CHAPTER-3

Materials and Methods

In this chapter, methodology adopted for xylanase production in submerged and solid state fermentation and in bioreactor is described. Biochemical characterization was carried out after purification of xylanase. Quality of xylanase-supplemented bread was analyzed with respect to rheology, color, texture, thermal properties, XRD measurements, sensory evaluation, FTIR spectroscopy, and scanning electron microscopy (SEM). Mathematical models for enzyme kinetics and dough rheology have been described.

3.1 Materials

3.1.1 Chemicals

Different media constituents were purchased from Merck (Bangaluru, India), Hi-Media (Mumbai, India), SD Fine (Mumbai, India) and CISCO (Bangaluru, India). Gel chromatography chemicals and markers were obtained from Bangalore Genei, (Bangaluru, India). The ion exchange matrices were obtained from Pharmacia Biotech (Uppasala, Sweden). N, N', N'-Tetramethylene diamine (TEMED) was purchased from Merck (Bangaluru, India). All chemicals used were of analytical grade.

3.1.2 Reagents and media

Various nutrient media were used for the isolation of microorganisms from pulp and paper industry waste as well as from soil. Composition of various media are given below.

3.1.2.1 Minimal medium

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.5 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>5.0 g</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.1 g</td>
</tr>
</tbody>
</table>
Xylan 1.0 g
All the above chemicals were dissolved in distilled water. Final pH of the medium was adjusted to 5 to 7.

3.1.2.2 Xylanase screening medium

Positive isolates from the minimal medium were grown in xylanase screening medium separately.

KCl 0.5 g
MgSO₄·7H₂O 0.5 g
NH₄NO₃ 5.0 g
MnSO₄·H₂O 0.1 g
FeSO₄·7H₂O 0.1 g
Xylan 10 g
Agar powder 15 g

All the above chemicals except agar powder were dissolved in distilled water. The pH of the medium was adjusted to desired pH range using 6N HCl and 1N NaOH prepared using sterile distilled water. Required amount of agar powder was added for plating.
Positive isolates from xylan screening medium agar plates were allowed to grow in nutrient broth medium and Czapek yeast extract medium to ascertain the compatibility of the microorganisms for optimum growth and yield of targeted products.

3.1.2.3 Nutrient broth medium

Solid medium 13 g
Distilled water 1 L
Agar powder 15 g
3.1.2.4 Czapek yeast extract medium

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>30 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Czapek concentrate</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

Czapek concentrate was prepared using

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaN03</td>
<td>300 g</td>
</tr>
<tr>
<td>KCl</td>
<td>50 g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>50 g</td>
</tr>
<tr>
<td>FeSO₄.7H₂O</td>
<td>1.0 g</td>
</tr>
</tbody>
</table>

All the above chemicals were dissolved in sterile distilled water. Czapek concentrate was prepared separately and added according to the requirement in the medium. pH of the medium was adjusted by adding 6N HCl or 1N NaOH.

3.1.3 Maintenance medium

3.1.3.1 Potato dextrose agar

The fungal isolates were maintained in potato dextrose agar (PDA) plates of the following composition:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato infusion</td>
<td>250 g</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
</tbody>
</table>

3.1.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS–PAGE was used for the determination of molecular weight. Reagents for SDS-PAGE are as follows:
### 3.1.4.1 30% Acrylamide bisacrylamide (ABA)

- Acrylamide: 30 g
- Bisacrylamide: 0.8 g

All the above chemicals were dissolved in 100 mL sterile distilled water. During storage, acrylamide and bisacrylamide are slowly converted to acrylic acid and bisacrylic acid. The deamination reaction is catalyzed by light and alkali. Therefore, the reagent was stored in dark brown bottle.

### 3.1.4.2 10% Sodium dodecyl sulphate (SDS) reagent

Stock solution of 10% (w/v) SDS was prepared in deionized water and stored at room temperature.

### 3.1.4.3 Upper Tris: 1.0 M, pH 6.8

- Tris-HCl: 12.12 g
- Distilled water: 100 mL

Final pH was adjusted to 6.8 with 6N HCl.

### 3.1.4.4 Lower Tris: 1.5 M, pH 8.8

- Tris-HCl: 18.17 g
- Distilled water: 100 mL

Final pH was adjusted to 8.8 with 6N HCl.

### 3.1.4.5 Reserver buffer (4X stock)

- Tris-HCl: 3.0 g
- Glycine: 14.4 g
- Distilled water: 250 mL

Working solution was prepared by diluting it to 1X using distilled water and adding SDS solution at 0.1% final concentration.
3.1.4.6 Ammonium persulphate 10% (w/v)

10 g ammonium persulphate (APS) was dissolved in 100 mL deionized water. Freshly prepared APS reagent was used before running the gel.

3.1.4.7 N,N,N',N' - Tetramethylethlenediamine (TEMED)

TEMED was used directly without any dilution.

3.1.4.8 Sample buffer (5X)

Glycerol 2.0 mL
β-mercaptoethanol 1.25 mL
Upper Tris 3.0 mL
20% SDS 2.5 mL
Distilled water 1.25 mL

3.1.5 Staining of SDS-PAGE gel

3.1.5.1 Coomassie brilliant blue staining

(A) Staining solution

Acetic acid 10 mL
Methanol 40 mL
Distilled water 50 mL
Coomassie blue (R-250) 0.1 g

(B) Destaining solution

Acetic acid 10 mL
Methanol 40 mL
Distilled water 50 mL
3.1.5.2 Silver staining

(A) Reducing solution

Reducing solution was prepared by dissolving 20 mg sodium thiosulphate in 100 mL distilled water.

(B) Silver nitrate solution

Stock solution of silver nitrate (20%, w/v) was prepared by dissolving 20 g silver nitrate in 100 mL double distilled water. It was diluted to 0.1% using double distilled water before use.

(C) Fixative

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>10 mL</td>
</tr>
<tr>
<td>Ethanol</td>
<td>40 mL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

(D) Developer

50 µL formaldehyde was added in 100 mL 6% sodium carbonate solution.

(E) Stopping reagent

1% acetic acid (v/v) was prepared in distilled water.

