CHAPTER 2

EXPERIMENTAL METHODOLOGY

The general materials and methods used for the extraction and assay of Xylanase from *Trichoderma reesei* as well as recombinant *E. coli*, characterization of xylanase from *Trichoderma reesei* and recombinant *E. coli* by SDS-PAGE and zymography have been explained in this chapter. The experimental design for the in-gel digestion for LC-MS/MS and the detailed procedure for protein sequencing by homology driven proteomics approach has also been discussed in this chapter.

2.1 MATERIALS NEEDED

2.1.1. Microorganism

The fungal organism *Trichoderma reesei* was obtained from Microbial Type Culture Collection and Gen Bank (MTCC) at the Institute of Microbial Technology (IMTECH), Chandigarh, India.

2.1.2. Chemicals

The commonly used chemicals were procured locally from various companies such as Fischer Scientific (Chennai), S.D. Fine Chemicals (Chennai) and Hi-Media (Mumbai). They were of analytical and HPLC grade. Analar grade organic solvents and chemicals were obtained from S.D. Fine chemicals. The LC-MS grade water, acetonitrile and Formic acid were obtained from Fluka (Switzerland). The commercial protein markers and kits were purchased from Aristogene Biosciences, Bangalore.
2.1.2. **Isolation of recombinant Xylanase**

After incubation period, the culture was centrifuged at 7000 rpm for 10 min. The supernatant was decanted and the cell pellet was collected for enzyme extraction. The cell pellets were crushed with the alumina powder using a mortar and a pestle at 4 °C for 30 min with 100 mM PMSF (phenyl methane sulfonyl fluoride) dissolved in isopropyl alcohol. The PMSF is a serine protease inhibitor commonly used in the preparation of cell lysates to block the activity of protease. Then the mixture was suspended in 1 X phosphate buffer and cell debris was removed by centrifugation at 9500 rpm for 30 min. The clear supernatant was collected and it containing the \( xyn2 \) gene product. This purified xylanase enzyme was used for all subsequent enzyme assays.

2.1.3. **Phosphate buffer**

0.2 M Sodium Phosphate monobasic (\( \text{NaH}_2\text{PO}_4 \)) solution was prepared by dissolving 27.8 g in 1000 ml distilled water. Similarly 0.2 M Sodium Phosphate dibasic (\( \text{Na}_2\text{HPO}_4 \)) solution was prepared by dissolving 53.65 g in 1000 ml distilled water. Phosphate buffer (pH 7.0) was made by mixing 39 ml of freshly prepared monobasic solution with 61 ml of the dibasic solution and making the volume upto 1000 ml.

2.1.4. **Sodium Citrate buffer**

0.1 M tri-sodium citrate solution was prepared and the pH was adjusted to 6.5 with 0.1 M citric acid.

2.1.5. **Substrate Preparation**

1.0 % birchwood 4-O-methyl glucuronoxylan was prepared in 0.05 M Sodium citrate buffer, pH 5.3. 1.0 g of xylan was homogenized using a kitchen blender in 80 ml buffer at 60 °C and heated to boiling point on a heating magnetic stirrer. The mixture was chilled with continued stirring, covered and stirred slowly overnight. The total volume was made upto 100 ml with buffer and stored at 4°C for a maximum of 1 week.

2.1.6. **Separating gel Preparation**

The separating gel mix was prepared as per components given in table 2.1. This separating gel mixture was transferred using a pipette to the gel cassette by running the solution carefully down one edge between the glass plates. This solution was added continuously until it reaches a position 1 cm from the bottom of the comb. The setting of gel with a smooth surface was made sure by very running distilled water carefully down one edge into the cassette using a pipette. On account of the great difference in density between the water and the gel solution, the water will spread across the surface of the gel without serious
mixing. Water was added continuously until a layer of about 2 mm exists on top of the gel solution. The gel was left to set and heat is evolved as the gel sets which can be detected by carefully touching the gel plates. A very clear refractive index change was seen between the polymerized gel and overlaying water as soon as the gel was set. (Walker 2002)

