Chapter 3

Materials and Methods
MATERIALS AND METHODS

CHEMICALS

Carboxymethyl-cellulose (low viscosity), birch wood xylan, oatspelt xylan, larchwood xylan, lichenan, laminarin, xyloglucan, aryl-glycosides (pNP-β-D-cellobioside, oNP-β-D-galactopyranoside, pNP-β-D-glucoside, oNP-β-D-xylopyranoside, pNP-β-D-galactopyranoside, pNP-α-galactopyranoside, pNP-β-D-xylopyranoside, pNP-β-D-glucopyranoside, pNP-α-D-glucopyranoside, pNP-α-L-arabinofuranoside, α-L-arabinopyranoside, pNP acetate, pNP myristate, pNP palmitate, pNP butyrate, pNP ferulate, pNP stereate, 4-methylumbelliferyl acetate, 4-methylumbelliferyl-β-D-xylopyranoside, 4-methylumbelliferyl-α-L-arabinofuranoside, mercaptoethanol, Coomassie brilliant blue G-250, xylose, Sephacryl HR 200 were purchased from Sigma chemical Co., USA. pNP ferulate was procured from Institute of Chemistry, Bratislava, Slovakia. Other chemicals used were rye arabinoxylan, wheat arabinoxylan, xylobiose, xylotriose, xylotetraose and xylopentaose (Megazyme, Ireland), ampholine carrier servalytes (SERVA, Germany), DEAE-sepharose, phenyl sepharose 6 Fast Flow, PBE-94 poly buffer exchanger (Amersham Biosciences), metal ions, sodium dodecyl sulphate, agarose, ethidium bromide (Sisco Research Laboratories, India), Congo red, sodium azide, all saccharides, glycerol, and other routine chemicals (Himedia Laboratory, India), standard protein markers (BIORAD), PCR mix, restriction enzymes, 100 bp ladder and 1 Kb ladder (Bangalore GENEI, India). All the chemicals were of analytical grade.

3.1 ISOLATION AND SCREENING OF FUNGAL CULTURES

The soil samples for isolation of fungal strains were collected from composting materials/industrial solid waste/soils from region in and around Amritsar and Ahemdabad (India). Ten grams of soil was suspended in 99 ml of sterile distilled water and homogenized in vortex mixer. The resultant suspension was serially diluted with sterile water and 100 μl of suitably diluted suspension was spread on yeast starch agar (YPSS) of the following composition (% w/v): starch 1.5, yeast extract 0.4, KH₂PO₄ 0.23, K₂HPO₄ 0.2, MgSO₄ 7H₂O 0.05, citric acid 0.057, and agar 2.0, pH adjusted to 7.0
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(Cooney and Emerson, 1964) containing 100 μg/ml of ampicillin to restrict bacterial growth and incubated at 45-50°C for 7-10 days. The purified cultures were further plated on YpSS medium containing remazol brilliant blue xylan (0.5 % w/v) instead of starch and incubated at 45°C for 5 days. The clear zones formed around the colonies were visualized in order to screen for xylanase producers. The xylanase producing fungal isolates were further screened for xylanase production on rice straw containing solidified culture medium.

3.2 CULTURES

The thermophilic fungal isolates included in the present study (Acrophialophora mainiana MTCC 6662, Aspergillus caespitosus MTCC 6326, Chaetomium thermophile MTCC 4891, Corynascus sepedonium MTCC 6490, Emericella nidulans var. lata MTCC 6327, Emericella nidulans var. nidulans MTCC 6339, Humicola fuscoatra MTCC 6329, Humicola insolens MTCC 4617, Malbranchea flavida 4889, Melanocarpus albomyces MTCC 3922, Mucor indicus MTCC 6333, Myceliophthora sp. MTCC 6661, Penicillium janthinellum MTCC 6564, Rhizomucor pusillus MTCC 4895, Thermoascus aurantiacus MTCC 4890, and Thermomyces lanuginosus D2W3) were identified on the basis of colony morphology, microscopic examination (Cooney and Emerson; 1964) and on the basis of rDNA sequence of ITS 1-5.8S-ITS2 region. The identified cultures used in the present study have been deposited with Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India and their accession numbers are shown above.

3.3 MORPHOLOGY AND IDENTIFICATION

3.3.1 Slide culture

In a petri-plate wet filter paper was placed at the base with two glass rods over it which formed the platform for glass slide and this assembly was steam sterilized. A thin layer of YpSs agar was cut into small squares (5 mm size) and was aseptically placed on the glass slide. The agar block was inoculated with the culture and a sterile cover slip was placed over it gently. The assembly was incubated at 45°C for 24-72 h and was examined using bright field and phase contrast microscope (Olympus BX 60; 40X/0.65 Ph2) for morphological study.
3.3.2 Molecular characterization of fungi

3.3.2.1 Extraction of DNA: The DNA was extracted from 40 mg of lyophilized mycelium (ground to fine powder) that was suspended in 550 μl of extraction buffer (50 mM Tris HCl pH 8.0, 0.7 M NaCl, 10 mM EDTA, 1% β-mercaptoethanol, 1% SDS) and 300 μl of equilibrated phenol. Upon homogenization, the tubes were incubated for 15 min at 65°C (Kim et al. 1999). The DNA in the aqueous phase was purified with repeated extractions using equal volumes of saturated phenol chloroform iso-amyl alcohol (PCI) mixture (25:24:1). The resultant DNA was precipitated with 9 parts of ice-cold isopropyl alcohol and 1 part of sodium acetate (3.0 M; pH 8.0). The tubes then were kept at -20°C for 2 h, followed by centrifugation for 15 minutes at 6708×g. The resultant DNA pellets were rinsed with 70% ethanol, air dried suspended in 50 μl of sterilized double distilled water and stored at 4°C until use (Sharma et al. 2008).