3.1.6 Estimation of reducing sugar (DNSA method)

3.1.6.1 DNSA reagent

Dinitro salicylic acid (10.6 g) and sodium hydroxide (19.8 g) were dissolved in 1416 mL distilled water and then following chemicals were added:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium potassium tartarate</td>
<td>316 g</td>
</tr>
<tr>
<td>Phenol</td>
<td>7.6 g</td>
</tr>
<tr>
<td>Sodium metabisulphite</td>
<td>8.3 g</td>
</tr>
</tbody>
</table>

The reagent was stored in brown bottle at 4°C.
3.1.6.2 Standard solution
Stock Solution: Xylose (0.1M) was prepared in distilled water. For the standard curve, 0.01M to 0.09M of xylose was prepared using distilled water.

3.1.7 Estimation of total sugar (Anthrone method)
3.1.7.1 Anthrone reagent (AR)
Anthrone 200 mg
Concentrated H₂SO₄ 100 mL
The reagent was chilled for 2 h prior to use.

3.1.7.2 Glucose standard
Stock solution: Glucose (1mg/mL) was prepared in distilled water.

3.1.8 Estimation of protein (Lowry method)
Reagent A: 2% sodium carbonate in 0.1N NaOH.
Reagent B: 1% CuSO₄·5H₂O in distilled water.
Reagent C: 1% sodium potassium tartrate in distilled water.
Freshly prepared working solution
98 mL reagent A + 1mL reagent B + 1mL reagent C
Folin Cio-calteau reagent
Folin Cio-calteau reagent was diluted with distilled water in 1:1 ratio.

3.2 Methodology
3.2.1 Isolation and screening of xylanase producing organism
Paper pulp industry waste (1g) and soil samples (1g) were suspended in 10 mL sterilized distilled water separately; vortexed thoroughly to prepare uniform slurry and plated on to minimal media containing 0.1% birchwood xylan agar plates. The plates were incubated at 30, 40, 50 and 60°C respectively and checked for appearance of colony of xylan degrading organism after every 24 h upto 10 days. The positive isolates were further
purified by re-streaking the individual colony on xylanase screening medium agar plate and incubating at 30-50°C upto 10 days. Isolation was also carried out by enrichment technique. Xylanase producing organisms were obtained by suspending various soil and wastes samples at 1% concentration in 100 mL minimal medium and incubating at selected temperatures (30-50°C) in an orbital shaker (200 rpm). After 24-48 h of incubation, 1% (v/v) inoculum was serially transferred to screening medium and incubated at various temperatures (30-50°C). After the appearance of desired growth, samples were spread plated onto xylanase screening medium (agar plates having pH 4, 5, 6, 7, 8) to obtain xylanase positive isolates. The isolates were simultaneously transferred to production medium at 1% final concentration. The process was repeated several times to obtain potent xylan degrading organisms. All the isolated strains were identified on the basis of standard biochemical methods. The isolates were screened for the production of xylanase by inoculating in xylanase screening media, minimal media, nutrient broth and Czapek yeast extract media. Production of xylanase by each organism was checked in these media at different pH's (4.5–7.5) by incubating at 30 and 50°C in an orbital shaker at 200 rpm. Samples were withdrawn at 24 h interval and centrifuged at 10,000 rpm for 20 min to obtain clear supernatant for determination of xylanase activity at 50°C, pH 5.

3.2.2 Identification of selected isolate

Preliminary studies on the xylanase production were carried out for all the isolates by measuring the enzyme activity. The strain exhibiting maximum xylanase activity was isolated for further optimization and characterization studies. Morphological study of the isolated strain was carried out by scanning electron microscopy (SEM). Best strain was deposited at Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh.

3.2.3 Scanning electron microscopy (SEM)

The isolated strain was grown on Czapek yeast extract agar at 30°C and allowed to sporulate. The spores were then transferred in the Czapek yeast extract medium and
allowed to grow for 5 days, filtered and lyophilized. SEM was performed on lyophilized powder of cells which was mounted on double side sticky tape on aluminum stubs and sputter coated with gold and palladium alloy. The samples were then visualized and the representative areas were photographed under SEM (JSM-6100, JEOL, Tokyo, Japan) at an accelerated voltage of 10KV and magnification in the range of 100-2000X.

3.2.4 Growth curve and culture conditions in submerged fermentation in shake flask

Growth pattern of selected isolate was observed at 5 pH, (30°C for 10 days) in Czapek yeast extract medium containing 1% birchwood xylan. Spore suspension was prepared by scraping spores in sterile distilled water and vortexing vigorously to prepare uniform suspension. Serial dilutions of spores were then prepared in sterile distilled water and 20 µL each dilution was spreaded on plates containing Czapek yeast extract agar medium with 1% birchwood xylan. Number of spores grown on each plate were counted after 3 days of incubation at 30°C. The xylanase production was studied by inoculating 1mL spore suspension containing 3x10^8 spores in 250 mL conical flask containing 50 mL sterile Czapek yeast extract media. The culture flasks were incubated at 30°C in an orbital shaker at 200 rpm for 12 days and samples were withdrawn after every 24 h. Samples were centrifuged at 10,000 rpm for 20 min and the supernatant was used to estimate xylanase activity, pH profile, extracellular protein, total reducing sugar and residual sugar.

3.2.5 Culture conditions in solid state fermentation in shake flask

To study the growth pattern and xylanase production in solid state fermentation, ground powder (<0.5 mm particle size) of selected lignocellulosic materials (5 g each) were taken in shake flask and the solid materials were moistened with different moistening media in different solid to moisture ratio (1:1–1:8) in 250 mL conical flask. After sterilization, flasks were inoculated using spore suspension described in submerged fermentation process (section 3.2.4). The culture flasks were incubated at 30°C in incubator for 12 days and samples were withdrawn after every 24 h. Citrate buffer (0.05 M, pH 5.5, 50 mL) was used for extraction of the enzyme. Samples were filtered using
sterilized muslin cloth and the supernatant was centrifuged at 20,000 rpm for 30 min and was used to measure xylanase activity, pH profile, extracellular protein, residual sugar.

3.2.6 Analytical procedures

3.2.6.1 Xylanase assay

Birchwood xylan (1%, w/v) was used as substrate in 0.05 M citrate buffer pH 6.0 in xylanase activity assay. After incubation at 50°C for 5 min, the reaction was terminated by adding dinitro-salicylic acid reagent followed by 5 min boiling (Miller, 1959). Absorbance of the mixture was measured at 540 nm. Standard curve was prepared using 0.01 M xylose stock. One unit of enzyme activity was defined as the quantity of enzyme liberating 1 µmol of xylose/min (Bailey, 1992).

