MATERIALS AND METHODS
3. MATERIALS AND METHODS

Isolation of mycoflora

Collection site

Mycoflora were isolated from the Siruvani forest of the Western Ghats, Coimbatore, Tamil Nadu, India. The Siruvani forest is located at the foot hills of Western Ghats extension at 76°73’N and 10°58’E and above 500 ± 50 MSL. The climate in this area is typically monsoonic, characterized by three seasons, viz., warm and moist rainy season (July - October), cold winter season (November - February) and dry and hot summer season (March - June). Data on the climatological conditions prevailed during the study period were recorded and presented in Table 1.

Litter mycoflora

Litter mycoflora was isolated by nylon net litter bag technique (Adhikari and Tiwari, 1991). Paddy straw, maize stover, coir pith, *Eucalyptus globulus* leaves and *Tectona grandis* leaves were dried at 80°C and used as baits. Each of the dried material (5.0 g) was placed in a nylon net litter bag (1 mm mesh; 20 x 20 cm size) with coloured beads (to identify the materials) and burried in the aerable forest soil at 10 cm depth. For each sample, six replicates were maintained. Samples were collected at regular monthly intervals in presterilised polyethylene bags. The litter samples were powdered and suspended in sterile distilled water (0.1 g per 50 ml). This suspension (1.0 ml) was poured into the Petriplates containing rose-bengal-streptomycin-agar medium.

Soil mycoflora

Soil samples in and around the litter bags were collected in presterilised polyethylene bags. Soil mycoflora were isolated by dilution plate technique (Waksman, 1922) at 10^4 dilution, on rose - bengal-streptomycin-agar medium in Petriplates.
The culture plates were incubated at 37°C for seven days. After the incubation period, total number of colonies, total number of species and number of colonies of each species in a Petriplate were recorded. The fungal species were subcultured on rose-bengal-streptomycin-agar slants and maintained in a refrigerator. Since the rose-bengal is fungistatic, it will restrict the overgrowth of fast growing fungi and enables the growth of slow growing fungi also on the plates.

Rose-bengal-streptomycin-agar medium (g/l) (Martin, 1950):

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextrose</td>
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</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Rose bengal (1% solution)</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.03</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

For identification, the fungi were stained with cotton blue, mounted on canadabalsm and observed under high power (100x) light microscope.

For statistical analysis, each Petriplate was considered as a quadrate as that of the quadrate in the study of higher plant ecology (Udaiyan, 1981).

Fungal population in the dry soil was enumerated by the formula

\[
\text{Population of fungi} / \text{g dry soil} = \frac{\text{Average number of colonies per plate}}{\text{Dry weight of the soil sample}}
\]

The frequency of occurrence of each species was calculated by the formula.

\[
\text{Frequency (\%)} = \frac{\text{Number of plates in which the species occurred}}{\text{Total number of plates sampled}} \times 100
\]

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Based on the frequency of occurrence the fungi were classified as follows:

- **Dominant**: 81 - 100%
- **Common**: 61 - 80%
- **Frequent**: 41 - 60%
- **Occasional**: 21 - 40%
- **Rare**: 01 - 20%

The abundance of each species was calculated by the formula:

\[
\text{Abundance (\%)} = \frac{\text{Total number of colonies of a particular fungal species in all replicates}}{\text{Total number of colonies of all fungal species in all replicates}} \times 100
\]

Based on the abundance, the fungal species were classified into 5 classes:

- **Class 1**: Species constituting 1 - 3\% of the total number of fungi in a Petriplate
- **Class 2**: Species constituting 4 - 8\% of the total number of fungi in a Petriplate
- **Class 3**: Species constituting 9 - 15\% of the total number of fungi in a Petriplate
- **Class 4**: Species constituting 16 - 25\% of the total number of fungi in a Petriplate
- **Class 5**: Species constituting 26 - 100\% of the total number of fungi in a Petriplate.
Litter analysis

Physical and biochemical properties of the litter samples were analysed before and after biodegradation.

**Physical properties**

**Weight loss**

The weight loss of the degraded litter sample was calculated by the formula

\[
\text{% weight loss} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where,  
- W1 - Initial dry weight of the sample before biodegradation  
- W2 - Final dry weight of the sample after biodegradation

**Mean relative decomposition rate (MRD)**

The litter samples, before and after biodegradation, were dried to a constant weight at 70°C and the mean relative decomposition rate was calculated by the formula

\[
\text{MRD g/g/day} = \frac{\text{Weight loss (g)}}{\text{Weight of the litter sample (g)}} \times \frac{1}{\text{Incubation period (days)}}
\]

**Moisture content**

Weighed amount of the litter sample was dried to a constant weight at 70°C, and reweighed. The moisture content of the litter was determined by the formula

\[
\text{Litter moisture (%)} = \frac{W_1 - W_2}{W_1} \times 100
\]

Where,  
- W1 - Initial weight of litter sample  
- W2 - Final dry weight of litter sample

**Biochemical properties**

The samples were dried to constant weight at 70°C and ground in a Willey mill to fine powder. Powdered sample was used for biochemical analysis.
Total sugars

The powdered sample (100 mg) was extracted twice with 5 ml of 80% hot ethanol. Non-reducing sugar present in the ethanol extract was evaporated to dryness on a water bath. The residue was dissolved in 10 ml of distilled water (Inman, 1962) and the reducing sugar content was estimated by Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952).

Reagents:

1. Copper reagent: Anhydrous sodium carbonate (24g) and potassium sodium tartrate (24g) were dissolved in about 250 ml of water. To this carbonate solution, copper sulphate solution (4g in 40 ml of water) was added with stirring and then 16g of sodium bicarbonate was added. Anhydrous sodium sulphate (180 g) was dissolved in about 500 ml of water and boiled to expel air. After cooling, the two solutions were mixed and diluted to 1000 ml.

2. Nelson's chromogenic reagent (arsenomolybdate reagent)

Ammonium molybdate (25 g) was dissolved in 450 ml of water and 21 ml of concentrated sulphuric acid was added and mixed. To this 3 g. of sodium hydrogen arsenate (Na$_2$HAsO$_4$.7H$_2$O) dissolved in 250 ml of water was added. After mixing, the solution was incubated at 37°C for 48 h. The reagent was stored in a glass stoppered brown bottle.

To 1 ml of the filtrate, 1 ml of alkaline copper reagent was added and boiled for 10 min. After cooling down for 1 min, 1 ml of arsenomolybdate reagent was added and the solution was made upto 5 ml with distilled water. The intensity of the colour developed was read at 540 nm in a spectrophotometer. Quantities of sugar present in the samples were determined with reference to a standard graph prepared with known quantities of glucose.
Carbohydrate

The carbohydrate content of the litter sample was estimated by the method of Hodge and Hofreiter (1962). In this method, carbohydrates were converted into simple sugars by acid hydrolys.