3.3.2.2 PCR amplification of ITS-I-5.8S-ITSII, 18S and D1/D2 hyper-variable region of 26S rDNA

The ITS-I, ITS-II and the intervening 5.8S coding rDNA was amplified by PCR using ITS1 (5’TCCGTAGGTGAACCTGCGG3’) and ITS4 (5’TCCTCCGCTTATTGATATGC3’) primer pair. The 18S rDNA region was amplified using primers NS1 (5’GTAGGTATATGCTTGTCTC3’) and NS8 (5’TCCGCAGGGTCACCTACGGA3’). Whereas, the primer pair LR0R (5’ACCCGCTGAACTTAAGCG3’) and LR3 (5’CCGTGTATTCAGGACGG3’) was used for amplification of hyper variable D1/D2 region of large subunit rDNA (26-28S) as described by White et al. (1999).

Amplification reaction mixture (50 μl) contained 25 μl of Hot start PCR mix containing high sensitivity and high specificity Taq polymerase, nucleotides and optimum reaction buffer (Genei, Bangalore, India), 2.5 μl of DMSO, 100 pmoles of each primer and 100 ng of DNA template. Thermal cycling (Personal master cycler, Eppendorf) consisted of initial denaturation of 4 min at 95°C, followed by 30 cycles: denaturation step at 94°C for 50 seconds, annealing step at 51°C for 1 min, primer extension at 72°C for 1 min, followed by final extension step for 10 min at 72°C. Amplification products were electrophoretically resolved in 1.4% (w/v) agarose gel containing ethidium bromide, using 1X TAE buffer at 70 V.
3.3.2.3 Restriction digestion of amplified DNA and analysis

Amplified 18S rDNA (0.5 µg) was digested with 2 units each of *RsaI*, (Biozymes, Espoo, Finland), *Hinfl* and *Mbol* (Genei, Bangalore, India) as per manufacturer's instructions. The digested products were resolved on 1.8% (w/v) agarose gels at 70 V and their molecular weights were determined using 100 bp/1 Kb ladder (Genei, Bangalore, India). Gels were photographed using gene snap (Gene Genius, Cambridge, UK) and analyzed by gene tool software to calculate the molecular weight and amount of DNA followed by matching of the DNA bands. The RFLP data were archived from the gene tool and further analysed using gene directory software, using restriction pattern of *R. pusillus* amplified product as reference track. A combinatory cluster analyses of *Mbol, RsaI* and *Hinfl* digested products was performed by UPGMA (Unweighted Pair Group Method with Arithmetical Average) with Dice coefficient at 5% level to derive the dendrogram (Gene Genius, Cambridge, UK).

3.3.2.4 Sequence analysis

The purified amplified ITS region and D1/D2 hyper-variable region of 26–28S rDNA were sequenced by SPA services (Genei, Bangalore, India). The amplified sequences of different fungi were aligned, to each other as well as the sequences retrieved from NCBI databases, using multiple sequence alignment software (ClustalX). Dendrograms were generated using neighbour joining (NJ) plot and the boot strapping was carried using 100 replications. The ITS and D1/D2 variable region of 26S rDNA sequences were deposited with NCBI and their accession numbers were obtained.

3.4 SOLID SUBSTRATE CULTURING FOR ENZYME PRODUCTION

3.4.1 Inoculum development

For preparation of inoculum, a spore suspension prepared (2 ml) from 7 days old culture was used to inoculate 50 ml of glucose pre-culture medium containing (% w/v); glucose 1.5, yeast extract 0.4, K₂HPO₄ 0.2, MgSO₄ 0.1 (pH 7.0) and incubated at 45°C under shaking conditions (150 rpm) for 24 h.

3.4.2 Culture conditions

Solid state fermentation was carried out in Erlenmeyer flasks (250 ml) that contained ground rice straw as a carbon source (5 g) and basal medium (15 ml) of
following composition KH$_2$PO$_4$ 0.4%, CH$_3$COONH$_4$ 0.45%, and (NH$_4$)$_2$SO$_4$ 1.3%. Prior to sterilization, the initial pH and moisture content of the medium were adjusted to 7.0 and 75%, respectively. The culture medium then was inoculated with a mycelial suspension (2 ml) of 24 h old culture grown on glucose pre-culture medium. The flasks were incubated in water saturated atmosphere at 45°C for 5 days in an incubator. Thereafter, the enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM; pH 6.0) to the flasks and kept at 45°C for 1 h at 100 rpm. The resultant slurry was filtered through muslin cloth and centrifuged at 8800 × g for 10 min and the extracts were used for enzymatic assay.

3.5 BIOBLEACHING OF PULP

Decker pulp was obtained from ABC paper mills (Hoshiarpur, India). The pulp (10% w/w consistency) was subjected to alkaline extraction (NaOH, 6.3 % w/v) for 30 min. at 50°C, and then was thoroughly washed with water and air dried. The alkali treated Decker pulp (pH 9.5) was subjected to bio-bleaching with xylanases from different fungal sources. The enzyme dose of 10 units/g pulp dry weight was used to treat pulp (6% w/v consistency) at 55°C for 120 min at 120 rpm. After enzymatic treatment, pulp (10% w/v consistency) was subjected to next alkaline extraction (2% w/v NaOH) at 65°C for 60 min at 120 rpm.