Table 2.1 Resolving Gel Preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>2 ml</td>
</tr>
<tr>
<td>30 % Acrylamide-0.8 % bis-acrylamide</td>
<td>1.67 ml</td>
</tr>
<tr>
<td>1.5 M Tris (pH 8.8)</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>20 % SDS</td>
<td>25 μl</td>
</tr>
<tr>
<td>10 % Ammonium per sulphate</td>
<td>25 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.5 μl</td>
</tr>
</tbody>
</table>

Table 2.2 Stacking Gel Preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>2.088 ml</td>
</tr>
<tr>
<td>30% Acrylamide-0.8 % bis-acrylamide</td>
<td>0.506 ml</td>
</tr>
<tr>
<td>1 M Tris (pH 6.8)</td>
<td>0.375 ml</td>
</tr>
<tr>
<td>20 % SDS</td>
<td>15 μl</td>
</tr>
<tr>
<td>10 % Ammonium persulphate</td>
<td>15 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>1.5 μl</td>
</tr>
</tbody>
</table>

2.1.7. Stacking gel Preparation

Stacking gel mix was prepared using the components given in table 2.2. Water was poured off as soon as the separating gel has set and the stacking gel solution was added to the gel cassette until the solution reaches the cutaway edge of the gel plate. The well-forming comb was placed into this solution and
L1 and P1, located at the 3′ ends of the xyn gene, were used for cDNA synthesis. The products of each RT reaction were used as templates for amplification of unique segments of individual genes by PCR using primer sets. With RNA isolated from *E. coli* cells and cDNA synthesized using the P1 primer, indicating that xylanase gene was synthesized as one transcript. As a control, RT reactions lacking reverse transcriptase were used and no PCR product was detected. Contamination of genomic DNA was eliminated by treating RNA with RNase-free DNase I before performing. The graph showing the amplification with respect to increase in cycle number is provided in figure 4.10.

The successful cloning of gene encoding xylanase enzyme will lead to the development of a new novel effective bleaching strategy to achieve significant reduction in the consumption of polluted resources like phenolic chlorinated chemicals. Further studies are anticipated involving the optimization of the experimental conditions necessary for achieving maximum gene expression for production of xylanase enzyme.

2.1.8. Solutions for zymogram

- Solution-A containing 50 mM Sodium Phosphate dibasic (Na$_2$HPO$_4$) and 50 mM Sodium Phosphate monobasic (NaH$_2$PO$_4$) at pH 7.2 along with isopropanol.
- Solution-B consisting of 50 mM Sodium Phosphate dibasic (Na$_2$HPO$_4$) and 50 mM Sodium Phosphate monobasic (NaH$_2$PO$_4$) at pH 7.2.
- Solution-C having 50 mM Sodium Phosphate dibasic (Na$_2$HPO$_4$) and 50 mM Sodium Phosphate monobasic (NaH$_2$PO$_4$) at pH 7.2 along with 5 mM β-mercaptoethanol and 1 mM EDTA

2.1.9. Reagents for LC-MS

- 100 mM Ammonium Bicarbonate in water: 0.791 g of Ammonium bicarbonate was dissolved in 100 ml of HPLC grade water. Buffer was prepared daily in large volumes in the range of 100 ml and discarded after use.
- 10 mM DTT in 100 mM Ammonium Bicarbonate: 0.0154 g DTT was dissolved in 10 ml of 100 mM Ammonium bicarbonate.
- 55 mM Iodoacetamide in 100 mM Ammonium Bicarbonate: 0.1018 g of iodoacetamide was dissolved in 10 ml of 100 mM Ammonium bicarbonate.
- 5 % (vol/vol) Formic acid in water: 1 ml of Formic acid was dissolved in 20 ml of HPLC grade water.
- Ammonium bicarbonate / acetonitrile: 10 ml of 100 mM Ammonium bicarbonate was mixed with 10 ml of Acetonitrile.
- Formic acid / Acetonitrile: 10 ml of 5 % Formic acid was mixed with 20 ml of Acetonitrile.
- Trypsin: 1.5 ml of 10 mM ammonium bicarbonate containing 10 %
(vol/vol) acetonitrile was added to the content of 20 μg vial and mixed well.