3.2.6.2 Cellulase assay

Cellulase activity was assayed using 50 mg filter paper (Whatman No.1) as substrate in 0.5 mL, phosphate buffer (0.05M, pH 6.0) by incubating it with 0.5 mL crude enzyme extract at 50°C for 1h. The reaction was quenched by adding 2 mL dinitro-salicylic acid reagent followed by 5 min boiling. Absorbance was measured at 540 nm. Standard graph was made using 1mM glucose stock. One unit of cellulase activity was defined as the quantity of enzyme that released 1 µmol of glucose/min (Ghosh, 1994).

3.2.6.3 Estimation of protein content

Extracellular protein content was estimated by Lowry method using bovine serum albumin (BSA) as standard (Lowry, 1951). Standard curve was prepared using BSA (1 mg/mL) dissolved in distilled water. The absorbance was monitored at 680 nm.
3.2.6.4 Estimation of sugar content

(A) Total sugar

Total sugar content of the culture broth was estimated by Somogyi’s (1945) method using Anthrone reagent. The Anthrone reaction is very rapid and a convenient method for determination of hexoses, aldopentoses and hexouronic acids present in free form or in polysaccharides. A suitably diluted 0.5 mL sample was taken in 10 mL test tube, 1.5 mL distilled water was added and the tubes were chilled for 15 min in ice, and 4 mL chilled Anthrone reagent was added by layering with pipette. The contents were thoroughly mixed by vortexing and tubes were incubated at room temperature for 10 min. The samples were then incubated in boiling water bath for 10 min and cooled in ice-bath for 2-3 min. The absorbance was measured at 625 nm against a reagent blank. Standard curve using glucose stock solution was prepared in 10-100 μg/mL range. The sugar content was expressed in %.

(B) Total reducing sugar

Reducing sugar content in the culture broth was estimated by di-nitrosalicic acid method (Miller, 1959) and expressed as % using glucose (1mg/mL) as standard. In 1mL of suitably diluted supernatant, 1mL distilled water and 3 mL DNSA reagent were added. The samples were then incubated in a boiling water bath for 10 min followed by cooling at room temperature. The samples were further diluted by adding 5 mL distilled water and the contents were thoroughly mixed by vortexing. The absorbance was measured at 600 nm. Reducing sugar content was estimated from the standard curve of glucose prepared in 0.2-2.0 mg/mL range.

3.2.6.5 Estimation of cell mass

The samples were centrifuged at 10,000 rpm for 20 min and the precipitated cell mass was washed with distilled water. Centrifugation and washing were repeated twice. The cell mass was dried in pre-weighed aluminium cups at 100°C till constant weight achieved. The amount of dry cell mass was expressed in g/L of culture broth.
3.2.7 Optimization of xylanase production in submerged fermentation in shake flask

The effect of medium composition and environmental factors on xylanase production by selected isolates was studied in shake flask to maximize the xylanase yield.

3.2.7.1 Carbon source

Various carbon sources (xylan, xylose, glucose, lactose, maltose, glycerol, starch, sorbitol, and mannitol) were used at 1% level in the production medium, which essentially consisted of sucrose medium without any carbon source. In addition, varying combinations of sucrose:xylan ratio (3:1, 1:1, 1:3, 0:4) were used to study the xylanase production pattern at 30°C, pH 5.5 in an orbital shaker at 200 rpm.

3.2.7.2 Nitrogen source

Effect of organic and inorganic sources on xylanase production, extracellular protein and cell mass yield was determined at 0.5% final concentration by inoculating 3x10^8 spores/mL in 250 mL Erlenmeyer flask containing 50mL Czapek yeast extract medium. The organic nitrogen sources e.g. peptone, meat extract, tryptophan, soya peptone, brain and heart infusion, soyabean meal, beef extract, yeast extract, protease peptone, and inorganic nitrogen source like urea, sodium nitrate (NaNO₃), ammonium chloride (NH₄Cl), ammonium sulphate (NH₄)₂SO₄, etc. were used in the basal medium having the composition of sucrose medium without any nitrogen source. All the experiments were carried out in duplicate at 30°C, pH 5.5 in an orbital shaker at 200 rpm.

3.2.7.3 Effect of inducer on xylanase production

Xylan is the inducer for the production of xylanase in submerged fermentation. Different concentrations of xylan (0.5 to 2.0%) were used in the production medium which essentially consisted of sucrose medium without any carbon source.
3.2.1 Environmental factors on the growth and xylanase production by *P. citrinum*

Effect of environmental factors on the fungal growth and xylanase production was studied. To study the effect of temperature on xylanase production, incubation was carried out at 25, 30, 35, 40, 45, and 50°C by inoculating $3 \times 10^8$ spores/mL in optimized medium on a rotary shaker at 200 rpm. Samples were withdrawn at regular intervals, centrifuged at 10,000 rpm for 20 min and analyzed for the estimation of xylanase activity, extracellular protein, cell mass, total sugar, and residual substrate. Similarly, the production pattern of xylanase was studied in the medium of different pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0) at 30°C at 200 rpm. Effect of agitation was studied at 100, 150, 200, 250 and 300 rpm.

3.2.8 Optimization of xylanase production in solid state fermentation

Effect of medium composition and environmental factors of xylanase production by selected isolates was studied in solid state fermentation in shake flask to maximize the xylanase yield.

3.2.8.1 Effect of different lignocellulosic substrate on xylanase production

Wheat straw, wheat bran, rice straw, rice bran, sugarcane bagasse, xylan, sawdust and corn cobs were used as lignocellulosic substrates for xylanase production in solid state fermentation. Each substrate (5 g) was transferred into 250 mL Erlenmeyer flask and moistening media containing 3.0% sucrose, 0.5% yeast extract, 0.1% K$_2$HPO$_4$, 3.0% NaNO$_3$, 0.5% KCl, 0.5% MgSO$_4$, and 0.01% FeSO$_4$ was added such that the final solid to moisture ratio was 1:5. The flasks containing substrate and moistening media were then sterilized for 15 min at 15 psig and 121°C. One loop full of organism was suspended in 10 mL sterilised distilled water. Effect of substrates on xylanase production was evaluated by adding the inoculum $10^8$ spores/g of dry substrate at 1:5 substrate to moisture ratio and 5.0 pH. The flasks were incubated at 30°C for 5 days.
3.2.8.2 Effect of treatment of lignocellulosic substrate on xylanase production

All the lignocellulosic substrates were dried at 40°C till constant weight in a tray dryer (Basic Technology Private Limited, Kolkata), ground in a mixer (Philips, India), passed through a sieve of 0.5 mm aperture size and used as substrate in SSF.