The enzyme-mediated release of chromophoric material from pulp was measured spectrophotometrically at 237, 254, 280 and 465 nm in pulp free filtrates using UV-VIS spectrophotometer (Shimadzu, UV mini 1240). Reducing sugars released from pulp were measured using dinitrosalicylic acid method (Miller, 1959).

The hand-sheets of bleached pulp were prepared according to TAPPI method (T205 om-88) recommended by Technical Association of the Pulp and Paper Industry using hand sheet former (Model SCA Type, Universal Engineering Corporation, India), and air dried (sheet diameter; 165 mm). The brightness of hand sheets was determined using Elrepho 070 (Lorentzen & Wettre, Sweden).
3.6 OPTIMIZATION OF CULTURE CONDITIONS FOR ENZYME PRODUCTION BY *Malbranchea flava*

The production of xylanase, β-xylosidase, α-L arabinofuranosidase and acetyl xylan esterase was optimized using ‘one factor at a time’ approach with following parameters using culture conditions as described in section 3.4.2.

### 3.6.1 Effect of different carbon sources

The effect of different carbon sources namely, rice straw, wheat straw, wheat bran, bagasse, corncob, saw dust, sorghum straw and filter paper on the production of xylanase was studied.

### 3.6.2 Effect of media types

The effect of fourteen different production media types (Appendix I) for production of xylanase, β-xylosidase, α-L arabinofuranosidase and acetyl esterase was studied in presence of sorghum straw as carbon source.

### 3.6.3 Effect of nitrogen sources

The effect of organic (peptone, malt extract, beef extract, tryptone, casein, soybean meal, corn steep liquor) and inorganic (ammonium sulphate, NH₄NO₃, (NH₄)₂HPO₄, sodium nitrate, ammonium acetate, urea) nitrogen sources on the production of xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase was studied by replacing yeast extract with equivalent amount of nitrogen in medium H (Appendix I).

### 3.6.4 Effect of nitrogen concentration on the production of enzymes

The effect of different concentrations of casein (1.0-6.0% w/v) in the medium on production of xylanase, β-xylosidase, α-L arabinofuranosidase and acetyl esterase was studied.

### 3.6.5 Effect of inoculum age

The effect of inoculum age i.e., 24, 36, 48, 60, 72, 84 and 96 h old culture and freshly prepared spore (3×10⁷ spores/ml) suspension (i.e., 0h culture) on the production of xylanase, β-xylosidase, α-L arabinofuranosidase and acetyl esterase was studied.
3.6.6 Effect of inoculum level

The influence of inoculum level on xylanase production was studied by inoculating medium in each flask (5 g) with 1, 2, 3, 4 and 5 ml inoculum of freshly raised (24-96 h) culture of *M. flava* strain.

3.6.7 Effect of temperature

Incubation temperature from 25-50°C was adjusted to observe its effect on the production of xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterases.

3.6.8 Effect of pH of medium

The effect of initial pH of the medium (3.0-10.0) on the production of xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase was studied. The pH of the basal medium was adjusted using dilute solutions of HCl (1N) and NaOH (1M).

3.6.9 Effect of moisture level and particle size of sorghum straw

The effect of initial moisture level (66.6-80%) and particle size (1mm-3mm) on xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl esterase production was studied. All experiments were carried out in triplicates and standard error @ 5% was calculated.

3.7 STATISTICAL MODEL: Methodology and Design of Experiments

Response Surface Methodology (RSM) using the Box-Behnken design (Box and Behnken 1960) of experiments was used to develop a mathematical correlation between three independent variables on the production of xylanase, β-xylosidase, α-L-arabinofuranosidase and acetyl xylan esterase by *M. flava*. Three independent variables, casein concentration (*X_1*), inoculum age (*X_2*) and inoculum level (*X_3*) were chosen to study their effect on enzyme production by SSF.

The models were studied using a range of variables designated as low (-1), middle (0) and high (+1) concentrations (Tables 3.1). The mathematical relationship of response *G* (enzyme production) and variable *X* was approximated by the quadratic model equation:

\[
G = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3
\]

Where, *G* is the predicted response, \( \beta_0 \) intercept, \( \beta_1, \beta_2 \) and \( \beta_3 \) linear coefficients, \( \beta_{11}, \beta_{22}, \beta_{33} \) squared coefficients and \( \beta_{12}, \beta_{13}, \beta_{23} \) interaction coefficients. The STATEASE, USA Design-Expert statistical software was used to obtain optimal working parameters.
and to generate response surface graphs. All experiments were carried out in triplicates and standard error @ 5% was calculated.

3.8 REGULATION OF ENZYME PRODUCTION

3.8.1 Induction and repression of xylanase and acetyl xylan esterase in Malbranchea flavia

The induction of xylanase and acetyl esterase was studied in a medium containing yeast extract, 0.4%, KH₂PO₄, 0.24%, MgSO₄, 0.05%, CaCl₂, 0.01% along with 1% (w/v) of monosaccharides (glucose, fructose, arabinose, xylose); disaccharides (lactose and cellobiose); polysaccharides (oat spelt xylan); lignocelluloses (rice straw, sorghum straw, wheat bran, corn cob and bagasse) or alcohols (ethanol, propanol, and methanol), and glycerol 1% (v/v) as carbon source. The synergistic/negative effects of sugars and alcohols (fructose, glucose, cellobiose, lactose, xylose, cellobiose, arabinose (@1% w/v), glycerol, methanol, propanol and ethanol (@1% v/v) to the inducer oat spelt xylan containing medium were studied. The flasks were inoculated with 2 g wet weight of 60 h old washed mycelium of M. flavia and incubated at 40°C /120 rpm for 120 h in an orbital shaker. The sampling was carried out at 12 h intervals up to 120 h, the contents were centrifuged (8000 × g for 10 min), and the supernatants were assayed for xylanase and acetyl xylan esterase activities. All experiments were carried out in triplicates and standard error @ 5% was calculated.