Reagents

(1) 2.5 N - Hydrochloric acid
(2) Anthrone reagent - Anthrone (200 mg) dissolved in 100 ml of ice cold 95 % sulphuric acid.

The powdered sample (100 mg) was hydrolysed in 5 ml of 2.5 N hydrochloric acid in a boiling water bath for 3 h and then cooled. The hydrolysate was neutralised with solid sodium carbonate (until the effervescence ceases) and made upto 100 ml with distilled water. To 0.5 ml aliquots, 4 ml of anthrone reagent was added and boiled for eight minutes. The sample was cooled rapidly and the green colour developed was read in spectrophotometer at 630 nm. Quantities of carbohydrates present in the samples were determined with reference to a standard graph prepared by using known quantities of glucose.

Cellulose

The cellulose content of the litter sample was determined by Updegroff (1969) method.

Reagents

(1) Acetic-nitric reagent - 15 ml of concentrated nitric acid in 150 ml of acetic acid (80 %).
(2) Anthrone reagent - Anthrone (200 mg) dissolved in ice cold sulphuric acid (100 ml)
(3) 67 % sulphuric acid

The powdered sample (100 mg) was acetolysed with 3 ml of acetic-nitric reagent in a boiling water bath for 30 min and then cooled and centrifuged. The supernatant was discarded. The residue was washed with distilled water and treated with 67 % sulphuric acid (10 ml) for 1 h. The volume was made upto 1000 ml with distilled water. To 1 ml of diluted solution, 5 ml of anthrone reagent was added and boiled in a water bath for 10 min. The samples were cooled and then read at 630 nm in a spectrophotometer. The calibration curve was plotted using cellulose as standard.
Hemicellulose and lignin

Hemicellulose and lignin content of the samples were estimated by Goering and van Soest method (1970).

Hemicellulose

Hemicellulose content was estimated as the difference between neutral detergent fibre (NDF) and acid detergent fibre (ADF) content of the samples.

Neutral detergent fibre (NDF)

Reagents

(1) Solution A - 18.61 g of disodium ethylene diamine tetra acetate and 6.81 g of sodium borate were dissolved in 200 ml of distilled water.

(2) Solution B - 30 g of sodium dodecyl sulphate was mixed with 10 ml of 2-ethoxyethanol and made up to 100 ml with distilled water.

(3) Solution C - 4.5 g of disodium hydrogen phosphate was dissolved in 100 ml of distilled water.

(4) Cold neutral detergent solution - solutions A, B and C were mixed in the order and the volume was made up to 1000 ml. pH of the solution was adjusted to 7.0.

Weighed amount of sample (1 g) was taken in a refluxing flask and added with 10 ml of cold neutral detergent solution, 2 ml of decahydonaphthalene and 0.5 g of sodium sulphate. The flask was heated to boiling temperature and refluxed for 60 min. The contents were cooled, filtered through sintered glass funnel (G-2) and washed with hot water and acetone. The residue was dried at 100°C for 8 h and weighed. The difference in weight gave the NDF content.
**Acid detergent fibre (ADF)**

**Reagent**

Acid detergent solution - 20g of cetyl trimethyl ammonium bromide was dissolved in 1000ml of 1N sulphuric acid.

In a refluxing flask, to 1.0g of powdered sample 100ml of acid detergent solution was added. The contents were refluxed gently for 5-10 min to avoid foaming as boiling begins by the onset of boiling temperature. Refluxing was done for 1h. The content was filtered through G-2 sintered glass funnel and washed with hot water and acetone until the residue became colourless. The residue was dried at 100°C for 8h, cooled and then weighed. The loss in weight denoted the ADF content. From these two fibre contents, hemicellulose content of the sample was calculated by the formula,

\[
\text{Hemicellulose (\%)} = \frac{\text{Neutral detergent fibre} - \text{Acid detergent fibre}}{\text{Weight of the sample}} \times 100
\]

**Lignin**

Lignin was determined in terms of acid detergent lignin. The acid detergent fibre was dissolved in 50 ml of 72% sulphuric acid. To this 1g of asbestos was added and allowed to stand for 3h with intermittent stirring with a glass rod. The acid was diluted with distilled water and filtered through a preweighed Whatman No.1 filter paper. The contents were washed several times until free from acid. The filter paper was dried at 100°C and after cooling in a dessicator it was weighed. The filter paper was transferred to a preweighed silica crucible and ashed in a muffle furnace at 550°C for about 3h. The crucible was cooled in a dessicator and weighed. For blank, instead of sample, the asbestos was treated with 72% sulphuric acid and the other procedures were similar as for the sample.

\[
\text{Lignin (\%)} = \frac{\text{Residue after extraction with 72\% } H_2SO_4 - \text{Ash}}{\text{Weight of the sample}} \times 100
\]

\[
= \frac{(\text{Test - Blank}) \text{ residue} - (\text{Test-blank}) \text{ Ash}}{\text{Weight of the sample}} \times 100
\]
Pectic substances

The amount of pectic substances present in the samples were determined by the method of Ranganna (1979).

The sample (1g) was treated with 30ml of 0.01N hydrochloric acid in a boiling water bath for 30 min. and filtered. The filtrate was collected separately. The residue was again boiled with 10ml of 0.3 N hydrochloric acid for 10 min and filtered. The filtrate was collected. The filtrates were pooled together, cooled and made upto 250ml with distilled water. The filtrate was then neutralized with 1N sodium hydroxide using phenolphthalein indicator. After neutralization, an excess of 10ml of 1N sodium hydroxide solution was added with constant stirring and left undisturbed for overnight. To this, 50ml of 1N acetic acid and 25ml of 1N calcium chloride solution were added at 5 min interval with stirring. The solution was allowed to stand for 1 h and then boiled in a water bath for 2 min. After cooling, the solution was filtered through Whatman No.1 filter paper. The precipitate was washed with boiling water until the filtrate was free from chloride and the paper was dried at 100°C for overnight, cooled in a dessicator and weighed. The pectin content is expressed as % calcium pectate.

% Calcium pectate = \[
\frac{\text{Weight of calcium pectate} \times 500 \times 100}{\text{Vol. of filtrate (ml)} \times \text{Weight of the sample (g)}}
\]

Crude fibre

Crude fibre content was estimated by the method of Maynard (1970). The ground material (1 g) was extracted with petroleum ether. After extraction, the residue was boiled with 50ml of 0.255 N sulphuric acid for 30 min. The contents were filtered through a muslin cloth and the residue was washed with boiling water until the washings were no longer acidic. The washed residue was boiled with 50ml of 0.313 N sodium hydroxide for 30 min and filtered through muslin cloth and again washed with boiling 1.25% sulphuric acid followed by water washing and alcohol washing. The residue was transferred to a preweighed ashing dish (W,) and dried at 130 ± 2°C for 2h, cooled in a dessicator and reweighed (W2). Then the content of the dish was ignited for 30 min at 600°C, cooled in a dessicator and reweighed (W3).