The selected substrate was cleaned using distilled water, dried, and treated. The treatment included: (i) alkali treatment using 1N NaOH followed by neutralization with 1N HCl and repeated washing with distilled water; (ii) acid treatment with 1N HCl followed by neutralization with 1N NaOH and washing with distilled water; and, (iii) steam treatment at 121°C for 2 h. The substrate was dried at 50°C till constant weight, ground to 0.5 mm particle size, packed in polyethylene bags and stored until use. Untreated substrate was used as control.

3.2.8.3 Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) study of the treated and untreated lignocellulosic residues

Treated and untreated lignocellulosic residues were tested under scanning electron microscope. SEM was performed on dried powdered samples which were mounted on double side sticky tape on aluminium stubs and sputter coated with gold and palladium alloy. The samples were then visualized and the representative areas were photographed under SEM (JSM-6100, JEOL, Tokyo, Japan) at an accelerated voltage of 10KV and magnification in the range of 100-2000X.

Fourier Transform Infrared Spectroscopy (FTIR) is a spectroscopic technique based on absorption of infrared photons that excite vibrations of molecular bonds. A spectrum of characteristics bands is obtained that can be used as a fingerprint to help in identification and characterization of molecular bonding present in the samples. FTIR offers a fast and non-destructive alternative to chemical measurement techniques of qualitative characterization. IR spectra were recorded at room temperature (28± 2°C) with a Tensor-27 spectrophotometer (Bruker, Germany) in the range of 600-4000 cm⁻¹ by accumulating 16 scans at 4 cm⁻¹ resolution. For spectral measurement of lignocellulosic residue platinum ATR (attenuated total reflectance) from Bruker was used. About 2 mg of dried treated and untreated powdered lignocellulosic residues were placed on ATR for
measurement. The peak intensity of each samples were measured and this entire spectra acquisition procedure took 1 min per sample.

3.2.8.4 Nitrogen source

The organic nitrogen sources (peptone, meat extract, tryptophane, soya peptone, brain and heart infusion, soya bean meal, beef extract, yeast extract, protease peptone), and inorganic nitrogen source like urea, sodium nitrate, ammonium chloride, ammonium sulphate were used in the basal medium (Czapek yeast extract) without adding any nitrogen source.

3.2.8.5 Optimization of different environmental factors using response surface methodology

The substrate giving the maximum xylanase production was subsequently used to investigate the effect of selected process variables (pH, temperature, solid to moisture ratio, and incubation time) on xylanase production. After the addition of inoculum, flasks containing substrate and moistening agent at the ratio of 1:4-1:6. pH 5.0-7.0 were incubated at 25-35°C for 4-6 days.

3.2.8.6 Experimental design and data analysis

Optimum operating conditions were determined using RSM with 2^4 factorial designs for the process variables A, B, C, D (pH, temperature, substrate to moisture ratio, and incubation time) with three levels each. Enzyme activity (U/mL) after desired period of incubation was measured as the response (equation 3.1).

\[ Y = b_0 + \sum b_i X_i + b_{ij} X_i^2 + \sum b_{ij} X_i X_j \]  

(3.1)

where, \( Y \) is predicted response (xylanase activity); \( X_i, X_i^2, X_i X_j \) are the variables representing linear, quadratic, and interactive effect; \( b_i \) is regression coefficient at the centre of the regression model; and \( b_i, b_{ij}, b_{ij} \) are coefficients estimated by the model, representing the linear, quadratic, and interactive effects of the coded variables (A, B, C, D).
Design Expert ver. 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN) was used to carry out the statistical analysis. Data was analyzed to estimate whether a given term has a significant effect (p≤0.05) on enzyme activity using the analysis of variance (ANOVA) combined with the Fischer test. Graphical and mathematical analysis was carried out using Design Expert program to determine optimum intensity level of the variables.

The overall second order polynomial relationship between Y and the variables can be represented as:

\[ Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_1A^2 + b_2B^2 + b_3C^2 + b_4D^2 + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD + \ldots \]  

(3.2)

where, A, B, C, D are the coded variables for pH, temperature (°C), substrate to moisture ratio, and incubation time (days); and, \( b_0, b_1, b_2, b_3, b_4, b_{11}, b_{12}, b_{13}, b_{14}, b_{22}, b_{23}, b_{24}, b_{33}, b_{34}, b_{44} \) are the coefficients.

3.2.9 Optimization studies for the production of xylanase in a laboratory fermenter

The production pattern of xylanase by the selected isolates was studied in a 5L laboratory fermenter (B-lite, Sartorious, Germany) with a working volume of 3L. The medium and production conditions were selected on the basis of the studies carried out in shake flask. The fermenter was run at 30°C with an aeration and agitation rates of 1.0 vvm (volume volume per min) and 300 rpm, respectively. The inoculum was prepared by inoculating three 500 mL flasks each containing 100 mL sterile Czapek yeast extract medium with \( 1 \times 10^8 \) spore/mL and incubating at 30°C, 200 rpm for 120 h. Fermenter vessel containing Czapek yeast extract medium was sterilized with all attachments in autoclave (PSM, Vertical Autoclave, Peenya, Bangaluru, India) for 20 min at 121°C, 15 psig pressure. The fermenter was equipped with pH, temperature, dissolve oxygen, agitation and antifoam control system.
3.2.9.1 Production studies at different substrate concentrations

Effect of substrate concentration on the growth and xylanase production was studied without pH control in a 5L fermenter with a working volume of 3L using Czapek yeast extract medium of varying carbon concentrations (5-15 g/L) at 30°C with aeration and agitation of 1.0 vvm and 300 rpm, respectively. Samples were withdrawn aseptically after every 24 h, centrifuged at 10,000 rpm for 20 min and analyzed for xylanase activity, extracellular protein, cell mass and residual substrate concentration.

3.2.9.2 Production studies at different temperatures

Effect of temperature on the growth and xylanase production was studied without pH control in the fermenter using czapek yeast extract medium of varying temperatures (25, 30, 35, 40°C) with aeration and agitation of 1.0 vvm and 300 rpm, respectively. Samples were withdrawn aseptically at every 24 h, centrifuged at 10,000 rpm for 20 min and analyzed.

