CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Methods

3.1.1 Microorganism and culture media

Aspergillus niger (MTCC - 16404) used in this study is purchased from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH) Chandigarh, India. The stock culture is maintained on agar slants at 5°C. The culture is maintained on Potato Dextrose Agar (PDA) and subcultured at regular intervals.

Table 3.1 Composition of PDA medium for A.niger

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>10.0</td>
</tr>
<tr>
<td>Dextrose</td>
<td>10.0</td>
</tr>
<tr>
<td>Agar</td>
<td>20.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
</tbody>
</table>

Aspergillus fumigatus (MTCC – 343) used in this study is purchased from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The stock culture is maintained on agar slants at 5°C. The culture is maintained on Czapek Dox medium and subcultured at regular intervals.

Table 3.2 Composition of Czapek Dox medium for A.fumigatus and A.terreus

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Czapek Concentrate</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.0</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>30.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
</tbody>
</table>

Table 3.3 Composition of Czapek Concentrate:

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO₃</td>
<td>30.0</td>
</tr>
<tr>
<td>Kcl</td>
<td>5.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>5.0</td>
</tr>
<tr>
<td>FeSO₄·7H₂O</td>
<td>0.1</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.0 L</td>
</tr>
</tbody>
</table>
Aspergillus terreus (MTCC - 1782) used in this study is purchased from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH) Chandigarh, India. The stock culture is maintained on agar slants at 5°C. The culture is maintained on Czapek Dox Medium (Table 3.2 and Table 3.3) and subcultured at regular intervals.

3.1.2 Inoculum preparation

The strains are subcultured on PDA/Czapek Dox slants and incubated for 120 hrs at a temperature of 30°C. After the incubation period, the spore suspensions are prepared by adding 10 ml of sterile water to the PDA slant containing sporulated slant cultures. The spores on the surface of the medium are dislodged using inoculation needle under aseptic conditions. The spore suspensions are filtered using sterile muslin cloth into sterile flasks. The filtered spore suspension is transferred into 250 ml Erlenmeyer flask containing 100 ml of potato dextrose broth and incubated for three days at 30°C for A. niger and A. terreus, 35°C for A. fumigatus. 2-3 ml from 2 days old culture is used as inoculum for the sterilized production medium.

3.1.3 Substrate Preparation

In this study, two agricultural byproducts viz. wheat bran, sugarcane bagasse are utilized as substrates. They are collected from nearby areas of Chidambaram, Tamilnadu, India. Since these agricultural byproducts are not available in completely dried form, it is necessary to dry these substrates prior to use them in the fermentation process. In the present study, substrates are dried by keeping them in oven at 60°C for 8 hours. After drying, the substrates are powdered in a laboratory grinder and sieved using a 40 mm sieve and stored for further use. The Composition of wheat bran and sugarcane bagasse are given in Table 3.4 and Table 3.5 (Gawande and Kamat, 1999) respectively.

Table 3.4 Composition of wheat bran

<table>
<thead>
<tr>
<th>Components</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>30</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>27</td>
</tr>
<tr>
<td>Lignin</td>
<td>20</td>
</tr>
<tr>
<td>Ash</td>
<td>7.5</td>
</tr>
</tbody>
</table>
Table 3.5 Composition of Sugarcane bagasse

<table>
<thead>
<tr>
<th>Components</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>43.5</td>
</tr>
<tr>
<td>Hemicellulose</td>
<td>22.9</td>
</tr>
<tr>
<td>Lignin</td>
<td>19.0</td>
</tr>
<tr>
<td>Ash</td>
<td>4.75</td>
</tr>
</tbody>
</table>

3.1.4 Preparation of mixed substrate

A varied combination of mixed substrates has been tried for the production of xylanase. In the preparation of mixed substrate, the quantity of wheat bran is kept constant at 5 g and the quantity of sugarcane bagasse is varied from 0.5 g to 7.35 g in order to obtain the wheat bran: sugarcane bagasse ratios of 0.52:1, 0.8:1, 1:1, 1.2:1, 1.47:1.