3.8.2 Native polyacrylamide gel electrophoresis and zymogram

Crude enzyme preparations (protein 70 µg) were fractionated by native polyacrylamide gel electrophoresis (PAGE) using 10% acrylamide gel with 4% stacking gel (Laemmli, 1970). For observing xylanase activity the gel was incubated for 15 min in 50 mM sodium citrate buffer (pH 6.0) and overlaid on polyacrylamide gel containing xylan (1.0%, w/v) for 2 h at 50°C. The overlay gel was removed and stained with 0.2% Congo red. Bands corresponding to xylanase appeared as clear zone against a dark background after destaining with NaCl (1.0 M) followed by treatment with 10% (v/v) acetic acid solution.

Esterase activity in native PAGE was detected using 4-methylumbelliferyl acetate (5 mM) as substrate. The substrate solution was prepared in dimethyl sulfoxide. Upon completion of electrophoresis, the gel was incubated in phosphate buffer (0.1 M,
pH 6.0) for 30 min and then the substrate solution was poured on gel and esterase bands were observed under UV light using gel documentation system (Gene Genius, Cambridge, UK).

β-xylosidase and α-L-arabinofuranosidase activity was detected by developing zymograms using 4-methylumbelliferyl-β-D-xylopyranoside and 4-methylumbelliferyl α-L-arabinofuranoside (10 mM) as substrate (prepared in 50 mM sodium citrate buffer pH 6.0). Upon completion of electrophoresis, the gel was incubated in sodium citrate buffer (50 mM, pH 6.0) for 30 min and then the substrate solution was poured on the native gel and the β-xylosidase and α-arabinofuranosidase bands were observed under UV light using gel documentation system (Gene Genius, Cambridge, UK).

3.8.3 Quantification of xylanases by IEF profiling

After fractionating the proteins on IEF, the gel in each lane was sliced (1.25 mm thickness). Each slice was incubated in 500 µl sodium citrate buffer (50 mM, pH 6.0) for 72 h at 4°C. The eluted protein in each fraction was assayed for endoxylanase against birchwood xylan (BWX), rye arabinoxylan (RAX), wheat arabinoxylan (WAX) and 4-O-methyl glucouronoxylan (MGX) as described in section 3.13.1.

3.9 2-D GEL ELECTROPHORESIS

3.9.1 2 Dimensional gel electrophoresis of secretome

The desalted enzyme extract was concentrated using ultrafiltration Amicon cell fitted with PM-10 membrane (10 kDa cut off). Protein (150 µg) samples were loaded by passive in-gel rehydration at 20°C in 125 µl rehydration buffer (8 M urea, 2% CHAPS, Destreak reagent, 1% IPG buffer pH 3-5.6 and 0.005% bromophenol blue in Milli Q grade sterilized water). The IPG strips (7 cm) were rehydrated for 16 h at room temperature in rehydration buffer. The isoelectric focusing (IEF) was performed using Ettan IGPhor 3 system (GE, Healthcare Biosciences) in a stepwise manner using voltage hour program that increased linearly in following steps: 100 V, 2 h; 300 V, 2 h; 1000 V, 2 h; 5000 V, 3 h (gradient); 5000 V, 6 h (step), with a total of 51000 Vh. Prior to SDS-PAGE, the IPG strips were incubated for 15 min in 6 ml of 0.05 M Tris-Cl (pH 8.8), 8 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 60 mM Dithiothreitol (DTT) and traces of bromophenol blue followed by incubation for 15 min in the same buffer except
that DTT was replaced with 50 mM iodoacetamide. The equilibrated IPG strips were transferred onto 12% polyacrylamide gels without stacking gel and overlaid with molten low melting agarose (0.5%). The second dimension was run at constant of 25 mA. The electrophoresis was carried out using a Hoefer mini VE system (GE, Healthcare Biosciences) and the gels were stained using silver nitrate.

3.9.2 Protein identification

The purified protein spots (silver nitrate stained) resolved by 2DE/SDS PAGE were excised for peptide mass fingerprinting (PMF). The PMF of the samples was carried out at TCGA (The Centre for Genomic Application, New Delhi), for peptide mass spectrometry analysis by LC/MS (Agilent 1100 series 2D Nano LCMS). Mass spectrometry data were compared with data in the NCBI and Swiss Prot databases using the Mascot search algorithm.