% Crude fibre = \[
\frac{\text{Loss in weight on ignition} [(W_3 - W_1) - (W_2 - W_1)]}{\text{Weight of the sample}} \times 100
\]
**Ash Content**

The samples were ignited at 600°C for 2h in a preweighed silica crucible. After ashing, the crucible was reweighed. The difference in the weight denoted the ash content of the sample.

**Soil analysis**

**Sample preparation**

The soil samples collected in and around the litter bags were air dried and sieved through a 2 mm sieve. For determination of available nitrogen content, the 2 mm sample was ground to pass a sieve of 0.5 mm diameter.

**Physical properties**

For determination of physical properties of the soil, the methods proposed by Piper (1950) were followed. Soil temperature was recorded using a thermometer in the field itself uniformly at 10 AM. For determining the pH and electrical conductivity, the soil sample was dissolved in distilled water (1:2 w/v) and mixed thoroughly. After one hour, pH and electrical conductivity of the soil suspension were read in a pH meter and a conductivity meter respectively. To determine the soil moisture content, the samples were dried to a constant weight at 105°C and the loss in weight was recorded as moisture content.

**Chemical properties**

**Organic carbon**

The organic carbon content of the soil sample was estimated by the colorimetric method of Sims and Haley (1971).

**Reagents**

1. Potassium dichromate solution - 1N
2. Concentrated sulphuric acid
Weighed amount of soil (1 g) was taken in a 250 ml Ehrlenmeyer flask. To this, 10 ml of 1.0 N potassium dichromate and 20 ml of concentrated sulphuric acid were added. The contents were mixed by stirring and kept for oxidation for 20 min. After the oxidation process, the mixture was made up to 100 ml with distilled water and filtered. The optical density of the filtrate was measured at 600 nm in a spectrophotometer. To draw the standard curve, sucrose was subjected to the same process as the soil sample. The sucrose samples were prepared such that without oxidation and at 100 ml volume, they would have been at $2.92 \times 10^{-3}$, $5.84 \times 10^{-3}$, $8.76 \times 10^{-3}$, $11.68 \times 10^{-3}$ M. These concentrations are equivalent to organic matter contents of 1, 2, 3, 4, 5, 6 and 7 % in 1 g of soil. After the oxidation was complete, optical density was measured at 600 nm against a sucrose-free reagent blank.

Available nitrogen

Available nitrogen content of the soil was estimated by the method of Subbiah and Asija (1956).

Reagents

(1) Potassium permanganate solution - 0.32 % (w/v)
(2) Sodium hydroxide solution - 2.5 %
(3) Boric acid - 2 %
(4) Double indicator - 0.5 % bromocresol green and 0.1 % methyl red in 100 ml ethanol.
(5) Sulphuric acid - 0.1 N

Weighed amount of soil (20 g) was taken in a distillation flask. To this, 100 ml of 0.32 % potassium permanganate and 100 ml of 2.5 % sodium hydroxide were added. In an ice tumbler, 25 ml of 2 % boric acid was taken and a few drops of double indicator were added. The ice tumbler was kept at the delivery end of the distillation flask and the delivery tube was immersed in the boric acid solution and distilled. About 30 ml of the acid was titrated with N/10 or N/50 sulphuric acid. From this, the amount of available nitrogen in the soil was calculated as follows:
Weight of the soil taken = 20 g
1 ml of N/10 sulphuric acid = 0.0014 g of nitrogen
Therefore 'X' ml = 0.0014 X g of nitrogen

This is present in 20 g of soil. So

\[ \text{in 100 g} = \frac{0.0014 X}{20} \times 100 \]

In terms of Kg/ha

\[ = \frac{0.0014X}{20} \times 10^6 \times 2 \]

= 140 X Kg/ha

Available phosphorus

Available phosphorus content of the soil sample was estimated colorimetrically by the method of Olsen et al. (1954).

Reagents

(1) Sodium bicarbonate solution - 2.5 M; pH 8.5
(2) Activated charcoal - Dargo G-60
(3) Chloromolybdic acid: Ammonium molybdate [(NH₄)₆Mo₇O₂₄·4H₂O-15.0g] was dissolved in about 300ml of distilled water, warmed to about 50°C and the solution was filtered. The molybdate solution was cooled and 410ml of 10 N HCl was added slowly with rapid stirring. This solution was cooled again to room temperature, diluted to 1000 ml with distilled water, mixed thoroughly and stored in an amber glass stoppered bottle.
(4) Chlorostannous acid reductant (0.1M): Stannous chloride (18.96g) was dissolved in 50ml of concentrated HCl. This solution was diluted to (with rapid stirring) 500ml with recently boiled distilled water. This gave a standard solution of 0.2 M Sn⁺⁺ and the molar concentration was determined by titration of a 5 ml aliquot with 0.1N standard iodine solution. This solution was diluted with 1.2N HCl to 1000ml to get 0.1M Sn⁺⁺.
(5) Standard phosphate solution (50mg/l): Potassium dihydrogen phosphate was dried at 40°C and 0.2195g was dissolved in about 400ml of distilled water. Then, 25ml of 7N H₂SO₄ was added and the solution was made upto 1000ml and mixed. This stock solution was diluted to prepare a working standard ranging from 2.0 to 20 mg/l.
To the weighed amount of soil (5 g), 50 ml of 0.5 M sodium bicarbonate (pH 8.5) and a pinch of Dargo G-60 were added and shaken well in a rotary shaker for 30 min. The solution was filtered through Whatman No. 40 filter paper. To 5.0 ml of the filtrate, 5.0 ml of chloromolybdic acid and 2.0 ml of diluted stannous chloride solution were added. The volume was made up to 25 ml and the contents were mixed thoroughly. Within 10 minutes, the intensity of blue colour developed was read at 680 nm in spectrophotometer. The calibration curve was plotted using phosphate standards.

**Available potassium**

The available potassium content of the soil sample was estimated by the method of Jackson (1967).

**Reagents**

1. Neutral ammonium acetate solution - 7.7%
2. Stock potassium solution (1000 mg/l K) - Potassium chloride (1.907 g) was dried at 110°C and dissolved in distilled water to give 1000 ml solution.
3. Standard potassium solution (10 mg/l K) - The stock solution was diluted 100 times with distilled water.