3.2.9.3 Production studies at different agitation rates

The effect of shear stress on xylanase production was studied in the fermenter at different agitation rates (200, 300, 400, 500 rpm) at 30°C, 1.0 vvm aeration, without pH control in Czapek yeast extract medium. Samples were withdrawn aseptically after every 24 h interval and analyzed.

3.2.9.4 Production studies at different aeration rates

To study the effect of dissolve oxygen concentration (5-20%) on growth and production of xylanase, fermenter was run at various aeration rates (0.5, 1.0 and 1.5 vvm), keeping all other parameter constant. Samples were withdrawn aseptically at every 24 h and centrifuged at 10,000 rpm for 20 min to obtain a clear supernatant for the measurement of xylanase activity, extracellular protein, residual substrates, cell mass and pH profile using standard assay methods.
3.2.9.5 Production studies without pH control

As the pH of the medium significantly affects the productivity of xylanase, various fermenter runs were carried out using Czapek yeast extract medium of varying initial pH from 4, 5, 6, and 7 at 30°C with aeration and agitation rates of 1.0 vvm and 400 rpm, respectively. The pH of the medium was adjusted initially to the desired level after which it was only monitored and not controlled. Samples were withdrawn aseptically after every 24 h and centrifuged at 10,000 rpm for 20 min to obtain a clear supernatant for the measurement of xylanase activity, extracellular protein, residual substrates, cell mass and pH profile using standard assay methods.

3.2.9.6 Production studies with pH control

To get the better understanding of the effect of pH on the xylanase production, various experiments were carried out at controlled pH. The fermenter containing sterile Czapek yeast extract medium was aseptically inoculated with \(1 \times 10^8\) spores/mL and run at 30°C, with aeration and agitation of 1.0 vvm and 400 rpm, respectively. The initial pH of the medium was adjusted to 4-7 and programmed such that any change in pH was compensated by automatic addition of 6N HCl and 1 N NaOH. Bottles containing 6N HCl and 1N NaOH were sterilized along with the fermenter at 121°C, 15 psig pressures for 20 min. Various fermenter runs at different controlled pH (4, 5, 6, and 7) were taken. The samples were withdrawn aseptically after every 24 h, centrifuged at 10,000 rpm for 20 min and the supernatant was analyzed for the estimation of xylanase activity, extracellular protein, cell mass and residual substrate using standard assay process.

3.2.9.7 Estimation of volumetric oxygen transfer coefficient (\(K_{L,a}\))

Volumetric oxygen transfer coefficient (\(K_{L,a} \text{ h}^{-1}\)) in the fermenter was determined by dynamic gassing out method using respiratory action of growing culture by varying the oxygen concentration in the broth. The air supply was switched off to reduce the oxygen concentration in the broth due to the respiratory activity of the cell mass. After a time, the air supply was switched on and oxygen concentration will rise again until it returns to the initial steady state value. The value of \(K_{L,a}\) may be calculated as follows:
\[
\frac{dC_l}{dt} = K_{L,a}(C_l^* - C_l) - XQ_0,
\]
(3.3)

where, \(K_{L,a}(C_l^* - C_l)\) is oxygen transfer rate (OTR, kg.L\(^{-1}\).h\(^{-1}\)); \(XQ_0\) is oxygen uptake rate (OUR, kg.L\(^{-1}\).h\(^{-1}\)); \(Q_o\) is specific rate of oxygen consumption (h\(^{-1}\)); and, \(X\) (kg.L\(^{-1}\)) is the cell mass concentration.

Rearranging the equation (3.3) yields

\[
C_l = -\frac{1}{K_{L,a}}[\frac{dC_l}{dt} + XQ_0] + C^*
\]
(3.4)

Thus, a graph of \(C_l\) vs \([\frac{dC_l}{dt} + xQ_0]\) gives a straight line, with slope \((-\frac{1}{K_{L,a}}\)) and \(C^*\) as the intercept.

### 3.2.9.8 Modeling of microbial growth

Kinetics of microbial growth was analyzed from the dry cell mass measured in different conditions in due course of fermentation in fermenter using following rate equations.

\[
\mu = \mu_n s/(K_s + s)
\]
(Monod, 1949)  
(3.5)

\[
\mu = \mu_n \left(\frac{k_s}{1 + (k_s)^n}\right)
\]
(Moser, 1958)  
(3.6)

\[
\mu = \mu_n \left(\frac{1}{1 + \frac{K_k s}{s}}\right)
\]
(Contois, 1959)  
(3.7)

\[
\mu = \mu_n \left(\frac{s}{K_s + s + \left(s^3/K_i\right)}\right)
\]
(Edward, 1970)  
(3.8)

where,

- \(\text{Bi}\) = An empirical coefficient, (dimensionless)
- \(k\) = Biological rate equation coefficient, (M\(^{-1}\).L\(^3\))
- \(K_i\) = Inhibition constant, (dimensionless)
- \(K_k\) = An empirical constant in Contois equation, (dimensionless)
- \(s\) = Substrate concentration (mass per unit volume)
3.2.10 Purification of xylanase

The extracted crude enzyme was partially purified by ammonium sulphate precipitation (60%) followed by dialysis for overnight in 0.05M citrate buffer. The concentrated fraction after dialysis was further concentrated using Amicon cones of 10 KDa cut off by centrifuging (Sigma, USA) at 5000 rpm for 5 min. It was then loaded into Q-sepharose anion exchange column of 2 mL capacity equilibrated with 0.1M phosphate buffer containing 1M NaCl (pH 7.0) at a flow rate of 0.1mL/min using Pharmacia® FPLC system. The column was washed with 50 mL loading buffer and eluted with linear gradient 1M NaCl. The active fractions containing xylanase were pooled and concentrated using Amicon cones (UFMC, NMWC, 10 KDa) by centrifuging (Sigma, USA) at 5000 rpm for 5 min. The purification profile of xylanase of different step was estimated by denaturing polyacrylamide gel electrophoresis. Purified fraction was used for characterization study.

3.2.10.1 Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% polyacrylamide slab gels with 0.1% sodium dodecyl sulphate as described by Lammeli (1970). The gel was allowed to run at 10 mA for 2 h in the electrophoresis chamber (Bangaluru Genci, India) filled with Tris-glycine buffer (pH 8.3). The protein bands were visualized by staining with coomassie brilliant blue R-250.