2.1.10. **Trypsin buffer Preparation**

20 μg of lyophilized enzyme was redissolved in 1.5ml ice cold 1 mM hydrochloric acid and 100 μl aliquots were stored at -20 °C. Trypsin vial was maintained on ice all throughout the preparation process. After thawing frozen aliquots, pH of the solution was adjusted by adding 15 μl of 50 mM ammonium bicarbonate shortly before use.

2.1.11. **Characterization Techniques**

Optical measurements were performed on a Systronics model 2201 UV-Visible spectrophotometer using a pair of quartz cuvette of path length 1cm. The readings were taken at 585 nm for enzyme assay and activity.

Agilent 1200 High performance liquid chromatography was used to separate the Complex tryptic peptide mixtures obtained as a result of in-gel digestion of the SDS PAGE gel. 100 % water with 0.1 % formic acid is used as solvent A and 80 % Acetonitrile + 20 % water with 0.1 % formic acid is used as solvent B. The sample was prepared by vortexing the tryptic digest with 0.1 % formic acid for 1 hr followed by centrifugation at 10,000 rpm for 5 min. The supernatant was taken onto Agilent auto sampler vials.

Automated nano-ESI source called Nanomate Triversa (Advion) was used to generate the ions for later mass spectrometric analysis. A specifically designed LC coupler is used to connect the flow from the LC to the ESI chip where the nano-ESI generated ions are transferred into the mass spectrometer. The spray current is monitored and at the beginning of a standard gradient, it should be above 50 nA.
The MS spectrum was obtained using LTQ Orbitrap Discovery, a hybrid type MS system with the ability to determine accurate m/z of intact precursors in the orbitrap analyzer.

2.2 METHODS

2.2.1 Extraction of Xylanase

After incubation period, the culture was centrifuged at 7000 rpm for 10 min. The supernatant was decanted and the cell pellet was collected for enzyme extraction. The cell pellets were crushed with the alumina powder using a mortar and a pestle at 4 °C for 30 min with 100 mM PMSF (phenyl methane sulfonyl fluoride) dissolved in isopropyl alcohol. The PMSF is a serine protease inhibitor commonly used in the preparation of cell lysates to block the activity of protease. Then the mixture was suspended in 1 X phosphate buffer and cell debris was removed by centrifugation at 9500 rpm for 30 min. The clear supernatant was collected and it containing the Xyn2 gene product. This purified xylanase enzyme was used for all subsequent enzyme assays.

2.2.2 Xylanase Assay

The method with the substrate 4-O-methyl-D-glucurono-D-xylan dyed with Remazol Brilliant Blue R (RBBxylan) was used to analyze the enzymatic activity of the xylanase obtained. The samples were incubated with the substrate in a bain-marie at a temperature of 50 °C for 30 min, at a pH of 6.00; adjusted with the aid of Sodium phosphate buffer. The reaction was stopped with the addition of a solution of 0.014 N hydrochloric acid diluted in ethanol.