A 50 g air-dried soil sample was weighed out into a 250 ml conical flask and 100 ml of (7.7%) ammonium acetate solution was added. The flask was stoppered and shaken for several minutes and then allowed to stand overnight. The contents of the flasks were then filtered through Whatman No. 42 filter paper. The soil was leached 4 to 5 times with excess of ammonium acetate solution. The filtrate and the leachates were pooled together and the final volume was acidified with nitric acid and evaporated to dryness on a water bath. To dried sample, 25 ml of concentrated nitric acid was added and heated to near boiling until the acid was evaporated to a small volume. When unoxidised organic matter were present in the sample, as evidenced by brown fumes, some amount of concentrated nitric acid (2 ml) and small quantities (2 ml) of hydrogen-peroxide (30%) were added for complete ashing of organic matter. The final residue was dried and dissolved in small amount of hydrochloric acid and
then in warm distilled water. The solution was filtered, neutralised with concentrated ammonium hydroxide and diluted to a known volume. The amount of potassium present in the clear filtrate was determined in a flame photometer by employing potassium filter. The calibration curve was prepared by using standard potassium solution.

**Soil cellulase activity**

Cellulase activity in the soil was determined by the method of Pancholy and Rice (1973). To 5.0 g of soil, 0.5 ml of toluene was added and mixed thoroughly. After 15 min, 10 ml of acetate buffer (0.01 M, pH 5.9) and 10 ml of 1% carboxy methyl cellulose were added. The reaction mixture was incubated for 24 h at 30°C and then approximately 50 ml of distilled water was added. The suspension was filtered through Whatman No. 30 filter paper. The filtrate was made up to 100 ml with distilled water. The reducing sugar content of the filtrate was determined by Nelson-Somogyi (Nelson, 1944; Somogyi, 1952) method (Page No. 34). Soil without carboxymethylcellulose and sterilised soil with carboxymethylcellulose were run simultaneously as controls. The cellulase activity was expressed in terms of glucose released /g of soil/day.

**Ethanol production from cellulosic wastes.**

**Screening of litter degrading fungi for production of saccharification enzymes**

The fungi isolated in the present study were screened for the production of enzymes involved in saccharification process viz., amylase, cellulases and xylanases.

*Aspergillus awamori* NRRL 2276 obtained from United States Department of Agriculture (USDA), Illinois, USA was used as reference fungus.

**Inoculum**

The isolated fungi were grown on Czapek's (Dox) agar (Purvis *et al.*, 1964) plates for six days at room temperature. After the incubation period, the plates were flooded with sterile distilled water and the surface of the fungal growth was brushed with sterile camel hair brush. The suspension was filtered over a sterile filter
system. The spore concentration of the suspension was adjusted to $10^6$ spores/ml using sterile distilled water and then used as inoculum for further studies.

_Czapek (Dox) agar medium (g/l)_

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium chloride</td>
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</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
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</tr>
<tr>
<td>Sodium nitrate</td>
<td>2.0</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
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<tr>
<td>Magnesium sulphate</td>
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<tr>
<td>Dextrose</td>
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<tr>
<td>Yeast extract</td>
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<tr>
<td>Streptomycin</td>
<td>0.03</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
</tbody>
</table>

pH of the medium was adjusted to 6.5 to 7.0

_Enzyme production_

The ability of the fungi to produce saccharifying enzymes was assayed in minimal salts medium of Carter and Bull (1969).

_Minimal salts medium (g/l)_

<table>
<thead>
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<th>Ingredient</th>
<th>Amount</th>
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<tbody>
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<td>Urea</td>
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<tr>
<td>Magnesium sulphate</td>
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</tr>
<tr>
<td>Calcium chloride</td>
<td>0.05</td>
</tr>
<tr>
<td>Zinc sulphate</td>
<td>0.02</td>
</tr>
<tr>
<td>Manganese sulphate</td>
<td>0.02</td>
</tr>
<tr>
<td>Copper sulphate</td>
<td>0.005</td>
</tr>
<tr>
<td>Ferrous sulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>1.0</td>
</tr>
<tr>
<td>Ethylene diamine tetra acetate</td>
<td>0.6</td>
</tr>
<tr>
<td>Sodium dihydrogen orthophosphate</td>
<td>1.56</td>
</tr>
<tr>
<td>Peptone</td>
<td>7.5</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0</td>
</tr>
</tbody>
</table>
For screening studies, to the minimal medium, 10 g/l of starch or cellulose or xylan was added for amylase, cellulase and xylanase enzyme assays respectively. The experiments were conducted with 50ml medium inoculated with 10% (v/v) spore suspension (10⁶ spores/ml). The culture flasks were incubated for seven days on an orbital shaker (150 rpm) at room temperature. After the incubation period, the cultures were filtered over a Whatman No.1 filter paper. The residue was used for growth determination. The filtrate was centrifuged at 10,000 rpm for 20 min at 4°C and the clear supernatant was used as enzyme. Medium amended with 5% glucose served as control.

**Growth**

Growth of the fungus was determined in terms of mycelial dry weight, and total protein and chitin content of the mycelium.

**Dry weight**

For dry weight determination, the mycelial biomass was collected on a pre-weighed Whatman No.1 filter paper, dried to a constant weight at 60°C and reweighed. The difference in weight denoted the mycelial growth of fungus.

**Protein content**

For protein estimation, weighed portions of dry mycelium were digested overnight at 39°C in 0.66 N NaOH (1:6 w/v) (Phillips and Gordon, 1989). The digested samples were filtered through glass microfiber filters and the protein content of the filtrate was estimated by Lowry *et al.* (1951) method taking bovine serum albumin as standard.

**Reagent A**

Sodium carbonate 2% in 0.1N sodium hydroxide.

**Reagent B**

Copper sulfate 0.5% in 1% sodium tartrate.
Reagent C

To 50ml of solution A, 1 ml of solution B was added.

Folin's phenol reagent

The commercial Folin's phenol reagent was diluted in glass distilled water in the ratio of 1:2. This reagent was always prepared fresh whenever needed.

To 0.5 ml of the supernatant, 2ml of 10% trichloroacetic acid was added and incubated for 60 min. After the incubation period, the sample was centrifuged at 15,000 rpm for 30 min. The supernatant was discarded and to the pellet 5ml of solution C was added. After 10 min, 0.5ml of diluted Folin's phenol reagent was added and the tubes were shaken vigorously in a cyclomixer and incubated in dark for 30min for colour development. The intensity of colour developed was read at 500 nm in a spectrophotometer.

Chitin content

For chitin estimation, dried mycelia were hydrolyzed with 6N HCl for 4 h at 100°C (Phillips and Gordon, 1989). The acid-water was filtered and evaporated to dryness at 45-50°C. The dry hydrolysates were then redissolved in 10ml of distilled water. The resultant aminosugars in the hydrolysate solution was estimated by the method of Chen and Johnson (1983).