Table 3.1: The Box-Behnken design for the three independent variables chosen for enzyme production by *Malbranchea flav*a under solid-state fermentation

<table>
<thead>
<tr>
<th>Trial</th>
<th>Casein (% w/v)</th>
<th>Inoculum age (h)</th>
<th>Inoculum level (ml)</th>
</tr>
</thead>
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<td>1</td>
<td>2.0 (0) *</td>
<td>36.00 (0)</td>
<td>3.00 (0)</td>
</tr>
<tr>
<td>2</td>
<td>2.0 (0)</td>
<td>72.00 (+1)</td>
<td>4.00 (+1)</td>
</tr>
<tr>
<td>3</td>
<td>2.0 (0)</td>
<td>0.00 (-1)</td>
<td>4.00 (+1)</td>
</tr>
<tr>
<td>4</td>
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<td>0.00 (-1)</td>
<td>3.00 (0)</td>
</tr>
<tr>
<td>5</td>
<td>0.5 (-1)</td>
<td>0.00 (-1)</td>
<td>3.00 (0)</td>
</tr>
<tr>
<td>6</td>
<td>2.0 (0)</td>
<td>36.00 (0)</td>
<td>3.00 (0)</td>
</tr>
<tr>
<td>7</td>
<td>3.5 (+1)</td>
<td>36.00 (0)</td>
<td>4.00 (+1)</td>
</tr>
<tr>
<td>8</td>
<td>2.0 (0)</td>
<td>36.00 (0)</td>
<td>3.00 (0)</td>
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<tr>
<td>9</td>
<td>3.5 (+1)</td>
<td>72.00 (0)</td>
<td>3.00 (0)</td>
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<td>36.00 (0)</td>
<td>3.00 (0)</td>
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<td>11</td>
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<td>2.00 (-1)</td>
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<tr>
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<td>72.00 (+1)</td>
<td>3.00 (0)</td>
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<tr>
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<td>36.00 (0)</td>
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<tr>
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<td>36.00 (0)</td>
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<tr>
<td>17</td>
<td>0.5 (0)</td>
<td>36.00 (0)</td>
<td>2.00 (-1)</td>
</tr>
</tbody>
</table>

*Coded values given in parentheses
3.10 PURIFICATION OF XYLANASES FROM *Malbranchea flava*

*M. flava* culture was grown on solidified sorghum straw culture medium under optimized culture conditions. The centrifuged (8000 × g for 20 min) culture extract was desalted and concentrated by ultrafiltration using Millipore stirred cell fitted with a PM-10 membrane (10 kDa cut-off) and used for purification of endoxylanases.

3.10.1 Ion-exchange chromatography

DEAE-Sepharose FF (Amersham Biosciences) slurry was prepared in Tris-Cl buffer (20 mM, pH 6.5) and degassed. The column (24 × 2.6 cm, Pharmacia) packed with DEAE-Sepharose was equilibrated with 3-5 volumes of Tris-Cl buffer (20 mM, pH 6.5) at a flow rate of 1 ml min⁻¹. Concentrated protein (800 mg) sample was loaded on to DEAE-Sepharose column and was eluted with 2 column volumes under isocratic conditions followed by linear gradient of 0-1.0 M NaCl in Tris-Cl buffer (20 mM, pH 6.5) at a flow rate of 1 ml min⁻¹. All the fractions (10 ml each) were analyzed for xylanase activity. Fractions corresponding to xylanase peaks obtained during NaCl gradient elution were pooled, concentrated using ultrafiltration PM-10 membrane (Millipore).

3.10.2 Phenyl sepharose 6FF (Amersham) hydrophobic interaction (HIC)

Concentrated sample corresponding to fractions eluted under isocratic conditions then was equilibrated with 1.0 M (NH₄)₂SO₄ in 50 mM acetate buffer (pH 5.0) and applied onto phenyl sepharose 6FF hydrophobic interaction (HIC) column (5 ml) (Amersham Biosciences) equilibrated with same buffer. The column was eluted with linear gradient of (NH₄)₂SO₄ (1.0 M-0 M) in 50 mM acetate buffer (pH 5.0) at flow rate of 1ml min⁻¹. The fractions containing xylanase activity were pooled separately and the purified enzyme designated as MFX I was characterized. The bound xylanase active fractions obtained during elution with ~0.45 M NaCl on DEAE-Sepharose column were pooled, concentrated, equilibrated with 1.0 M (NH₄)₂SO₄ in 50 mM acetate buffer (pH 5.0) and resolved on phenyl sepharose 6 FF hydrophobic interaction (HIC) column (5 ml) (Amersham Biosciences) as described above. The active fractions corresponding to xylanase (MFX II) were pooled, for further purification.
3.10.3 Poly-buffer exchanger (PBE-94)

The active fractions corresponding to xylanase (MFX II) were pooled, desalted and concentrated using Amicon cell fitted with a PM-10 membrane (10 kDa cut off) and applied onto polybuffer exchanger (PBE-94, Amersham Biosciences) column (10 × 0.75 cm) equilibrated with 20 mM acetate buffer (pH 5.0). The column was eluted with a linear gradient of NaCl from 0 to 1.0 M in 20 mM acetate buffer (pH 5.0) at a flow rate of 0.2 ml min⁻¹. The fractions containing purified MFX II as assessed by SDS-PAGE were pooled and characterized.

3.11 PURIFICATION OF ACETYL XYALN ESTERASE FROM Malbranchea flava

3.11.1 Ammonium sulphate precipitation of crude enzyme extract

M. flava culture was grown on solidified sorghum straw culture medium under optimized culture conditions. The centrifuged (8000 × g for 20 min) culture extract was precipitated by adding ammonium sulphate to 100% saturation. The pellet was recovered by centrifugation, dissolved in minimal amount of water and dialysed against deionised water with several changes.