For example, 1:1 ratio of Wheat bran : Sugarcane bagasse mixed substrate composition is prepared by taking 5 g of wheat bran with 5 g of sugarcane bagasse in a 250 ml Erlenmeyer flask containing 8 ml water along with the optimized most significant nutrients obtained from central composite design. The initial pH of the medium is adjusted by adding weak acid and weak base. The temperature, initial pH, initial moisture content and incubation time are fixed according to the experimental design.

3.1.5 Preparation of DNS (Dinitrosalycyclic Acid) solution

Different ingredients used for the preparation of DNS solution are as follows:

1) 3.5 Dinitrosalycyclic Acid 20 g
2) Sodium hydroxide 32 g
3) Distilled water 1500 ml

The above ingredients were dissolved gently in water bath at 80°C until a clear solution was obtained. Then the following chemicals are added.

4) Rochelle salt (sodium potassium tartarate) 600 g
5) Phenol (melted at 60°C) 10 ml
6) Sodium meta bisulphate 10 g

After dissolving the above ingredients, the solution was kept for 2 weeks at room temperature before use.
3.1.6 Preparation of Na-citrate buffer (pH-5.3)

To 25 ml of 0.1 % Citric acid, 25 ml of 0.2 % of NaH₂PO₄ was mixed and the pH was adjusted to 5.3 with further addition of 0.2 % NaH₂PO₄.

3.1.7 Preparation of 1% Xylan solution (%w/v)

1% Oat spelt xylan in 0.05 M Na-citrate buffer, pH -5.3

1 g of oat spelt xylan was homogenised in 80 ml of sodium citrate buffer at 70°C and heated to boiling point preferably on a magnetic stirrer. It is cooled slowly overnight. The volume is made up to 100 ml with the same buffer and stored at 4°C for a maximum of one week.

3.1.8 Solid state fermentation (SSF)

The xylanase production in batch solid state fermentation is carried out in 250 ml Erlenmeyer flasks (plugged with cotton) with 10 g of wheat bran, 0.1% (v/v) of Tween-80, 0.1% (w/v) of oat spelt xylan, supplemented with nutrients concentrations defined by the experimental design. 0.1 % of oat spelt xylan serves as an inducer for xylanase production. The initial moisture content is adjusted to 80% (Yasser Bakri et al., 2003). Each flask is covered with hydrophobic cotton and autoclaved at 121°C for 20 min. After cooling, each flask is inoculated with 2 to 3 ml of inoculum prepared from 6 day old slants of the culture grown at 30°C and the inoculated flasks are incubated at 30°C in an incubator.

*Aspergillus niger* and *A. terreus*

During the preliminary screening process, the experiments are carried out for 120 hours and it is found that at the 96th h, the maximum production occurs. Hence experiments are carried out for 96 h.

*Aspergillus fumigatus*

During the preliminary screening process, the experiments are carried out for 144 hours and it is found that at the 120th h, the maximum production occurs. Hence experiments are carried out for 120 h.

All the fermentation runs are conducted in triplicates and the average is recorded.

The effect of process parameters such as substrate concentration, Incubation Time, pH, temperature, % moisture content are studied using the respective optimized medium.
3.1.9 Determination of moisture Content

Moisture content was estimated by drying a known weight of sample to a constant weight at 105°C and dry weight was recorded. To fix the initial moisture content of the solid medium, the sample was soaked with desired quantity of water. After soaking, the sample was again dried as described above and moisture content was calculated as follows (Adhinarayana et al., 2004):

% of Initial moisture content of the solid medium

\[
= \frac{\text{weight of sample after soaking} - \text{Dry weight}}{\text{Dry weight}} \times 100
\]

**Model calculation for determination of Initial moisture content**

Dry weight of wheat bran = 10 g
Weight of wheat bran after (soaking in 8 ml of water & drying) = 18.0 g
Therefore,

% of Initial moisture content of the solid medium

\[
= \frac{(18 - 10)}{10} \times 100
\]

\[= 80 \%
\]

3.2 Analytical Methods

3.2.1 Extraction of xylanase from the fermentation medium

At the end of the fermentation period, 50 ml of 0.05M citrate buffer (pH – 5.3) is added to the fermented matter and the contents are agitated for 30 minutes at 200 rpm in an orbital shaker at 30°C and filtered through a cotton cloth by squeezing. The extract is centrifuged at 15,000 rpm for 20 minutes and the supernatant is used for determination of enzyme activity.