Reagents

(1) Acetyl - acetone reagent - 4% acetyl acetone in 1.25 N sodium carbonate (v/v).

(2) Ehrlich reagent - 1.6 g of N-N dimethyl para aminobenzaldehyde in 60 ml mixture of ethanol and conc. HCl (1:1 v/v).
To 1 ml of the hydrolysate in a teflon lined screw cap tube, 1 ml of acetylacetone reagent was added and heated at 90°C for 10 min. After cooling, 4 ml of 96% ethanol and 1 ml of Ehrlich reagent were added and left undisturbed for 45 min. Then the sample was read at 530 nm. D-glucosamine was used as standard.

**Enzyme assay**

**Amylase**

The culture filtrate obtained from starch supplemented basal medium was used as enzyme for amylase assay. To 0.5 ml of enzyme, 0.5 ml of 1% starch in 0.05 M, (pH 5.0) sodium citrate buffer (w/v) was added and incubated at 50°C for 30 min. The reaction was terminated by the addition of 1 ml of Nelson-Somogyi reagent.

**Cellulases**

The culture filtrate from cellulose amended basal medium was used for assay of cellulases.

**Filter paper activity**

For the assay of filter paper activity, Whatman No.1 filter paper strips were used as substrate. To a prewarmed suspension of 100 mg of substrate in 1 ml of 0.05 M sodium citrate buffer (pH 5.0), 1 ml of enzyme was added. This mixture was incubated in a waterbath at 50°C for 60 min. At the end of the reaction, 1 ml of Nelson-Somogyi reagent was added.

**Endoglucanase**

Carboxymethylcellulose (1% in 0.05 M sodium citrate buffer, pH 5.0) was used as substrate for endoglucanase assay. The substrate (0.5 ml) was incubated with enzyme (0.5 ml) at 50°C for 30 min and then the reaction was terminated by the addition of 1 ml of Nelson-Somogyi reagent.
Exoglucanase

For exoglucanase assay, 0.5 ml of 1% cellulose in 0.05 M sodium citrate buffer (pH 5.0) and 0.5 ml of enzyme were incubated at 50°C for 30 min. After 30 min, 1 ml of Nelson-Somogyi reagent was added to stop the reaction.

β-Glucosidase

β-glucosidase activity was measured by incubating 0.5 ml of 0.1% cellobiose in 0.05 M sodium citrate buffer (pH 5.0) with 0.5 ml of enzyme at 50°C for 30 min. The reaction was terminated by adding 1 ml of Nelson-Somogyi reagent.

Xylanase

The culture filtrate from oat spelt xylan amended basal medium was used for xylanase assay.

Endoxylanase

The endoxylanase activity was determined by measuring the amount of xylose released from oat spelt xylan. The assay mixture contained 0.5 ml of enzyme and 0.5 ml of 1% xylan suspension in 0.05 M sodium citrate buffer (pH 5.0). The assay mixture was incubated at 50°C for 30 min. The reaction was stopped by adding 1 ml of Nelson-Somogyi reagent.

In all the above enzyme assays, the amount of reducing sugars released was estimated by Nelson-Somogyi (Nelson 1944; Somogyi 1952) method. In amylase assay, maltose was used as standard and the enzyme activity was expressed as micromoles of maltose released /ml of culture filtrate (IU/ml). In cellulase assay, glucose was used as standard and the enzyme activity was expressed as micromoles of glucose released /ml of culture filtrate (IU/ml). In xylanase assay, xylose was used as standard and the enzyme activity was expressed as micromoles of xylose released /ml of culture filtrate (IU/ml).
**β-D xylosidase**

The β-D xylosidase activity was determined by measuring p-nitrophenol released from p-nitrophenyl β-D-glucopyranoside. The reaction mixture containing 0.5ml of p-nitrophenyl β-D-glucopyranoside (0.1% in 0.05 M sodium citrate buffer, pH 5.0) and 0.5 ml of enzyme was incubated for 30 min at 50°C. At the end of the reaction, 2 ml of 1M sodium carbonate solution was added and this mixture was made upto 5ml with distilled water. The developed yellow colour was measured at 430 nm. The enzyme activity was expressed as micromoles of p-nitrophenol released /ml of culture filtrate (IU/ml).

**Protease**

The reactants, 2ml of haemoglobin (1% in 0.05M sodium acetate buffer, pH 5.0) and 2ml of enzyme were incubated for 30 min at 50°C. The reaction was terminated by the addition of trichloroacetic acid (2ml, 10%). After mixing on a cyclomixer, the tubes were centrifuged and the protein content of the supernatant was assayed by Lowry et al. (1951) method (Page Nos. 45 and 46). The unit activity is defined as the absorbance measured at 450 nm by the action of 1 µg L-tyrosine under the assay conditions used.

**Production of ethanol from cellulosic wastes**

**Saccharification of cellulosic wastes**

**Preparation of enzymes**

Based upon the screening test, out of fifty three fungi, five fungi were selected for saccharification of cellulosic wastes. The fungi selected were *Aspergillus ficum, A.japonicus, A.oryzae, A.tamarii* and *Trichoderma koningii*. *Aspergillus awamori* NRRL 2276 was used as reference fungus. The cellulosic wastes, paddy straw, sugarcane bagasse, vegetable waste, municipal solid waste and leaf litter were used as substrates for ethanol production. The substrates were pretreated with 1N NaOH for 1h and washed with distilled water, dried at room temperature and stored.
in a dessicator. For saccharification of cellulosic wastes, the pretreated substrates (1% w/v) were amended as sole carbon source in the basal medium of Carter and Bull (1969) in place of glucose. The amended medium was inoculated with the spore suspension of test fungi (spore concentration - 10^6 spores/ml; inoculum volume - 10% v/v) and incubated on an orbital shaker (150 rpm) at room temperature for seven days. After the incubation period, the culture broth was filtered and centrifuged at 10,000 rpm for 20 min at 4°C. The clear supernatant was used as saccharification enzyme. The enzyme obtained from a particular substrate was used for the saccharification of the same substrate. For example, enzyme obtained from paddy straw amended medium was used for saccharification of paddy straw. The pretreated substrate suspended in 0.05M sodium citrate buffer, pH 5.0 (1% w/v) was treated with the enzyme (1 IU of FPA activity / ml) at 50°C. At regular time intervals samples were withdrawn and assayed for amount of reducing sugars released. The reducing sugar content was assayed by Nelson-Somogyi method (Page No.34). The rate of saccharification was calculated by the formula (Tewari et al., 1988).

\[
\text{Saccharification \%} = \frac{\text{Reducing sugars formed} \times 0.9}{\text{Cellulose content of the pretreated substrate}} \times 100
\]

**Fermentation**

The hydrolysates obtained after saccharification processes were further fermented to ethanol by employing the yeast *Saccharomyces cerevisiae* and the bacterium *Zymomonas mobilis*.