3.2.10.2 Molecular characterization of purified xylanase

The purified xylanase was characterized in terms of its molecular weight.
For the estimation of subunit relative molecular weight of purified xylanase protein, 10% SDS-PAGE was run with the known protein standards under denaturing condition as described earlier. The standard proteins (Bangaluru Genei, India) used were bovine serum albumin (66KDa); ovalbumin (45KDa); glyceraldehydes-3- phosphate dehydrogenase (36 KDa); carbonic anhydrase (29 KDa); trypsinogen (24 KDa); trypsin inhibitor (20.1 KDa); α-lactalbumin (14.2 KDa); and, aprotinin (6.5 KDa). Proteins were visualized by staining with coomassie brilliant blue R250.

3.2.11 Biochemical characterization of purified xylanase

3.2.11.1 Determination of pH and temperature optima and pH and temperature stability

The optimum pH was determined by measuring the xylanase activity at 50°C over a range of pH from 5 to 11 using the following buffers: (50 mM) citrate buffer, pH 3 to 6; phosphate buffer, pH 6.5 to 8; and, glycine NaOH buffer, pH 8.5 to 11. The pH stability was determined after incubating the enzyme in these buffers for 24 h at room temperature (30±2°C) and the residual activity was measured. The optimum temperature was determined by assaying the xylanase activity for 5 min at temperatures ranging from 25 to 90°C at pH 6 using standard protocols. Thermal stability was determined by measuring the residual enzyme activity after incubation of enzyme solution at 50°C for 24 h at pH 6 without substrate.

3.2.11.2 Effect of additives on xylanase activity

Effect of various additives on xylanase activity was determined by incubating xylanase with different additives at room temperature (30± 2°C). Selected monovalent and divalent metal ions (Na⁺, Mg²⁺, Ca²⁺, Mn²⁺, Fe³⁺, Zn²⁺, Cu²⁺, Co²⁺, Hg²⁺), Tween 80, EDTA (10 mM) were used as additives. Xylanase activity with different additives (Yₐ,r) was monitored at every 15 min for 1 h.
\[ Y_{res} = \frac{Y_{ad}}{Y_c} \times 100 \]  

(3.9)

where, \( Y_{res} \) is residual activity (%); and \( Y_c \) represent xylanase activity in the absence of any additives.

3.2.11.3 Estimation of kinetic constants

The enzymatic reactions are modeled by Michaelis-Menten equation (3.10)

\[ v = \frac{V_{max} [S]}{K_m + [S]} \]  

(3.10)

where, \( V_{max} \) is maximum rate of product formation (U/mL.min); \( K_m \) is substrate concentration corresponding to \( (V_{max}/2) \) (mg/mL); and, \( K_m \) and \( V_{max} \) are kinetic parameters. Usually, the first step is to calculate the reaction rate versus the substrate concentration, \([S]\), from the batch reaction data. Typically, only initial rate data are used. Several batch experiments are to be conducted with different initial substrate concentrations. From each data, the reaction rate is evaluated at zero time, i.e. at initial concentration of the substrate. The data are plotted to estimate \( K_m \) and \( V_{max} \). The Michaelis-Menten equation can be linearized by taking the reciprocal of equation 3.10 This plot is known as Lineweaver-Burk plot.

\[ \frac{1}{v} = \frac{K_m}{V_{max}} \left( \frac{1}{s} \right) + \frac{1}{V_{max}} \]  

(3.11)

A plot of \( 1/v \) vs \( 1/s \) will yield straight line with \( K_m/V_{max} \) as slope and \( 1/V_{max} \) as intercept. Xylanase activity was estimated over a range of substrate (xylan) concentration (1–10 mg/mL), and kinetic constants \( K_m \) and \( V_{max} \) were determined using equation (3.11).
3.2.12 Application of xylanase in bread manufacturing

3.2.12.1 Proximate analysis of whole wheat flour

Moisture content was determined according to the American Association of Cereal Chemists (AACC) method (AACC, 2000). Fat, ash, acid insoluble ash, crude fibre, dry gluten and protein content were determined according to the methods described in American Organization of Analytical Chemists (AOAC) method (AOAC, 1984).

3.2.12.2 Manufacturing of bread

Dough was made by mixing wheat flour (100 g); instant dry yeast (3 g); sugar (6 g); salt (1.5 g); shortening (3 g); partially purified xylanase (10 μL); and, water according to requirement. Wheat flour, instant dry yeast, shortening, xylanase and water were mixed together in an automatic mixing bowl (Continental, Ambala, India) for 15 min and the dough was kept for fermentation at 37°C and 80% relative humidity (RH) for 1 h. After 1 h, it was mixed with salt and sugar for 15 min in the automatic mixing bowl and proofing was carried out for 1 h at 37°C in proofing cabinet (Continental, Ambala, India). Baking was carried out at 200°C for 20 min in baking oven (Continental, Ambala, India). After 1 h cooling at room temperature, bread was sliced and stored in self-sealed polyethylene bags at room temperature (25±2°C) and refrigerator (4±1°C), respectively for up to 15 days for staling study. Measurements were taken after 1 h of baking and continued at 24 h interval. Each time two loaves were examined.

3.2.12.3 Rheological properties of dough

Rheological properties of bread dough were measured using a controlled stress rheometer (Bohlin, Gemini, UK) using a 25 mm parallel geometry with 2 mm gap between plates. The dough samples were placed between the plates and the edges were carefully trimmed with spatula. The flow experiments were conducted under steady-state condition with shear rate ranging from 0.01 to 100 s⁻¹. The dough was rested at room temperature for 20 min before testing to release residual stresses. In case of the dynamic oscillatory experiments, first linear viscoelastic region of the samples was determined. Frequency
sweep experiments were then carried out at 0.5% strain rate between 0.01 to 10 Hz. All the rheological experiments were performed at least twice and their averages were used in analysis.

3.2.12.4 Modeling of the dynamic rheology data

Oscillation measurements
Data obtained from frequency sweep for dough with and without xylanase were plotted as $G'(\omega)$ or $G''(\omega)$ vs. frequency ($\omega$) in a log-log plot.

Power law model
The power law describes the rheological behavior of dough within the linear viscoelastic region because the frequency dispersions of the dynamic mechanical spectra ($G'$, $G''$) are more or less straight lines with different slopes. Therefore, each set of data can be fitted in power law equations.

$$G'(\omega) = G'_{\omega 1} \omega^x \text{ (Pa)}$$

$$G''(\omega) = G''_{\omega 2} \omega^y \text{ (Pa)}$$

(3.12)

(3.13)

The coefficients $G'_{\omega 1}$ (Pa) and $G''_{\omega 2}$ (Pa) are the storage and loss moduli, respectively, extrapolated to the values of the initial measuring frequency $\omega_1$ or $\omega_2$; and, $x$ and $y$ are the power law index (dimensionless) for storage modulus and loss modulus respectively.