3.11.2 Phenyl sepharose 6FF (Amersham) hydrophobic interaction (HIC)

The dialysate was equilibrated with 1.0 M (NH₄)₂SO₄ in 50 mM acetate buffer (pH, 5.0) and applied onto phenyl sepharose 6FF hydrophobic interaction (HIC) column (5 ml) (Amersham Biosciences) equilibrated with same buffer. The column was eluted with linear gradient of (NH₄)₂SO₄ (1.0 M-0 M) in 50 mM acetate buffer (pH 5.0) at flow rate of 1ml min⁻¹. The fractions containing esterase activity were pooled separately and concentrated using ultrafiltration PM-10 membrane (Millipore).

3.11.3 Chromatofocussing

The active fractions corresponding to esterase were pooled, desalted and concentrated using Amicon cell fitted with a PM-10 membrane (10 kDa cut off) and applied onto polybuffer exchanger (PBE-94, Amersham Biosciences) column (10 × 0.75 cm) equilibrated with 25 mM imidazole HCl buffer (pH, 7.4). The column was eluted with a linear pH gradient (7.4-4.0) with polybuffer HCl-74 (pH, 4.0) (Amersham
Biosciences) at a flow rate of 0.2 ml min\(^{-1}\). The fractions containing esterase activity were pooled and concentrated using ultrafiltration PM-10 membrane (Millipore).

3.11.4 Poly-buuffer exchanger (PBE-94)

The active fractions corresponding to esterase were pooled, desalted and concentrated using Amicon cell fitted with a PM-10 membrane (10 kDa cut off) and applied onto polybuffer exchanger (PBE-94, Amersham Biosciences) column (10 × 0.75 cm) equilibrated with 20 mM acetate buffer (pH 5.0). The column was eluted with a linear gradient of NaCl from 0 to 1.0 M in 20 mM acetate buffer (pH 5.0) at a flow rate of 0.2 ml min\(^{-1}\). The fractions containing purified esterase as assessed by SDS-PAGE were pooled and characterized.

3.12 CHARACTERIZATION OF PURIFIED XYLANASES AND ACETYL ESTERASE

3.12.1 Molecular weight determination by SDS-PAGE and zymogram development by renaturation of SDS-PAGE gels.

The homogeneity and molecular weight of purified enzymes was determined using 10% SDS-PAGE gel (Laemmli, 1970) employing Bio-Rad Mini-Protean II electrophoresis assembly. Tris-glycine buffer (Tris 25 mM, Glycine 192 mM, pH 8.0) containing 0.1% SDS was used as the electrode buffer. Samples to be analyzed were treated with sample buffer and boiled 5 min prior to application to the gel. Electrophoresis was carried for 15 min at 90 V till samples reached the resolving gel and then a constant voltage of 100 V was applied. The standard protein markers (Molecular weight SDS kit, Biorad) that included lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa) and phosphorylase b (97.4 kDa) were used as control. The \(M_r\) of enzymes was determined by plotting curve of log \(M_r\) of the standard protein markers verses their relative mobility of purified proteins.

Zymogram analysis was performed with a modification of the method of Taylor II et al. (2006). After SDS-PAGE, the gel was incubated in 80 ml of refolding buffer [20 mM piperazine-N, N-bis (2-ethanesulfonic acid), pH 6.8, 2.5% Triton X-100, 2 mM Dithiothreitol (DTT), and 2.5 mM \(CaCl_2\)] for 1 h at room temperature and held
overnight in fresh refolding buffer at 4°C. The gel was thoroughly washed with distilled water and placed on 0.5% Remazol Brilliant Blue (RBB)-xylan (Sigma) containing gels for 4 h at 50°C. For detection of acetyl esterase activity in the gel of 4-methylumbelliferyl acetate was used as a substrate. The stock solution 4-methylumbelliferyl acetate (10 mM) was prepared in dimethyl sulfoxide and then diluted to 5 mM with phosphate buffer (100 mM, pH 6.0) containing 2.5% Triton X-100. After thoroughly washing the renatured gel, 4-methylumbelliferyl acetate (5 mM) was poured on the gel and esterase bands were observed under UV light using gel documentation system (Gene Genius, Cambridge, UK).

3.12.2 Determination of pI by IEF

IEF was performed according to the instructions provided by Novex, using 5% acrylamide gel containing 2.4% broad pH range (3-10.0) ampholine carrier servalyte (SERVA, Germany) in a Mini-protein II system (Biorad). The cathode buffer contained 0.35% (w/v) arginine and 0.29% (w/v) lysine, whereas, 10 mM phosphoric acid was used as anode buffer. IEF was carried out for 1 h each at constant 100 and 200 V followed by 500 V for 30 min. After fractionating the proteins on IEF, the gel was fixed in TCA (10% v/v) for 2 h and silver staining was performed. The pI of the purified enzyme was determined by plotting the curve of relative mobility of standard protein markers verses their pI.

For detection of xylanases, after electrophoresis the gels were incubated for 15 min in sodium acetate buffer (50 mM; pH 5.0) and overlaid on polyacrylamide gel containing xylan (1%, w/v) for 2 h at 50°C. The overlay gel was removed and stained with 0.2% Congo red. Bands corresponding to xylanase appeared as clear zone against a dark background after destaining with 1M NaCl followed by treatment with 10% (v/v) acetic acid solution.