The yeast strain *S. cerevisiae* was obtained from Sakthi Sugars Ltd., Bhavanisagar, Tamil Nadu, India and was maintained on YPD agar slants. (Tantirungkij et al., 1994).
**YPD agar medium (g/l)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

For fermentation with yeast, the liquid medium proposed by Barron et al. (1995) having the following composition was used.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen</td>
<td></td>
</tr>
<tr>
<td>phosphate</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Hydrolysate from</td>
<td></td>
</tr>
<tr>
<td>saccharification process</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The bacterial strain *Z. mobilis* ATCC 10988 was obtained from microbial type culture collection, Chandigarh, India. The stock culture was maintained on RM agar slants (Gunasekaran and Kamini, 1991) having the following composition (g/l).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>20.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen</td>
<td></td>
</tr>
<tr>
<td>phosphate</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

**pH of the medium was adjusted to 6.0**

For fermentation studies, the medium having the following composition was used (IMTECH, 1990).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Diammonium sulphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Potassium dihydrogen</td>
<td></td>
</tr>
<tr>
<td>phosphate</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Hydrolysate from</td>
<td></td>
</tr>
<tr>
<td>saccharification process</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>
Fermentation was carried out by batch process employing free cells and immobilised cells.

Fermentation by free cells

For fermentation of hydrolysates by free cells of *S. cerevisiae* and *Z. mobilis*, to 90 ml of the fermentation medium 10 ml of the primary inoculum (100 mg dry cells / 100ml) was added and the fermentation flasks were incubated at 30°C. At every 24 h interval, samples were removed and analysed for ethanol (Caputi et al., 1968) and residual sugar contents (Page No.34).

Ethanol estimation

To 1ml of the hydrolysate, 24 ml of distilled water was added and the diluted hydrolysate was distilled at 78°C. The distillate (3ml) was collected in a flask containing 25ml of chromic acid. This mixture of acid and alcohol was made up to 50 ml and kept in a water bath at 60°C for 30 min. After 30 min, the contents were cooled and the colour intensity was measured at 600nm in spectrophotometer. Calibration curve was drawn using ethanol as standard.

Fermentation by immobilised cells

*S. cerevisiae / Z. mobilis* (1.5g dry weight) were thoroughly mixed with sodium alginate (3.5%, w/v) and sodium chloride (0.1%, w/v) and the resulting slurry was made into spherical beads of 3 to 4 mm diameter by injecting the slurry into calcium chloride solution (4%, w/v). The beads formed were kept in the calcium chloride solution for 1h for gelatination and then washed with sterile distilled water. Before use, the beads were activated by incubating in the corresponding fermentation medium containing 10% dextrose, instead of hydrolysate, for 2 h at 30°C.

For fermentation of the hydrolysates, varying concentrations (2 to 10% w/v) of the immobilised beads were inoculated into the corresponding fermentation medium and the fermentation flasks were incubated at 30°C. At every 12 h interval,
samples were drawn from the flasks to estimate the ethanol content (Page No.52) and residual sugar content (Page No.34).

Biobleaching and delignification of Hardwood Kraft Pulp (HWKP) by xylanases

*Production and purification of xylanase*

For the production of xylanase enzyme, the selected fungi were grown in Carter and Bull (1969) medium amended with oat spelt xylan for seven days at room temperature on an orbital shaker (150 rpm). After the incubation period, the mycelial mat was filtered out and the culture filtrate was centrifuged at 10,000 rpm for 20 min at 4°C. The clear supernatant was the crude enzyme preparation. The crude enzyme was purified by the method proposed by Keskar *et al.* (1989) in a stepwise process.

*Step-I*

The culture filtrate (200ml) was concentrated by precipitating the enzyme with 3 volumes of chilled ethanol. The precipitate was collected by centrifugation at 9000g for 30 min. and dissolved in 40ml of 10mM sodium phosphate buffer, pH 8.0. The undissolved solid particles were removed by centrifugation.

*Step-II*

The clear supernatant was further purified on DEAE cellulose by batch treatment. A 130 ml portion of DEAE cellulose (40mg/ml; equilibrated with 10mM sodium phosphate buffer, pH 8.0) was suspended in 40 ml of enzyme solution for 30 min. The slurry was filtered through Whatman No.1 filter paper. The filtrate was collected and the DEAE cellulose 'cake' was resuspended twice, each time with 10ml of the same buffer. The filtrate and washings were pooled together.

*Step-III*

A 15 ml of the pooled enzymes was applied to Sephadex G-50 column (1.5 x 45 cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.2 and eluted with the same buffer. The fractions were collected at the rate of 10ml/h.
Step-IV

Xylanase activity (Page No.48) and protein content (Page Nos.45 and 46) in each fractions were determined and the active fractions were pooled and concentrated by freeze drying.

Enzyme characterization

Optimum pH

To determine the optimum pH for maximum enzyme activity, universal buffers (citrate/phosphate) of pH 3.0 - 9.0 were used. The enzyme activity was estimated in this pH range.

Optimum temperature

Xylanase activity was measured in the temperature range of 20-90°C to determine the optimum temperature for maximum enzyme activity.

Thermostability

Thermostability of the enzyme was estimated at 37° and 60°C

Determination of Vmax and Km

A constant volume of enzyme was incubated with different concentrations of oat spelt xylan (0-32 mg/ml in 0.05 M sodium citrate buffer; pH 5.0) at 50°C for 30 min. Initial reaction rate was measured at each concentration. The values of maximum velocity (Vmax) and Michaelis constant (Km) were determined from Line Weaver - Burk plot (Line Weaver and Burk, 1934) and Eadie Hofstee plot.

Determination of pI value

The pI values of the active enzyme fractions were determined by isoelectric focussing (IEF) technique (O' Farrell, 1975).
Reagents

Monomer for focusing gel (40% T, 5% C)

- Acrylamide - 8.0 g
- Bisacrylamide - 2.0 g
- Water to - 25 ml

Stored at 4°C in the dark

Gel solution

- Urea - 5.5 g
- Monomer solution - 1 ml
- NP 40 (10%) - 2 ml
- Ampholyte 3/10 - 0.5 ml
- Water - 2.5 ml

Ammonium persulphate solution - 10%

-N,N,N',N' - Tetramethyl ethylene diamine (TEMED)

2-Mercaptoethanol

Sample overlay solution (9M urea, 2% ampholytes)

- Urea (ultrapure) - 5.5 g
- Ampholytes 3/10 - 0.5 ml

(Pharmalyte 2D, 40%)

- Water to - 10 ml

IEF sample buffer

- Urea - 2.85 g
- Nonidet P 40 (10%) - 1 ml
- Pharmalyte 2D - 250 μl
- 2-Mercaptoethanol - 250 μl
- Water to - 5 ml

Anode solution

10mM phosphoric acid
**Cathode solution**

10mM sodium hydroxide solution, degassed and stored in vacuum.

