CHAPTER II

MATERIALS AND METHODS
Methods:

Organism and its maintenance:

The fungus used in this study was *Monocillium indicum* Saxena. The culture was maintained on a modified Sabouraud's agar medium containing glucose, 2%; peptone, 1%; NaCl, 0.25%; agar, 3%; pH 5.5.

Screening for phenol oxidase producing fungi:

Fungal cultures were isolated from a paper mill soil sample on the basis of a positive Bavendamm reaction (Nobles, 1965) on tannic acid containing malt agar plates (malt extract, 1.5%; tannic or gallic acid, 0.5%; agar, 3.0%). Cultures producing phenol oxidase exhibit a dark brown zone around them. An identical reaction was obtained when the fungi were grown on Sabouraud's agar plates containing o-dianisidine (0.01%). One of them, showing a significant zone of coloration was selected for further studies and was identified as *Monocillium indicum* Saxena by International Mycological Institute, Surrey, U.K. (IMI Herbarium No. 329787).

Culture Conditions:

The basal medium routinely used for growth and laccase production contained (gm/l): KH$_2$PO$_4$, 0.2; CaCl$_2$, 2H$_2$O, 0.0132; MgSO$_4$, 7H$_2$O, 0.05; ammoniacal ferric citrate, 0.085; ZnSO$_4$, 7H$_2$O, 0.0462; MnSO$_4$, H$_2$O, 0.035; CoCl$_2$, 6H$_2$O, 0.007; CuSO$_4$, 5H$_2$O, 0.007; L-asparagine, 1.0; ammonium nitrate, 0.5; thiamine-HCL,
The medium was supplemented with Tween-80 (0.01% v/v) and buffered with 20 mM phthalate buffer pH 5.0. To obtain a suitable inoculum, 7 day old culture from a roux bottle was macerated using glass beads in 150 ml sterile saline and used at 20% (v/v) concentration. All experiments were carried out in 250 ml Erlenmeyer flasks containing 20 ml of medium and incubated at 28 ± 2°C under static conditions. In specific experiments, where the effect of various medium components was investigated, all test media were prepared in triplicates. Cultures were harvested at suitable intervals.

The individual carbon sources and lignin (Indulin AT, Sigma) were added at a concentration of 1% and 0.1% respectively. In experiments carried out to study the effect of nitrogen, basal medium did not contain ammoniacal ferric citrate and yeast extract; and only the specific nitrogen source was added to give a concentration of 30 mM nitrogen.

For studies on cellulase production by *M. indicum*, the fungus was grown in 100 ml of Mary Mandels medium (1969) containing 1% amorphous cellulose (Sigma) as the sole carbon source in 250 ml conical flasks on a shaker. Cultures were harvested periodically, filtered to remove the mycelium and the extracellular culture filtrate was used for assaying cellulases.

**Growth on lignin related aromatic compounds:**

The medium used to study response of *M. indicum* to lignin related aromatic compounds, contained the following mineral salts (gm/l) : \( \text{Na}_2\text{HPO}_4 \), 0.8; \( \text{KH}_2\text{PO}_4 \), 0.2; \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \),
0.2; (NH₄)₂SO₄, 0.5; CaCl₂, 2H₂O, 0.1; NaCl, 0.05; FeSO₄·7H₂O, 0.01, pH 5.5. The aromatic compounds were incorporated individually in the mineral medium at 0.1% (w/v) concentration. The aromatic compounds used were ferulic, vanillic, syringic, benzoic, cinnamic, caffeic, protocatechuic and p-hydroxy benzoic acids, catechol, vanillyl alcohol, syringaldehyde, veratraldehyde, guaiacol, vanillin, cinnamyl alcohol and cinnamaldehyde. Inoculated media were incubated on a shaker at 28 ± 2°C and growth was checked after 5 days.

Growth was determined in terms of mycelial dry weight after filtering the mycelium through a cheese cloth and drying the mycelium till constant weight was attained.

Enzyme Assays:

**Laccase (EC. 1.10.1.5)**: Laccase activity was assayed using 2.8 ml of 0.34 mM o-dianisidine (Sigma) as substrate in 0.1 M sodium acetate buffer, pH 5.0. The reaction was initiated by the addition of 200 μl of appropriately diluted enzyme and the increase in absorbance was monitored at 460 nm spectrophotometrically (Uvikon, Kontron).

With guaiacol (Aldrich) as substrate, 200 μl of enzyme was added to 2.8 ml of 0.02% guaiacol dissolved in 100 mM acetate buffer, pH 5.0, and the increase in absorbance at 440 nm was proportional to laccase activity.

One unit of laccase is defined as the amount of enzyme required to cause a change in absorbance of 1.0 per min at 28 ±
Cellulase Assay:

1. **Filter paper activity (Fp activity)**: Fp activity was assayed using 50 mg of Whatman filter paper No. 1 by the method of Mandels et al. (1976). One unit of Fp activity is defined as umoles of reducing sugar liberated per min per ml at 50°C.