3.12.3 Temperature and pH optima

The optimal temperature of purified xylanases and esterase were determined by assaying the activities against birchwood xylan and 4-nitrophenyl acetate, respectively, between 30 and 90°C. The optimal pH was determined by measuring the activity against birchwood xylan (1% w/v) between pH 2.0 and 10.0 using 100 mM HCl-KCl (pH 2.0), sodium citrate (pH 3.0-6.0), sodium phosphate (pH 7.0 and 8.0), and Glycine-NaOH
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(pH 9.0 and 10.0) buffers at 50°C. The optimal pH for purified esterase was determined by measuring the esterase activity against 4-nitrophenyl acetate between pH 3.0 and 7.0 using 100 mM sodium citrate (pH 3.0-5.0), sodium phosphate (pH 6.0-7.0) containing 2.5% (v/v) triton X-100.

3.12.4 Stability

For the determination of temperature and pH stability, the enzyme aliquots were pre incubated at different temperatures and pH range for 0-240 min and assayed for xylanase and esterase using birchwood xylan and 4-nitrophenyl acetate as substrate, at 50°C thereafter.

3.12.5 Effect of metal ions and other compounds

The purified xylanases were pre-incubated in 10 mM solution of MgCl2, NaCl, KCl, MnCl2, CuCl2, ZnCl2, FeCl3, CaCl2, EDTA, DTT, β-mercaptoethanol, NBS and SDS for 30 min at room temperature in sodium citrate buffer (50 mM, pH 6.5). The purified esterase was pre-incubated in 10 mM solution of MgCl2, NaCl, KCl, MnCl2, CuCl2, ZnCl2, FeCl3, CaCl2, NaCl, EDTA, DTT, SDS, β-mercaptoethanol NBS and PMSF for 30 min at room temperature in sodium phosphate buffer (100 mM, pH 6.0) with 2.5% (v/v) triton X-100. The residual activities of xylanases and esterase were determined using birchwood xylan and 4-nitrophenyl acetate, respectively, as substrates.

3.12.6 Substrate specificity

3.12.6.1 Activity against polysaccharides

Substrate specificity of purified xylanases was determined against polysaccharides replacing birchwood xylan in the assay mixture with oat spelt xylan (OSX), debranched arabinan (DAX), larchwood xylan (LWX), wheat arabinoxylan (WAX), rye arabinoxylan (RAX), carboxy methyl cellulose (CMC), 4-O-methyl glucouronoxylan (MGX), lichenin, laminarin, and xyloglucan (XG) at 1% (w/v). The reaction was carried by incubating 490 µl citrate buffer (50 mM, pH 6.0), 500 µl substrate (1% w/v) and 10 µl enzyme, at 50°C for 1 h. The reaction was stopped by the addition of 2 ml DNS reagent and tubes were kept in boiling water bath for 10 min. The developed colour was read at 540 nm using Novaspec II Spectrophotometer (Pharmacia).
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3.12.6.2 Activity against pNP substituted substrates

Activities of purified xylanases were determined towards 3 mM p-nitrophenyl derivatives, pNP-α-L-arabinofuranoside, pNP-α-L-arabinopyranoside, pNP-β-D-cellobioside, pNP-α-D-mannopyranoside, oNP-β-D-galactopyranoside, pNP-β-D-galactopyranoside, pNP-α-D-galactopyranoside, pNP-β-D-glucopyranoside, oNP-β-D-xylopyranoside and pNP-β-D-xylopyranoside prepared in sodium acetate buffer (50 mM, pH 5.0). A reaction mixture containing enzyme (25 µl), 50 µl of sodium acetate buffer (50 mM, pH 5.0) and 25 µl of substrate was incubated at 50°C for 1 h, the reaction was terminated by adding 100 µl of NaOH-glycine buffer (0.4 M, pH 10.8) and the developed yellow color was read at 405 nm using an ELISA Reader (MULTISKAN; Lab system).

The substrate specificity of purified esterase (25 µl) was determined towards 1 mM pNP-α-L-arabinofuranoside, pNP-β-D-cellobioside, pNP-α-D-mannopyranoside, pNP-β-D-galactopyranoside, pNP-β-D-glucopyranoside, pNP-β-D-xylopyranoside, pNP-acetate, pNP ferulate, pNP myristate, pNP butyrate, pNP palmitate, and pNP stearate prepared in sodium phosphate buffer (0.1 M, pH 6.0) containing 2.5% triton X-100. The reaction was initiated by adding 125 µl of substrate and incubated at 50°C for 1 h, the reaction was terminated by adding 100 µl NaOH-glycine buffer (0.4 M, pH 10.8) and the developed yellow color was read at 405 nm using an ELISA Reader (MULTISKAN; Lab system).

3.12.7 Enzymes kinetics

The Michaelis–Menten kinetic parameters (K_m and V_max) and K_cat were determined against BWX, OSX, LWX and RAX as substrates using Lineweaver-Burke plots. The apparent K_m, V_max, and K_cat for acetyl esterase were determined against pNP acetate using Lineweaver-Burke plot.