**Treatment buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris - HCl</td>
<td>62.5mM</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate</td>
<td>2%</td>
</tr>
<tr>
<td>Glycerol</td>
<td>10%</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>5%</td>
</tr>
<tr>
<td>pH</td>
<td>6.8</td>
</tr>
</tbody>
</table>

**Stain solution**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coomassie brilliant blue R-250</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>40 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Water</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

The dye was first dissolved in methanol and then mixed with acetic acid and water. The stain solution was filtered before use.

**Destainer**

As above without dye

**Standards**

A standard of Bio-Rad pI set was used as pI markers

**Preparation of IEF gel**

Acid cleaned glass tubes (3mm inner diameter) were sealed at one end with parafilm and placed on casting stand. The gel solution was mixed in a sidearm flask and deaerated under vacuum. 10μl of ammonium persulphate (10%) and 8μl of TEMED were mixed and gel mix was poured into the glass tubes leaving about 1cm space at top. The space left was carefully layered with water and allowed to polymerise for several hours.
Running of gel

The water on the top of the gel was removed and the tubes were placed in the upper chamber of the gel apparatus. The anode solution was poured into the lower buffer chamber and the cathode solution was poured in the upper chamber of the apparatus. Any air bubble if present at the bottom of the tubes were removed by a syringe with a long curved needle. A little base was squirited on top of the gel and 5μl of sample overlay was added. The gel was prerun at 200 volts for 15 min, at 300 volts for 30 min and at 400 volts for 30 min. The cathode solution was removed and fresh cathode solution was added on the upper chamber. The samples were mixed in sample buffer and loaded on the top of the gel. 5μl sample overlay was loaded on the top and the gel was run for 16h at 400 volts constant. Next day, the power was increased to 800 volts for one hour and then the power supply was disconnected. Water was squirited between the gel and glass using a syringe fitted with 26 gauge long needle, and the gels were carefully taken into screw cap tubes with 10 ml treatment buffer. The tubes were gently shaken on a rocking platform for about 2h to get proteins in the gel equilibrated with SDS and 2-mercaptoethanol.

Staining

The gels were immersed in the staining solution for overnight with uniform shaking. After staining, the gels were transferred to a plastic container with 200-300 ml of destaining solution and shaken gently and continuously. Dye that was not bound to proteins was thus removed. The destainer was changed frequently until the background of the gel become colourless. The proteins fractioned appeared as blue colour bands. The gels were photograhed and stored in polyethylene bags after drying in vacuum.

Determination of molecular weight

The molecular weights of the purified xylanase enzymes were determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).
Reagents

**Stock acrylamide solution**
- Acrylamide: 30 g
- Bisacrylamide: 0.8 g
- Water to 100 ml

**Separating gel buffer**
- Tris-HCl: 22.7 g
- Water to 100 ml
- pH: 8.8

**Separating gel (10%)**
- Stock acrylamide solution: 13.3 ml
- Separating gel buffer: 8.0 ml
- Water: 18.1 ml

The gel mixture was degassed on a water pump for 3-5 min and then following were added:
- Ammoniumpersulphate solution (5%): 0.2 ml
- Sodium dodecyl sulphate (SDS) solution (10%): 0.4 ml
- N, N,N',N" - tetramethylene diamine (TEMED): 20 μl

**Stacking gel buffer**
- Tris-HCl: 7.6 g
- Water to 100 ml
- pH: 6.8

**Stacking gel mixture (4%)**
- Stock acrylamide solution: 1.35 ml
- Separating gel buffer: 1.0 ml
- Water: 7.5 ml
The gel mixture was degassed for 3-5 min on a water pump and added with the following solutions:

- **Ammoniumpersulphate solution (5%)** - 50 μl
- **SDS solution (10%)** - 100 μl
- **TEMED** - 10 μl

**Electrode buffer**
- **Tris-HCl** - 12 g
- **Glycine** - 28.8 g
- **SDS** - 2 g
- **Distilled water** - 2 l
- **pH** - 8.2 - 8.4

**Sample buffer (5x concentration)**
- **Stacking gel buffer** - 5 ml
- **SDS** - 0.5 g
- **Sucrose** - 5 g
- **Mercaptoethenol** - 0.25 ml
- **Bromophenol blue**
  - (0.5% w/v solution in water) - 1 ml
- **Water to** - 10 ml

**SDS solution - 10%**

**Standard marker proteins**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (Daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Lactalbumin from bovine milk</td>
<td>14,200</td>
</tr>
<tr>
<td>Trypsin inhibitor from soybean</td>
<td>20,100</td>
</tr>
<tr>
<td>Trypsin PMSF from bovine pancreas</td>
<td>24,000</td>
</tr>
<tr>
<td>Carbonic anhydrase from bovine anthrocytes</td>
<td>29,000</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle</td>
<td>36,000</td>
</tr>
<tr>
<td>Albumin, egg</td>
<td>45,000</td>
</tr>
</tbody>
</table>
The above marker protein samples were dissolved (1 mg/ml) in sample buffer. For loading the well, 25 μl was used.

Silver staining

Washing solution

One ml of formaldehyde (37%), was mixed with 40 ml of methanol and 60 ml of distilled water.

Staining solution

Silver nitrate solution - 0.1%

Developer

Sodium carbonate solution
(3 g in 80 ml) - 80 ml

Sodium thiosulphate solution
(200 mg/l of water) - 1.0 ml

Formaldehyde - 1.0 ml

Water to - 100 ml

Stopper

Acetic acid solution - 5%

Thoroughly cleaned and dried glass plates and spacers were assembled properly and held together with bulldog clips in an upright position. White petroleum jelly or 2% agar (melted in a boiling water bath) was applied around the edge of the spacers to hold them in place and to seal the chamber between the glass plates.

The separating gel solution was poured in the chamber between the glass plates, layered on top with distilled water and left undisturbed for 30-60 min. for polymerisation. After polymerisation, the water layer was removed and the surface
was washed with a little stacking gel solution. Then the stacking gel mixture was poured on the top and comb was placed in the stacking gel. The gel was left for setting (30-60 min). Once the stacking gel was polymerised, the comb was removed without disturbing the well and the gel was installed in the electrophoresis apparatus after removing the clip and agar. The tanks were filled sufficiently with electrode buffer and any air trapped at the bottom of the gel was removed.