3.2.2 Enzyme Assay

Endo β-1, 4-Xylanase activity is measured by incubating 0.5ml of 1% (w/v) oat spelt xylan in 0.05M Na-citrate buffer (pH 5.3). And 0.5 ml of suitably diluted enzyme extract at 50°C for 30 min. The release of reducing sugar is measured by dinitro salicylic acid (DNS) method (Miller, 1959) and D-Xylose is used as the standard. One unit (IU) of xylanase activity is defined as the amount of enzyme releasing 1μmol of reducing sugar measured as xylose equivalents per minute.
under the assay conditions. Xylanase production in SSF was expressed as IU/g dry substrate (IU/gds).

Carboxy methyl Cellulase activity was assayed by adding 0.5 ml of appropriately diluted enzyme to 0.5 ml of 1 % (w/v) of carboxymethyl cellulose (CMC) in 50 mM Na-citrate buffer, pH 5.3 and incubating at 50°C for 30 min. The amount of reducing sugars released during the reaction was measured using the DNS method (Miller, 1959) and D-glucose was used as the standard. One unit of carboxy methyl cellulase activity was defined as the amount of enzyme that liberated 1 µmol of reducing sugar measured as glucose equivalents per minute under the assay conditions.

Reducing sugars namely xylose and glucose concentration is determined from a standard curve as shown in APPENDIX - I and APPENDIX - II under same condition using D-Xylose and D-Glucose with Bio-spectrophotometer.

Enzyme Activity is calculated using the formula,

\[
\text{Xylanase Activity (IU/gds)} = \frac{A \times 1000 \times D \times V}{30 \times 150 \times 10}
\]

\[
\text{CMCase Activity (IU/gds)} = \frac{A \times 1000 \times D \times V}{30 \times 180 \times 10}
\]

Where, 
A - Amount of reducing sugar (mg/ml) 
1000 - Conversion factor 
D - Dilution factor 
V - Total Volume of enzyme extract obtained (ml)

3.2.3 Calibration Chart for xylanase activity – D-Xylose standard

1. 1g of xylose was dissolved in 50 ml of 0.05 M Na-citrate buffer and made upto 100 ml in a standard flask using Na-citrate buffer.
2. From this 10 ml was taken and made upto 100 ml using distilled water.
3. This is the stock solution of xylose which contains 1 mg of xylose per ml.
4. Various dilutions from the above stock solution are made to contain 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mg of xylose per ml.
5. 3 ml of DNS solution was added to 1 ml of various standard diluted xylose solution and incubated at 50°C for 30 minutes.
1. 1 ml of Rochelle salt solution was immediately added at warm condition and allowed to cool.
2. Absorbance (optical density) of xylose solution was determined using uv-visible spectrophotometer at 540 nm.
3. A standard plot was prepared from the above readings and shown in Appendix I.

3.2.4 Xylanase Assay procedure
1. 0.5ml of 1% (w/v) oat spelt xylan in 0.05M Na-citrate buffer (pH 5.3) was mixed with 0.5 ml of enzyme suitably diluted solution.
2. 3 ml of DNS solution was added to the above mixture and incubated at 50°C for 30 min.
3. After incubation 1 ml of Rochelle salt solution was immediately added at warm condition and allowed to cool.
4. A blank was also prepared using the same steps, the only change is 0.5 ml of distilled water was added instead of 0.5 ml of enzyme solution.
5. The colour developed after 30 min incubation at 50°C was measured using UV-visible spectrophotometer at 540 nm.
6. The slope of the standard curve determined with xylose was used in the calculation of xylanase activity.