2. **Endo-β-glucanase or Cx activity (EC. 3.2.1.4.)**: Endo-β-glucanase activity was measured by the method of Mandels and Weber (1969) using 1% carboxymethyl cellulose (Sigma). The number of units equals to umoles of reducing sugar liberated per ml per min at 50°C.

3. **β-glucosidase (EC. 3.2.1.21.)**: β-glucosidase was assayed using PNPG (Sigma) as the substrate by the method of Berghem and Petterson (1973). One unit of β-glucosidase is defined as the amount of enzyme required to release one umole of PNP under assay conditions.

**Lignin peroxidase (LiP)**: LiP was assayed with veratryl alcohol (Aldrich) as the substrate (Tien and Kirk, 1984).

**Manganese-dependent peroxidase (MnP)**: MnP was assayed with phenol red by the method of Kuwahara et al. (1984).

**Protocatechuate 3,4-dioxygenase (EC. 1.13.11.3.)** was assayed by following the decrease in absorbance at 280 nm. The assay mixture consisted in μmoles: phosphate buffer (pH 7.0) 150; protocatechuate (Sigma) 0.5; and appropriate amount of enzyme in a final volume of 3.0 ml. Reaction was initiated by addition of
substrate. One unit was defined as the amount of enzyme required
to bring about a decrease of 0.1 OD/min at 290 nm at 28 ± 2°C.

**Protocatechuate 4,5-oxygenase** activity was assayed by the method

**Catechol 2,3-dioxygenase (EC. 1.13.11.2)**: The enzyme was assayed
for by the method of Nozaki et al. (1970). Again the unit was
defined as the amount of enzyme required to bring about a change
of 0.1 OD/min at 28 ± 2°C.

**Purification of Laccase**:

All operations of enzyme purification were performed at
10-12°C. Laccase from a 6 day old culture filtrate was
precipitated by ammonium sulphate between 50 to 85% saturation
and the precipitate was collected by centrifugation (20,000g for
30 min). The precipitate was dissolved in a small volume of 0.05
M acetate buffer pH 5.0 (buffer A) and dialyzed for 24 hr.
against two changes of similar buffer to remove the ammonium
sulphate.

**Ion-exchange chromatography**: The ammonium sulphate concentrate
was then loaded onto DEAE-cellulose (Sigma) which had been
previously equilibrated with buffer A for charging. The enzyme
was left for adsorption on DEAE cellulose for 2 hrs on a magnetic
stirrer, and then loaded onto a column. Several column volumes
of buffer A were then passed through to remove any unbound
enzyme. The enzyme was eluted with a linear 0 to 1.0 M gradient
of NaCl. 10.0 ml fractions were collected. The enzyme activity
and protein content of these fractions were determined as
mentioned elsewhere in materials and methods. The concentration of free chloride ions was determined using an ion selective electrode (Orion Research Inc., Cambridge, MA, USA) which had been standardized using different concentrations of KCL (10^{-4} to 10^{-2}) in buffer A. The fractions showing activity were pooled, dialyzed and concentrated by reverse dialysis.

**Preparative Polyacrylamide gel electrophoresis (Preparative-PAGE)**: For most of the studies the ammonium sulphate precipitate after dialysis was subjected to 8% preparative PAGE in an LKB vertical slab gel electrophoresis unit using 3 mm spacers. Proteins were separated at a constant current of 60 mA. Once the run was complete, a small section was cut from the length of the gel and stained for activity. When the activity bands had developed, the sliced gel was aligned with the remaining part and the portion corresponding to the activity band was cut and frozen. The enzyme was extracted by centrifugation after grinding the gel matrix with glass powder and suspending in buffer A. The enzyme was concentrated either by lyophilization or reverse dialysis with PEG 2000 (Sigma).

**Enzyme characterization**: The major laccase band purified by preparative PAGE was used for characterization.

**Relative molecular weight determination**: The molecular weight of the major laccase band was determined by Fast Protein Liquid Chromatography (Pharmacia, Sweden), using Sephacryl-S-200 column (48 x 1.3 cm) equilibrated with buffer A. Elution was carried
out at a flow rate of 1.0 ml/min. The standard molecular weight marker proteins (Sigma) used were β-amylase (200,000); alcohol dehydrogenase (150,000); bovine serum albumin (45,000); carbonic anhydrase (29,000) and cytochrome C (12,400). The void volume was 30 ml and the bed volume, 92 ml. Fractions of 3.0 ml were collected.