The protein content of the samples were made uniform by using 5x concentration of sample buffer. The samples were heated in a boiling water bath for 2-3 min. After cooling, 10 μl of sample was injected into the sample well using a microsyringe through the stacking gel and then run at 30mA until the bromophenol blue (marker dye) reaches the bottom of the gel. After the run was complete, the gel was removed carefully from the plates, transferred to a clean plastic container and washed with washing solution with slow shaking for 10 min. The wash solution was discarded and the gel was rinsed with plenty of water for 2 min and soaked in sodium thiosulphate solution (0.2%) for 1-2 min. Then the gel was washed twice with water, each time 1-2 min, drained and soaked in silver nitrate solution (0.1%) for 10 min with gentle shaking and then again washed twice in water. The washed gel was then soaked in developer solution with gentle shaking. The proteins reduced the silver nitrate to silver and yellow to dark brown colour bands appeared on the gel. When sufficient intensity of band developed, the reaction was stopped by adding acetic acid solution. The gel was then photographed and stored in polyethylene bags.

**Enzyme pretreatment of hardwood kraft pulp**

Unbleached pulp of *Eucalyptus grandis* wood was obtained from Tamil Nadu Newsprint Limited (TNPL), Kagithapuram, Tamil Nadu, India.

**Enzyme pretreatment**

The pulp was subjected to enzyme pretreatment before the conventional alkaline treatment and chlorine extraction sequence (EDED). The pulp was treated with various concentrations of xylanase enzyme (250, 500, 1000 IU/g dry pulp) at
2.5% pulp consistency and 50°C for 24h. After the treatment, the pulp was filtered and the supernatant was assayed for pH, reducing sugar (Page No. 34) and carbohydrate contents (Page No. 35). A portion of the residue was used for preparation of hand sheets. The kappa number and brightness of the handsheets were determined.

**Alkaline treatment**

After enzyme pretreatment, a portion of the pulp (2.5% consistency) was washed with excess of distilled water, dried and used for alkaline treatment. The pulp was treated with 0.03 g NaOH/g dry pulp at 80°C for 1h (Christov and Prior, 1994). After alkaline treatment, the pulp was filtered and dried. Handsheets were prepared from the dried pulp. The kappa number and brightness of the hand sheets were determined.

**Chlorine treatment**

Another portion of the enzyme pretreated pulp was subjected to conventional chlorine extraction sequence (EDED process) as per the following table (Buchert et al., 1992).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Consistency (%)</th>
<th>Temp (°C)</th>
<th>Time (min)</th>
<th>Final pH</th>
<th>Active chlorine charge</th>
<th>NaOH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D50/C50</td>
<td>3</td>
<td>45</td>
<td>0.5/44.5</td>
<td>1.8-4.9</td>
<td>5.14*</td>
<td>-</td>
</tr>
<tr>
<td>E₁</td>
<td>10</td>
<td>60</td>
<td>60</td>
<td>11-11.2</td>
<td>2.0*</td>
<td>2.6</td>
</tr>
<tr>
<td>D₁</td>
<td>10</td>
<td>60</td>
<td>180</td>
<td>3.7-4</td>
<td>2.0ᵇ</td>
<td>-</td>
</tr>
<tr>
<td>E₂</td>
<td>10</td>
<td>60</td>
<td>60</td>
<td>10.9-11.1</td>
<td>0.9</td>
<td>0.9ᵇ</td>
</tr>
<tr>
<td>D₂</td>
<td>10</td>
<td>70</td>
<td>180</td>
<td>3.7-4.0</td>
<td>0.8*</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.4ᵇ</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.94*</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.60ᵇ</td>
<td></td>
</tr>
</tbody>
</table>
The reference pulps were bleached with a chlorine multiple of 0.18 and enzymatically treated pulps with a chlorine multiple of 0.15. The chlorine multiple is the active chlorine charge (percentage) in prebleaching (D/C stage) condition divided by kappa number of the pulp. After every bleaching, the pulp was thoroughly washed. The chemicals were dosed as percentage per dry weight pulp. After bleaching, hand sheets were prepared and kappa number and brightness of the hand sheets were determined.

**Deinking of waste papers by xylanase enzyme**

The waste papers, inkpapers, photocopy papers and newspapers were made into pulp. The pulp with 2.5% consistency was treated with xylanase enzyme (500 IU/g dry pulp) at 50°C for 24 h. After the treatment, the pulp was filtered and hand sheets were prepared. Kappa number and brightness of the hand sheets were determined.

After each treatment, the pH, reducing sugar content (Page No. 34) and carbohydrate content (Page No.35) of the leachate were assayed.

**Preparation of hand sheets**

To prepare the hand sheets (2x4 cm size), the pulp suspension was filtered through a Buchner funnel under vaccum. The residue was blotted and air dried for 24h.

**Kappa Number (TAPPI, 1990)**

Kappa number is used as a criteria for the lignin content of pulps and is determined as the volume of 0.1 N potassium permanganate (ml) consumed by 1g of
moisture free pulp. Small pieces of hand sheets were weighed out which will consume approximately 50% of pottasium permanganate solution (1%). A second specimen was weighed out to determine its moisture content. The test specimen was disintegrated in 500ml of distilled water until free of fibre clots and undispersed fibre bundles. The disintegrated specimen was made upto 800 ml. To 100ml of pottasium permanganate solution (0.1N), 100ml of sulphuric acid (4N) was added, cooled to 25°C and immediately added to disintegrated test specimen. After 10 min, the reaction was stopped by adding 20ml of pottasium iodide solution (1N) and titrated against sodium thiosulphate solution (0.2N). Starch solution (0.2%) was used as the indicator. A blank titration was carried out in the same manner but without pulp.

The kappa number was calculated by the formula

\[ K = \frac{p \times f}{W} \]

and \[ p = \frac{(b-a) N}{0.1} \]

where
- \( K \) = kappa number
- \( p \) = amount of 0.1 N \( \text{KMnO}_4 \) consumed by the sample (ml)
- \( f \) = factor for correction
- \( w \) = weight of moisture free pulp in the specimen in g
- \( b \) = amount of thiosulphate consumed in blank determination (ml)
- \( a \) = amount of thiosulphate consumed by sample (ml)
- \( N \) = Normality of thiosulphate

Correction for reaction temperature

\[ K = \frac{pf}{W} \left[ 1 + 0.013 (25 - t) \right] \]

where,
- \( t \) = actual reaction temperature in degree celsius.

**Brightness**

Brightness of the handsheets were measured at 457 nm in a Perkin Elmer \( \lambda 3 \beta \) spectrophotometer equipped with reflectance sphere.