Week gel model
Week gel model provides a direct link between the microstructure of the material and its rheological properties. Gabriele et al. (2001) proposed power law relaxation modulus to describe the rheological behavior of dough, jam and yoghurt. Week gel model can be used because of its functional simplicity. Week gel model has been used to explore other types of deformation including creep relaxation and uniaxial and biaxial extension (Gabriele et al., 2004).

$$|G'| = \sqrt{(G'(\omega)^2 + G''(\omega)^2)} = A(\omega)^{1/2}$$

(3.14)

where, $A$ is a constant which can be interpreted as the interaction strength between the flow rheological units; and, $z$ is number of flow units interacting with each other to give the observed flow response, also called coordination number.
Creep measurements

Creep test was carried out by applying a constant shear stress (τ = 50 Pa) in the linear viscoelastic region (determined from strain sweep tests) for 60s and resultant strain was measured as a function of time. After 60s creep (under constant stress), the stress was removed and the recovery of strain was measured for 180s. The results were expressed in terms of instantaneous shear creep compliance (J).

During the creep recovery phase after the removal of load, the entire stored deformation energy is used up for the restoration process. The compliance value \( J = \gamma / \tau \) can be calculated and the recovery value \( J_e / J_{\text{max}} \) is equivalent to the solid properties. The flowability (fluidity) at the end of the applied load phase was obtained by the parameter, like zero shear viscosity \( \eta_o = \tau / \gamma \).

Following models were used for modeling of creep data

**Peleg model**

Peleg (1980) suggested that creep data can be modeled by a linear relation:

\[
\frac{J}{J_1} = k_1 + k_2 t
\]

where, \( k_1 \) (Pa s) and \( k_2 \) (Pa) are constants; \( 1/k_1 \) represents initial decay rate (1/(Pa s)); and, \( 1/k_2 \) is asymptotic strain (1/Pa).

**Burgers model**

The Burgers model (Steffe, 1996) in terms of compliance function is described by four parameter equation:

\[
J_e = f(t) = J_o + J_1 (1 - \exp\left(-\frac{t}{\lambda_{\text{ret}}}\right)) + \frac{t}{\mu_0}
\]

where, \( J_o \) is instantaneous compliance resulting from instantaneous stress applied; \( J_1 \) is time-dependent retarded elastic compliance (Kelvin component); \( \lambda_{\text{ret}} \) is retardation time of the Kelvin component; and, \( \mu_0 \) is asymptotic viscosity of the material (viscous flow of bond-free constituents). The instantaneous compliance \( (J_o) \) is the compliance at zero time and was determined by extrapolation of the compliance to zero time.

**Kelvin model**

Kelvin model (Steffe, 1996) is a six parameter mechanistic model and is an extension of Burger model:
where, \((\lambda_{ret})_1 = \mu_1 / G_1\); and \((\lambda_{ret})_2 = \mu_2 / G_2\); \(J_1\) and \(J_2\) are the retarded compliance of first and second Voigt-Kelvin element; and, \((\lambda_{ret})_1\) and \((\lambda_{ret})_2\) are the retardation times of first and second Voigt-Kelvin element respectively.

### 3.2.12.5 Determination of dough stickiness and uniaxial extensibility

Dough stickiness was determined in a Texture Analyser (TA-XT plus, Stable Micro System, Surrey, UK) using Chen and Hoseney cell (Hoseney and Chen, 1994). Uniaxial extensibility was assessed by Kieffer dough and gluten extensibility rig. The resistance to extension \((gf)\) and extensibility \((mm)\) were determined in tension mode by recording the peak force and the distance at the maximum and the extension limit (Smewing, 1995).

### 3.2.12.6 Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) study of treated and untreated dough

Lyophilized powder of untreated and xylanase treated dough were tested under scanning electron microscope. SEM was performed on lyophilized powdered samples which were mounted on double side sticky tape on aluminium stubs and sputter coated with gold and palladium alloy. The samples were then visualized and the representative areas were photographed under SEM (JSM-6100, JEOL, Tokyo, Japan) at an accelerated voltage of 10KV and magnification in the range of 100-2000X.

FTIR spectra were recorded at room temperature \((28\pm2^\circ C)\) with a Tensor-27 spectrophotometer (Bruker, Germany) in the range of 600-4000 cm\(^{-1}\) by accumulating 16 scans at 4 cm\(^{-1}\) resolution. For measuring the spectra of lyophilized powder of untreated and xylanase treated dough samples, platinum ATR (attenuated total reflectance) from Bruker, Germany was used. About 2 mg each of dried untreated and xylanase treated powdered dough samples were placed on ATR for measurement. After that the peak intensity of each sample was measured. This entire spectra acquisition procedure took one min per sample.
3.2.12.7 Determination of physical properties of bread

Quality analysis of fresh bread samples was carried out by measuring physical properties such as mass (m), volume (v), specific volume (m/v), height/width ratio of central slices and moisture content (%). Weight loss after baking and during storage was recorded. Mass was measured by weighing of fresh loaf as well as stored loaf. Volume was determined by seed displacement process (Demirkesen et al., 2010). Moisture content was determined by keeping the bread sample at 100°C in hot air oven till constant weight and expressed in % (wet basis).

3.2.12.8 Color measurement

The color of fresh and stored bread was measured in terms of L*, a*, and b* values using Hunter Lab ColorFlex spectrophotometer (Hunter Associate Laboratory, Reston, VA, USA) at three different locations on crust and crumb of bread. L* corresponds to lightness ranging from 0 to 100, indicating black to white; a* positive value corresponds to redness while negative value corresponds to greenness; and, b* positive value corresponds yellowness while negative value corresponds to blueness. The instrument (45°/0° geometry, D65 optical sensor, 10° observer) was calibrated against a standard white reference tile (L* = 93.80, a* = -0.88, and b* = 2.21). A glass petri dish containing the bread sample was placed above the light source and covered with a plate and post-process Hunter L*, a*, and b* values were recorded. Color measurements were taken in triplicate and average values were used for calculation. Total color difference (ΔE) was calculated as

\[
\Delta E = \left( (\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2 \right)^{1/2}
\]

(3.18)

