.4 um sintered glass filter (G-5) to remove suspended solids. The clear supernatant was then used for measurement of absorbance at 465nm against distilled water. Optical densities were transformed into colour units according to the equation

\[
\text{Colour units (CU)} = \frac{500 \cdot A_2}{A_1}
\]

where \( A_1 \) is absorbance of standard platinum cobalt solution having 500 colour units and \( A_2 \) is absorbance of effluent.

The standard platinum cobalt solution is prepared by dissolving 1.246g \( \text{K}_2\text{PtCl}_6 \) (equivalent to 500 mg Pt) and 1.00 g crystallised cobaltous chloride (\( \text{CoCl}_2 \cdot 6\text{H}_2\text{O} \), equivalent to 250 mg metallic Co) in distilled water with 100ml concentrated HCl (AR) and diluting to 1000ml.

3.11.5 REDUCING SUGARS ESTIMATION

The following reagents were used in the reducing sugar estimation by the method of Nelson (1944).

1. Alkaline copper sulphate

   i) Sodium pottassium tartarate 15g
      \( \text{Na}_2\text{CO}_3 \) (anhydrous) 30g
      \( \text{NaHCO}_3 \) 20g
      Distilled water 300ml
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂SO₄</td>
<td>180g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>500ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄·5H₂O</td>
<td>5g</td>
</tr>
<tr>
<td>Na₂SO₄ (anhydrous)</td>
<td>45g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>250ml</td>
</tr>
</tbody>
</table>

The alkaline copper reagent was made by mixing 4 parts of reagent (iii) with 1 part of reagent (iv).

2. Arsenomolybdate reagent:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>25g ammonium molybdate was dissolved in 450ml of distilled water and 21ml of concentrated H₂SO₄ was slowly added to it followed by gentle mixing.</td>
<td></td>
</tr>
<tr>
<td>3g sodium arsenate was dissolved in 25ml of distilled water.</td>
<td></td>
</tr>
</tbody>
</table>

Components (i) and (ii) were mixed with stirring and stored at 37°C for 24-48 hrs. The solution was stored in a dark brown bottle. The working arsenomolybdate reagent was prepared by diluting 1 volume of arsenomolybdate with 2 volumes of 1.5N H₂SO₄.

One ml of the sample was mixed with 1ml of alkaline copper reagent and boiled for 10 minutes. The mixture was cooled and 1ml of arsenomolybdate reagent was added and shaken quickly. The entire reaction mixture was centrifuged (5,000g x 10 min) and the absorbance of the supernatant was measured at 540nm. Control tubes contained 1ml of distilled water in place of sample. The reducing sugars were quantitated from the glucose standard curve prepared in the range of 20-200 µg.