**pH activity optimum**: The assay system consisted of 2.9 ml of 0.34 mM o-dianisidine in 0.1 M of HCL-KCl buffer, pH 1.0 and 2.0; citrate buffer, pH 3.0 and 4.0; acetate buffer, pH 5.0 and 6.0; or phosphate buffer, pH 7.0 and 8.0. Reaction was initiated by the addition of 100 μl (2.0 units) of enzyme and increase in absorbance was monitored at different pH values.

**Temperature stability**: Temperature stability of the laccase was investigated from temperatures between 20 to 80°C. Enzyme, 8.2 units in 1.0 ml of buffer A was incubated at each temperature tested for 10 min. Residual activity was then measured under standard assay conditions.

**Substrate specificity**: Activity of laccase towards a variety of substrates was determined polarographically by incubating 1.9 ml of 1.0 mM substrate in buffer A with 25 μl of the enzyme. The oxygen uptake during each of the reaction was monitored on an oxygraph (Gilson, France). Activities were calculated as ppm O₂ consumed/min/mg protein.

**Gel electrophoresis**: Samples containing 35-45 μg of protein unless otherwise stated, were subjected to 10% sodium dodecyl
sulphate (SDS)-PAGE or 8% native PAGE using the discontinuous Tris-glycine system of Laemmli (1970). Molecular weights on SDS-PAGE were calculated from Sigma SDS-6H standards. Lithium dodecyl sulphate gels (LDS) were run by the method of Evans et al. (1984). pI values of the major laccase band was determined by iso-electric focusing in 140 x 2.7 mm id tubes in 5% polyacrylamide gel rods (T 5% and C 3%) containing pharmalyte 3-10 (Pharmacia), with a Pharmacia gel electrophoresis apparatus GE-2/4 LS. Focusing was done for 10-12 hrs at 500 V. Gels were calibrated with a broad pI calibration kit of pI 3.5 to 9.3 (Pharmacia, Sweden). Casting, preparation of sample, focusing and staining of the gels were carried out as described in Pharmacia IEF manual.

Staining of electrophoresis gels: Protein bands on native PAGE, SDS-PAGE and IEF gels were stained with Coomassie brilliant blue R-250. Laccase on native and preparative PAGE was visualized by activity staining using 0.34 mM o-dianisidine as substrate in buffer A. Glycoprotein staining was carried out by the method of Zacharius et al. (1969) using Schiff's reagent. Peroxidase activity on LDS gels was visualized using either 3-amino-9-ethylcarbazole or 3,3',5,5'-tetramethylbenzidine as described by Evans et al. (1984).

Immunoblotting: The major laccase band of M. indicum was immunoblotted with specific antibodies to LiP of P. chrysosporium (Gallagher et al., 1989); laccase of A. bisporus (Gallagher, 1989) and C. versicolor (Evans, 1985) and to a synthetic
polypeptide sequence of 15 amino acids of the N-terminal end from
*A. bisporus* laccase (Gallagher, 1989). The sequence of the
synthetic polypeptide was DTKTFFNDLVNTRLA. The
proteins were separated on 10% SDS-PAGE and then transferred to
nitrocellulose by 400 mA for 2 hr in a Bio-Rad Trans Blot cell at
12-15°C. Blocking, incubation with first and second antibody and
washings were performed as described for an enzyme-linked
detection system (Bio-Rad Immuno Blot).

**Assay for [lignin-\(^{14}\)C] lignocellulose degradation:**

\[^{14}\]C\)-lignin labelled wheat straw (*Triticum aestivum*)
having a specific activity of 186 Bq/mg dry weight was a gift
from Dr. Etienne Odier, France. Extractive free lignocellulose
was prepared according to Crawford and Crawford (1978) wherein
the labelled plant material was extracted by the following
procedure to remove unincorporated phenylalanine and other
extractable compounds: (i) washed with water at 80°C for 4 hr;
(ii) refluxed in 1:1 benzene-ethanol for 4 hr, repeated once;
(iii) refluxed with ethanol for 2 hr, repeated until the ethanol
remained colorless; and (iv) washed with water at 80°C for 2
hrs, repeated once. Extracted lignocelluloses were dried
overnight at 50°C. Approximately 10 mg of [lignin-\(^{14}\)C]
extractive free lignocellulose (2.3 x 10\(^4\) cpm) and 10 mg of
similarly treated unlabelled wheat straw were added to 150 ml
conical flasks each containing 20 ml of basal medium. 20 ml of
macerated mycelia was added as inoculum. Flasks were sealed and
incubated at 28 ± 2°C. Cultures were periodically flushed with
sterile air and the evolved \(^{14}\)CO\(_2\) trapped in 5.0 ml of
ethanolamine containing scintillation cocktail. The cocktail consisted of equal parts of toluene (4.0 gm PPO + 0.1 gm of POPOP per litre of toluene) and anhydrous methanol:ethanolamine (4:1 v/v). Counting was done in an LKB B1000 liquid scintillation counter. The results shown are an average of 5 sets.