The substrate degradation into precipitated products is proportional to the endo-xylanase activity. The blue coloration of the supernatant is due to non precipitation of intact Remazol and is read in a spectrophotometer at 585 nm. One Unit of enzyme is defined as the sum of enzyme that releases 1 μmol of
reducing sugar equivalent D-xylose from arabinoxylan at pH 6.00, per minute, at 50 °C (Prozyn, 2008)

2.2.3 Isolation by SDS PAGE

The lyophilized precipitate was thawed and mixed with sample loading buffer. The samples were prepared with sample loading buffer as per the combination given in table 2.3. The sample loading buffer contains SDS, glycerol, β-mercaptoethanol, Tris buffer, and Bromophenol Blue. They were heated at 95 °C for 10 minutes and then spun in microfuge for 5 minutes. Bottom spacer was removed and the sandwich was clipped to the electrophoresis apparatus filled with 1X Tris-glycine-SDS Buffer in the lower chamber. Carefully remove the comb from the gel and fill the top of the apparatus with 1X Tris-glycine-SDS Buffer.

Table 2.3 Sample preparation for SDS-PAGE

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample</th>
<th>Sample Volume</th>
<th>Sample Loading Buffer (SLB) volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium Range Marker</td>
<td>10μl</td>
<td>10μl SLB for SDS-PAGE</td>
</tr>
<tr>
<td>2</td>
<td>Sample EY1</td>
<td>50 μl</td>
<td>20μl SLB for SDS-PAGE</td>
</tr>
<tr>
<td>3</td>
<td>Sample EY1</td>
<td>50 μl</td>
<td>20μl SLB for SDS-PAGE</td>
</tr>
<tr>
<td>4</td>
<td>Sample EY1</td>
<td>50 μl</td>
<td>20μl SLB for SDS-PAGE</td>
</tr>
</tbody>
</table>

The samples were loaded in different gels, into the bottom of the wells using microlitre syringe or micropipette fitted with long tip. The apparatus was connected to the power supply and electrophoresis was started at 50 V for the first 30 min and then increased the voltage to 100 V. When the dye front was at 0.5 cm above the bottom of the gel, power pack was turned off. The gel plates were removed and the gels were washed with distilled water for 30 seconds. After washing the gels were stained with Coomassie staining solution.
2.2.4 Confirmation by Zymography

The lyophilized precipitate was thawed and mixed with sample loading buffer. The samples were prepared with sample loading buffer for zymography that contains glycerol, β-mercaptoethanol, Tris buffer, Bromophenol Blue and not SDS, as per the combination given in table 2.4.

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample</th>
<th>Sample Volume</th>
<th>Sample Loading Buffer (zSLB) volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Sample EY1</td>
<td>30μl</td>
<td>10μl zSLB for zymography</td>
</tr>
<tr>
<td>2</td>
<td>Sample EY2</td>
<td>37μl</td>
<td>10μl zSLB for zymography</td>
</tr>
</tbody>
</table>

Bottom spacer was removed and the sandwich was clipped to the electrophoresis apparatus filled with 1X Tris-glycine-SDS Buffer in the lower chamber. The comb was removed carefully from the gel and the top of the apparatus was filled with 1X Tris-glycine-SDS Buffer. The samples were loaded into the bottom of the wells using microlitre syringe or micropipette fitted with long tip. The apparatus was connected to the power supply started electrophoresis at 50 V for the first 30 min and then increased the voltage to 100V.

When the dye front was at 0.5 cm above the bottom of the gel, power pack was turned off. The gel plates were removed and the gels were washed with distilled water for 30 seconds. The Gel was then transferred to a renaturation solution, and rocked for 30 mins at 37°C. After rinsing the gel is developed using developing buffer, till the dark brown coloration appears.

2.2.5 Optimum pH Determination

Using the enzymatic activity methods described above, the curves of optimum pH for the action of Xylanase enzyme studied were drawn. For the pH
curves, the temperature was set at the value of the method (50 °C), and readings of enzyme activity were made at pH 4.0 to 11.0 at the interval of 0.5 pH.