3.2.5 Model calculation for Xylanase Activity

Optical density of the unknown sample = 0.522

Equation for xylose standard: \( y = 1.161 \times (x \text{ is in mg/ml}) \)

\[
x = \frac{0.522}{1.161} = 0.4496 \text{ mg of xylose liberated}
\]

\[
\text{Xylanase Activity (IU/gds)} = \frac{A \times 1000 \times D \times V}{30 \times 150 \times 10}
\]

\( A = 0.450 \text{ mg of xylose} \)

\( D = \text{Dilution factor } = 500 \)

\( V = \text{Volume of the enzyme extract } = 50 \text{ ml} \)

\[
\text{Xylanase Activity (IU/gds)} = \frac{0.450 \times 1000 \times 500 \times 50}{30 \times 150 \times 10}
\]

\[
= 250 \text{ IU/gds}
\]
3.2.6 Calibration Chart for CMCase activity – D-glucose standard

1. 1g of glucose was dissolved in 50 ml of 0.05 M Na-citrate buffer and made up to 100 ml in a standard flask using Na-citrate buffer.
2. From this 10 ml was taken and made up to 100 ml using distilled water.
3. This is the stock solution of glucose which contains 1 mg of glucose per ml.
4. Various dilutions from the above stock solution are made to contain 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 mg of glucose per ml.
5. 3 ml of DNS solution was added to 1 ml of various standard diluted glucose solution and incubated at 50°C for 30 minutes.
6. 1 ml of Rochelle salt solution was immediately added at warm condition and allowed to cool.
7. Absorbance (optical density) of glucose solution was determined using uv-visible spectrophotometer at 540 nm.
8. A standard plot was prepared from the above readings and shown in Appendix II.

3.2.7 CMCase Assay procedure

1. 0.5 ml of 1 % (w/v) of carboxymethyl cellulose (CMC) in 0.05M Na-citrate buffer (pH 5.3) was mixed with 0.5 ml of suitably diluted enzyme solution.
2. 3 ml of DNS solution was added to the above mixture and incubated at 50°C for 30 min.
3. After incubation 1 ml of Rochelle salt solution was immediately added at warm condition and allowed to cool.
4. A blank was also prepared using the same steps, the only change is 0.5 ml of distilled water was added instead of 0.5 ml of enzyme solution.
5. The colour developed after 30 min incubation at 50°C was measured using UV-visible spectrophotometer at 540 nm.
6. The slope of the standard curve determined with glucose was used in the calculation of CMCase activity.
3.2.8 Model calculation for CMCase Activity

Optical density of the unknown sample = 0.757
Equation for xylose standard: \( y = 3.294 \times (x \text{ is in mg/ml}) \)
\[ x = \frac{0.757}{3.294} = 0.23 \text{ mg of glucose liberated} \]

\[ \text{CMCase Activity (IU/gds)} = \frac{A \times 1000 \times D \times V}{30 \times 180 \times 10} \]

\( A = 0.230 \text{ mg of glucose} \)
\( D = \text{Dilution factor} = 200 \)
\( V = \text{Volume of the enzyme extract} = 50 \text{ ml} \)

\[ \text{CMCase Activity (IU/gds)} = \frac{0.23 \times 1000 \times 200 \times 50}{30 \times 180 \times 10} \]

\[ = 42.59 \text{ IU/gds} \]

3.3 Experimental Design for Media Optimization

The medium optimization was carried out through a stepwise optimization strategy,

i) Screening of nutrients was done by Plackett- Burman design (PBD),
ii) the screened most significant nutrients from PBD were further analyzed by Central Composite design (CCD) and a regression model was established.

i) the experimental verification or validation of the model was carried out
ii) the process parameters were analyzed by Central Composite design (CCD), a regression model was established and experimental validation of the model was also carried out.