3.12.8 Thin layer (TLC) and high-pressure liquid chromatography (HPLC) of hydrolysis products of purified xylanases

For enzymatic hydrolysis of BWX, OSX, LWX, RAX and WAX, respectively, 900 µl of the respective substrate prepared in sodium citrate buffer (50 mM, pH 6.0) was incubated with 100 µl of purified xylanases (MFX I/MFX II) at 50°C for 72 h. Samples were withdrawn at interval of 24 h, freeze dried by lyophilization and
redissolved in methanol. Hydrolyzed products were detected by thin layer chromatography (TLC). Thin layer chromatography (0.25 mm layer of silica gel F-254, Merck, India) was performed using mixture of ethyl acetate: acetic acid: water (3:2:1 v/v) as solvent system. The resolved hydrolysis products were detected by spraying the plates with diphenylamine reagent followed by heating at 100°C for 10 min. A mixture of D-xylose (Sigma), xylobiose, xylotriose, xylotetraose and xylopentaose (Megazyme, Ireland) was used as standard. HPLC was carried out with the DIONEX system (USA) equipped with a P680 pump, a thermostatted column compartment (TCC) and a differential refractive index detector (RI-101, SHODEX). The PL HI-PLEX NA column (Varian 300 ×7.7 mm) used for separating sugars was maintained at 60°C. Water was used as mobile phase at a flow rate of 0.2 ml min⁻¹. Sugars in the hydrolysates were identified using xylose, xylobiose, xylotriose, xylotetraose and xylopentaose as standard.

3.13 ESTIMATION OF ENZYME ACTIVITIES

3.13.1 Endoxylanase

The solution of birch wood xylan (1%) prepared in sodium citrate buffer (50 mM, pH 6.5) was used as substrate for estimation of xylanase activity. Xylanase activity in SSF experiments was determined according to Bailey et al. (1992). The assay mixtures that contained 1.8 ml of substrate solution and 0.2 ml suitably diluted enzyme were incubated at 50°C for 5 minutes. The reaction in above assay was stopped by addition of 3 ml dinitrosalicylic acid (DNS) reagent and the contents were boiled for 15 minutes. Xylanase activity in purified fractions and fractions obtained after IEF profiling was determined by incubating 1ml reaction mixture containing 10 μl enzyme, 500 μl of substrate solution and 490 μl of sodium citrate buffer (50 mM, pH 6.5) at 50°C for 15 min. The reaction was stopped by addition of 2 ml dinitrosalicylic acid (DNS) reagent and the contents were boiled for 15 minutes. Xylanase assay of crude filtrate (in shake culture experiments) was performed by incubating reaction mixture that contained 500 μl of substrate solution and 500 μl of suitably diluted enzyme at 50°C for 5 minutes. The color developed in above assays was read at 540 nm using
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Novaspec II spectrophotometer (Pharmacia). The amount of reducing sugar liberated was quantified using xylose standards.

3.13.2 Avicel adsorbable xylanase

The purified xylanases were characterized for their ability to adsorb to avicel. The adsorption to avicel was studied by incubating 200 µl of enzyme with 0.1 g of avicel in 800 µl sodium citrate (50 mM, pH 6.5) buffer at 4°C for 15 min. The contents were centrifuged at 11000 × g for 10 min and the clear supernatant was assayed for residual xylanase activity using birch wood xylan (1% w/v) as substrate (Badhan et al. 2007). Avicel adsorbable xylanase activity was measured indirectly by subtracting avicel non-adsorbable xylanase activity from total xylanase activity.

3.13.3 β-xylosidase, α-L-arabinofuranosidase

The substrates, pNP-β-D-xylopyranoside and pNP-α-L-arabinofuranoside (10 mM) prepared in sodium citrate buffer (50 mM, pH 6.0) were used to assay β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55) activities, respectively. The reaction mixtures containing 25 µl of enzyme, 25 µl of substrate solution and 50 µl of sodium citrate buffer (50 mM, pH 6.5) were incubated at 50 °C for 30 min in dark. Activity of purified fractions against pNP-substrates (cited in section 3.11.6.2) was performed following above said protocol. The reaction in above cited assays was terminated using equal amounts of NaOH-glycine buffer (0.4 M, pH 10.8) and the developed color was read at 405 nm using ELISA Reader (MULTISKAN; Labsystems). The amount of p-nitrophenol released was quantified using the p-nitrophenol as standard.

3.13.4 Acetyl esterase

p-Nitrophenyl-acetate used as substrate for esterase activity was prepared by mixing 9 vol. of phosphate buffer (100 mM, pH 6.0) containing 2.5% Triton X-100 with 1 vol. of 10 mM pNP-acetate in dimethyl sulfoxide followed by immediate vortexing (Mastihuba et al. 2002). For estimation of acetyl esterases in the crude extracts obtained from shake flask culture and SSF experiments, the reaction mixture (150 µl) that contained appropriately diluted enzyme (25 µl) and substrate solution (125 µl) was incubated at 50°C for 30 min in dark. Acetyl esterase activity in purified fractions was also assayed by the above mentioned protocol. The developed color during assay was observed at 405 nm using Multiskan ELISA Reader (Labsystems) and
quantified using the pNP as standard. For all the assays, appropriate substrate blanks, reagent blanks and enzyme blanks were also prepared. All assays were carried out in triplicate.

3.14 ESTIMATION OF PROTEIN

The protein in the enzyme extracts was determined by protein dye binding method as described by Bradford (1976) as well at 280 nm in the samples fractionated during protein purification using UV Mini 1240 spectrophotometer (Shimadzu).

3.15 ENZYME UNITS

One unit of endoxylanase was defined as the amount of enzyme required to release 1 μmole of xylose equivalents from their respective substrate per minute under the assay conditions. One unit of β-xylosidase, α-L-arabinofuranosidase and acetyl esterase activity was expressed as the amount of enzyme required to release 1 μmole of pNP per minute under assay conditions. For SSF experiments the enzyme activity was expressed as units per gram of dry weight substrate (U/g of dry weight substrate). The specific activity was expressed in units/mg of protein.