The various buffer system used to determine optimum pH were Citrate Phosphate buffer for pH of 4.0, 4.5, 5.0 and 5.5, Phosphate buffer for pH of 6.0, 6.5, 7.0, and 7.5, Tris-HCl buffer for pH of 8.0, 8.5, 9.0 and 9.5, Glycine-NaOH buffer for pH 10.0, 10.5 and 11.0. The effect of pH on enzyme activity in the present research was investigated using 0.2 M sodium citrate buffer at different pH values. The optimum pH of the enzyme was determined under the standard assay conditions by measuring activity in the presence of buffers at different pH values.

One ml of Xylanase solution was mixed with 3 ml of buffer at various pH from 4.0 to 11.0 in different test tubes and add substrate for then the mixture was incubated at 50 °C for 10 minutes. After incubation period the solution read in a spectrophotometer at 585 nm.

2.2.6 Optimum Temperature Determination

The effect of temperature on enzyme activity was examined under standard assay conditions at different temperatures ranging from 15 to 65ºC and the buffer brought to relevant temperature before the assay.

1 ml of xylan solution was mixed with 3 ml of buffer (pH 7) in 6 different tubes and the mixture in each tube is maintained at different temperature range from 15 °C to 65 °C with 10 °C interval for 30 minutes to attain the respective temperature. After 30 minutes 0.5 ml of the enzyme extract from T. reesei was added to the tube containing substrate and buffer. The enzyme substrate mixture was immediately transferred into a quartz cuvette and placed in the spectrophotometer.
Absorbance values were read at 585nm and the experiment was repeated with the other set of 6 tubes of varying temperature using recombinant *E. coli* as enzyme source. The OD values of the assay solution were plotted against the reaction time to determine the optimum temperature of the enzymes.

### 2.2.7 Determination of Michaelis-Menten Kinetics

Michaelis-Menten constant (\(K_m\)) and maximum velocity (\(V_{max}\)) of xylanase was determined using xylan as substrate at concentrations ranging from 2 mgml\(^{-1}\) to 10 mgml\(^{-1}\) according to standard assay conditions. 1 ml of 1 % xylan at varying concentrations like 2, 4, 6, 8 and 10 mgml\(^{-1}\) was taken in 5 different tubes as shown in table 2.5. 3 ml of buffer (pH-7.0) is added to all tubes, mixed and allowed to stand for 10 minutes.

**Table 2.5 Substrate concentration variation**

<table>
<thead>
<tr>
<th>Test tube No.</th>
<th>Volume of 1 % xylan (in ml)</th>
<th>Volume of buffer (pH-7) (in ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>3 (blank)</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>2.6</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>0.8</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

0.5 ml of the *T. reesei* enzyme extract was added to the tube containing substrate and buffer. The enzyme substrate mixture was immediately transferred into a quartz cuvette placed in the spectrophotometer. Absorbance values were read at 585 nm and the experiment was repeated with the other set of 5 tubes of varying xylan concentration (2 to 10 mgml\(^{-1}\)) using recombinant *E. coli* as enzyme source. The OD values of the assay solution were plotted against the reaction time to determine the kinetic constants.
2.2.8 In-gel digestion for LC-MS

The CBB stained gel containing the corresponding band for xylanase was rinsed with water for few hours and the corresponding band was cut into cubes of 1 mm dimension. The gel pieces were destained with 100 mM ammonium bicarbonate-acetonitrile mixture at room temperature until gel pieces became white and shrunken. They were then dried in vacuum centrifuge. The dry gel pieces were added with ice cold trypsin buffer such that the gel pieces are completely covered by buffer and left in an ice bucket or a fridge. After 2 hrs, 20 μl of ammonium bicarbonate buffer is added and incubated at 37 °C overnight. The supernatant was discarded and 100 μl of formic acid-acetonitrile mixture was added into gel pieces. The tubes were spinned and the supernatant was collected. Peptide extracts in the supernatant were dried in a vacuum centrifuge and stored at -20 °C for further analysis (Shevchenko et al 2006).