**DNA extraction and Southern hybridization:** *M. indicum* genomic DNA was extracted from fresh mycelia harvested from 6 day old liquid culture by the method of Renno et al. (1990). Digestion of DNA with Bam HI (BRL, USA) was carried out according to the manufacturer’s manual. The digested DNA was transferred onto nylon membrane (Amersham Int., U.K.) by vacuum (Vacuo-Blot system, LKB, Sweden) from the agarose gel. Southern hybridization was performed as described by Renno et al. (1990). The random primer labelling kit (Rapid Multiprime DNA labelling, Amersham Int., U.K.) was used for labelling the probe according to the manufacturer’s instruction. The probe was obtained from Dr. Renno (Polytechnic of Central London, London) which comprised of a nucleotide sequence from 210 to 1770 from the gene for LiP isozyme H8 from *P. chrysosporium* (Accession No. for the gene bank, X 06689, Smith et al., 1988).

**Isolation and identification of ferulic/vanillic acid degradation pathway intermediates:** The culture supernatant of ferulic/vanillic acid grown *M. indicum* was acidified to pH 2.0 with 2N H₂SO₄ saturated with NaCl, extracted twice with diethyl ether and the organic phase was dried over anhydrous sodium sulfite.
Thin layer chromatography (TLC) analysis: The organic layer was evaporated to a small volume and spotted on 0.25 mm thick silica gel-G (E. Merck, India) plates for qualitative analysis. The solvent systems used were: benzene, toluene, acetic acid, [BzTo] (40: 40: 20) or benzene, dioxane, acetic acid [BzDo] (90: 25: 4). Following development, chromatograms were examined under UV light and then sprayed with diazotized sulfanilic acid (Walker and Taylor, 1983) or a mixture of 0.3% FeCl₃ and 0.3% K₃Fe(CN)₆ 1:1 (W/V).

Individual chromatogram spots were purified by preparative TLC, eluted with ether and their UV spectra compared to those of authentic standards.

High pressure liquid chromatography (HPLC) analysis: The residual concentration of ferllic acid and presence of intermediates was determined by reverse phase HPLC with a Zorbax -ODS C₁₈ column (4.65 mm x 25 cm, Shimadzu, Japan). The operations were carried out at 28 ± 2°C at a flow rate of 1.0 ml/min. For estimation of ferulic acid, a mixture of water:methanol (60:40), pH adjusted to 5.0 with dilute phosphoric acid was used and detection was done at 280 nm on a spectrophotometric detector (SPD detector, Shimadzu, Japan).

Preparation of cell-free extracts (CFE): Ferulic or vanillic acid grown pellets of M. indicum were harvested by filtering through a cheese cloth and washed with 20 mM phosphate buffer (pH 7.0). The pellets were ground using glass powder by repeated
freezing and thawing. The cell paste was suspended in a minimum volume of the above buffer and centrifuged at 25,000 g at 4°C for 30 min. The resulting supernatant was taken for assay.

Microscopic examination: For examination, the fungal mycelium was stained with lacto-phenol blue and observed under Polyvar microscope (Reichert Jung, Austria).

Analytical methods:

Protein was measured by the method of Bradford, (1976) using bovine serum albumin as standard.

Reducing sugar in the medium was measured by Bernfield's (1955) method using glucose as standard.

The carbohydrate content of the enzyme was determined by the phenol sulfuric assay (Dubois et al., 1956) using glucose as standard.

Kappa No.: Residual lignin in pulps was determined from permanganate consumption by use of a standard method (TAPPI, 1976), and is reported as "Kappa number" equal to about 6X the weight percent of kraft lignin in pulp.

Vanillic acid was estimated spectrophotometrically at 259 nm using methanol as the solvent system.

Estimation of β-ketoadipate: β-ketoadipate was determined by Rothera's color reaction (1908). 0.8 gm of (NH₄)₂SO₄ was added to 0.1 ml of cell free extract, followed by the addition of 0.2
ml of 1% (w/v) sodium nitroprusside and 0.5 ml of liquid ammonia. The color obtained was compared to that of acetone control.

Delignification of unbleached pulp: Experimental kraft pulp was prepared by treating saw dust with 0.87 M NaOH and 0.13 M sodium sulfite for 2 hr at 121°C. The pulp was washed several times with water to remove the alkali.

Delignification of the pulp was carried out in 250 ml conical flasks containing 100 ml of basal medium and 1.0% pulp. Flasks were inoculated with 20% macerated inoculum of *M. indicum* and incubated on a rotary shaker. Following incubation, the pulp with mycelium was harvested by filtration, stirred for 1 hr. in 50 ml of 1 N NaOH, filtered and washed to neutrality with water, dried at 100°C, and then weighed and analyzed. Controls were incubated without fungus and the pulps treated in the same way.