3.2.12.9 Textural properties of bread

Crumb texture was determined by Texture Analyzer (TA-XT plus, Stable Micro System, UK) using plunger type P 36R probe of 36mm diameter. Slices of 2-2.5 cm thickness were compressed to 50% of their original thickness, at 1mm/s speed test, with a 30s delay between first and second compression. Firmness (gf), cohesiveness, springiness, resilience, gumminess (gf) and chewiness (gf) were calculated from the texture profile.
analysis (TPA) data. Firmness/hardness is the maximum force necessary at the first compression cycle (first bite). Cohesiveness is defined as the rate of disintegration of bread during mechanical action. It can be measured by the ratio of the positive force area at the second compression to the area at the first compression. Springiness/elasticity is the height that the food recovers at the time that intervenes between the end of the first bite and the start of the second bite. It is measured by the ratio of time difference between second bite to time difference between first bite. Gumminess is defined as the product of hardness and cohesiveness. It is the characteristic of semisolid foods with a low degree of hardness and a high degree of cohesiveness. Resilience is a measurement of how the sample recovers from deformation both in terms of speed and forces derived. It is the ratio of areas from the first force turnaround point to the crossing of the time axis and the area formed from the first compression cycle. Chewiness is defined as the amount of energy required to masticate a solid food. It is the product of gumminess and springiness (which is equal to hardness x cohesiveness x springiness). Variation of firmness with time was computed using equation (3.19) and (3.20) (Kim and D’Appolonia, 1977) to determine rate constant and Avrami exponent.

\[ \theta = \frac{F_a - F_t}{F_a - F_0} = e^{-kt^n} \]  

(3.19)

Upon linearization of equation (3.19) yields

\[ \ln(-\ln(1-\theta)) = \ln\ln\left(\frac{F_a - F_t}{F_a - F_0}\right) = \ln(k) + n\ln t \]  

(3.20)

\[ \ln(-\ln(1-\theta)) = \ln\ln\left(\frac{F_a - F_0}{F_a - F_t}\right) = \ln(k) + n\ln t \]  

(3.21)

where, \( \theta \) is fraction of uncrystallized material at time \( t \) expressed in terms of firmness (dimensionless); \( F_0 \) is firmness of fresh bread, \( F_t \) is firmness (g.force) at time \( t \); \( F_a \) is limiting value of firmness at 15 days (g.force); \( k \) is rate constant (per day); \( n \) is constant (dimensionless); and, \( t \) is storage time (days).

3.2.12.10 Differential scanning calorimetry (DSC) of stored bread

Differential scanning calorimeter (DSC-Phox, Netzsch, Germany) was used to determine glass transition temperature and enthalpy. The equipment was calibrated for heat flow.
and temperature using distilled water (mp= 0°C, \( \Delta H_m = 334.5 \) J/g), indium (mp=156.6°C, \( \Delta H_m = 28.5 \) J/g Netzsch standard), and gallium (mp=29.8°C, \( \Delta H_m = 80 \) J/g Netzsch standard). An empty pan was used as reference. DSC cell was dried with a flush of dry nitrogen to prevent moisture condensation. About 20-30 mg bread sample was taken in the hermetically sealable aluminum pan and 20 µL distilled water was added. After sealing, bread samples were heated at 10°C/min from 20 to 200°C in the sealed pan. The parameters measured were the onset temperature (T_o), the peak temperature (T_p), and the conclusion temperature (T_c). Straight line was drawn between onset temperature (T_o) and conclusion temperature (T_c). The area enclosed by the straight line and the endothermic curve was calculated using proteus analysis software provided with the instrument. The area represents the change of enthalpy (\( \Delta H \)). Runs were taken in triplicate for each sample. At the time of retrogradation of starch, enthalpy value provides a quantitative measure of the energy change that takes place when recrystallized amyllopectin melts, consequently accurate magnitude of thermal transition events (i.e. T_o; T_p; T_c) are recorded. Midpoint of onset and conclusion temperature is considered as T_g.

3.2.12.11 X-ray diffraction (XRD) study of stored bread

X-ray diffraction (XRD) profile of lyophilized powder untreated and xylanase treated bread samples stored for different duration at room temperature and refrigeration temperature were studied in order to examine the crystallinity (retrogradation) of the stored bread samples. The samples were taken in powdered form and analyzed using Philips X'Pert Pro X-ray diffractometer system. The radiation was used Cu Ka (\( \lambda = 1.54060\) Å ) with 40 KV voltage and 40 mA intensity. Each time about 500 mg powdered samples were scanned from 5° to 60° 2-0.

3.2.12.12 Scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) of stored bread sample

Untreated and xylanase supplemented bread samples were tested under scanning electron microscope. SEM was performed on lyophilized powdered samples which were mounted on double side sticky tape on aluminium stubs and sputter coated with gold and palladium alloy. The samples were then visualized and the representative areas were
photographed under SEM (JSM-6100, JEOL, Tokyo, Japan) at an accelerated voltage of 10 KV and magnification in the range of 100-2000X.

FTIR spectra were recorded at room temperature (28±2°C) with a Tensor-27 spectrophotometer (Bruker, Germany) in the range of 600-4000 cm⁻¹ by accumulating 16 scans at 4 cm⁻¹ resolution.

For measuring the spectra of lyophilized powdered untreated and treated stored bread samples, platinum ATR (attenuated total reflectance) from Bruker was used. About 2 mg lyophilized powder stored bread samples were placed on ATR for measurement. After that the peak intensity of each samples were measured and this entire spectra acquisition procedure took 1 min per sample.

3.2.12.13 Sensory evaluation of bread

Sensory evaluation was carried out using nine-point Hedonic scale (9= extremely good, 8= very good, 7= moderately good, 6= slightly good, 5= neither good nor bad, 4= slightly bad, 3= moderately bad, 2= very bad 1= extremely bad) by a semi trained panel of 10 judges.

The sensory panel was selected among the students and faculty members of the University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh. Panel consisted of five male and five female (age group: 20-50 years with an average age of approximately 30 years).

Each panelist was asked to evaluate the product and award the scores with respect to overall symmetry, smoothness, stickiness, uniformity of crumb cells, appearance, color, aroma and taste. The average values of the sensory scores were used for analysis.

3.3 Statistical analysis

All the experiments were carried out in triplicate. Modeling and statistical parameters (coefficient of determination, standard error, analysis of variance, ANOVA) calculation were carried out using STATISTICA version 6.0 (Statsoft Inc., 2002, Tulsa, USA).