2.2.9 Nano-LC-MS/MS Analysis

The Complex tryptic peptide mixtures obtained were separated using Agilent 1200 High performance liquid chromatography which has a 1D nano-LC setup consisting of a capillary pump for loading the sample onto a trap column and a nano pump for the separation on the analytical column. The standard gradient of 70 min length was used (Junqueira et al 2008a). The sample was prepared by vortexing the tryptic digest with 0.1% formic acid for 1 hr followed by centrifugation at 10,000 rpm for 5 min. The supernatant was taken onto Agilent auto sampler vials. The ions for later mass spectrometric analysis were generated using an automated nano-ESI source called Nanomate Triversa (Advion). A specifically designed LC coupler connects the flow from the LC to the ESI chip where the nano-ESI generated ions are transferred into the mass spectrometer. The spray current is monitored and at the beginning of a standard gradient, it should be above 50 nA. The MS spectrum was obtained using LTQ Orbitrap Discovery, a hybrid type MS system with the ability to determine accurate m/z of intact precursors in the orbitrap analyzer. 12.5 fmol of BSA
digest was also loaded on column as control to check the performance of the experiment.

2.2.10 In silico analysis

Protein identification and analysis software performs a central role in the investigation of proteins from two dimensional (2D) gels and mass spectrometry. For protein identification, certain empirically acquired information was matched against a protein database to define a protein as already known or as novel. For protein analysis, information in protein databases is used to predict certain properties about a protein that might be useful for its empirical investigation.

![UniprotKB Analysis](image-url)
use with both Gram positive bacteria like Staphylococcus aureus and Mycobacterium avium and Gram negative bacteria like Escherichia coli and Salmonella typhimurium for providing instant stabilization of RNA.

15 ml culture was added into the tube and mixed immediately by vortex mixer for five seconds and incubated for 5 min at room temperature. After incubation the tubes were centrifuged for 10 min at 4500 rpm. The supernatant was decanted and the tubes were left inverted on a tissue paper for 10 seconds. The pellets were frozen ethanol-dry ice mix and stored at -20 °C for 2 weeks.

4.1.6.1 Isolation of RNA

2 μl of Proteinase K and 300 μl of Cell Lysis solution were used to resuspend cell pellet by mixing thoroughly. The mix was transferred to 1.5 ml tube and incubated at 65 °C for 45 min with vortexing at every 15 min in between incubation period. The cell sample was kept on ice bath for 5 min.

150 μl of MPC protein Precipitation Reagent was added to 300 μl of lysed sample and vortex mixed for 10 s. Then it was spun in a cooling microcentrifuge at 3000 rpm for 10 min in 4°C and the supernatant was transferred to a clean tube. 50 μl of MPC protein Precipitation Reagent was added and the same was repeated. 500 μl isopropanol was added to the recovered supernatant and the tube was shaken well for 30 to 40 times. RNA was obtained as pellets after centrifugation at 4°C for 10 min in a microcentrifuge. The isopropanol was carefully poured off without dislodging the RNA pellet. All the residual isopropanol was removed with a pipette and the pellet was allowed to air dry for 15 min.

4.1.6.2 Removal of contaminating DNA

10 μl of RNAse free DNAse I was diluted up to 200 μl with 1X DNAse buffer. The RNA pellet was completely resuspended in 200 μl of DNAse buffer. The RNA pellet was completely resuspended in 200 μl of DNAse buffer. The RNA pellet was completely resuspended in 200 μl of DNAse buffer.
Figure 2.4 MASCOT Analysis

The various tools used in in-silico analysis are UniprotKB/Swissprot analysis (Figure 2.1) for comparing the obtained sequence with databases, BLAST (Figure 2.2) to find local similarity regions between sequences that can be used to infer functional and evolutionary relationships between sequences, Protparam (Figure 2.3) to compute various physicochemical properties, MASCOT Analysis (Figure 2.4), MS BLAST Search and Peptide mass tool to assist in peptide mapping.