Step-I

3.3.1 Screening of medium components using Plackett-Burman design

The first step of optimization strategy is to identify the medium components that have significant effects on xylanase production. Placket-Burman design is an effective and efficient technique for the optimization of medium components and can be used to select the significant factors and to eliminate the insignificant one in order to obtain more manageable and smaller set of factors. Based on the
Plackett–Burman design, each factor is examined at two levels, low (−1) and high (+1). This design assumes that there are no interactions between the different media constituents, \( x_i \) in the range of variable under consideration [Abou-Zeid, AM, 1997]. In this model a linear approach is considered to be sufficient for the screening process and can be represented in equation 3.1:

\[
Y = \beta_0 + \beta_i x_i (i, \ldots, k)
\]  

(3.1)

where, \( Y \) is the estimated target function and \( \beta_i \) are the regression coefficients. The contrast coefficient, \( \beta_0 \), is calculated as the difference between the average of measurements made at the high (+) and the low (-) levels of the factors. This coefficient notifies the main effect of the studied factor.

To determine the variable significantly affect the xylanase production, Plackett-Burman design using statistical software package MINITAB (Release 15.1, PA, USA), is used. For screening purpose a total of twelve medium components are tested for their significance in 20 experimental runs and insignificant ones are eliminated.

**STEP -II**

3.3.2 Optimisation using Central Composite Design of Response surface methodology (nutrient optimization)

In order to enhance the production of xylanase, central composite design of Response Surface Methodology (RSM) is employed to optimize the most significant factors, identified by the Plackett–Burman design separately for the three strains with the three different substrates. This method can eliminate the drawbacks of single factor optimization [Plackett RL and Burman JP., 1946]. RSM is useful for small number of variables (up to five) but is impractical for large number of variables, due to high number of experimental runs required. CCD was used to obtain a quadratic model, consisting of factorial trails and star points to estimate quadratic effects and central points to estimate the pure process variability with xylanase production.

The effects of the most significant variables on xylanase production are studied by central composite design using Response surface Methodology. In the
present study the statistical model is obtained using the Central Composite Design (CCD) with three/four independent variables.

The total number of design points is calculated from equation 3.2,

$$N = 2^K + 2K + n_0$$  \hspace{1cm} (3.2)

Where K is the number of levels in a factorial design, $n_0$ is the number of replicates at the center point 

For 3 variables, $K = 3$

$$N = 8 + (2 \times 6) + 6 = 20$$

A $2^3$ factorial design is employed with 20 experimental runs for 3 variables. These 20 experiments are performed with different combinations of three dependent variables. Here $n_c = 2^K$, $n_a = 2 \times K$, $n_o = 6$:

$$n_c = 8 : (\pm 1, \pm 1, \pm 1), \hspace{1cm} n_a = 6 : (\pm \alpha, 0, 0), \hspace{1cm} n_o = 6 : (0,0,0)$$

Where the value of $\alpha = n_c^{1/4} = 8^{1/4} = 1.68$ to make the design rotatable.

For four variables, a $2^4$ factorial design was employed with 30 experimental runs. These 30 experiments were performed with different combinations of the four dependent variables.

$$n_c = 16 : (\pm 1, \pm 1, \pm 1), \hspace{1cm} n_a = 8 : (\pm \alpha, 0, 0), \hspace{1cm} n_o = 6 : (0,0,0)$$

Each factor in this design is studied at five different levels -2, -1, 0, +1, +2 and a set of 30 experiments are carried out. All the variables are taken at a central coded value considered as zero. The experiments with five different concentrations of each significant variable from minimum to maximum ranges are employed simultaneously covering the spectrum of variables for xylanase production in the central composite design. Batch experiments are conducted as per the central composite design for xylanase production in 250 ml Erlenmeyer flasks. All the experiments are carried out in triplicates and the average value is taken as the response. The CCD experiment is designed using the Design Expert Software package (Version 8.0.7.1, Stat-Ease, Inc., and Minneapolis, USA). The following equation 3.3 is used for coding the actual experimental values of the factors in the range of (-2 to +2):

$$x_i = \frac{X_i - X_0}{\Delta X_i}$$  \hspace{1cm} (3.3)
Where \( x_i \) is the coded value of the \( i^{th} \) independent variable, \( X_i \) the natural value of the \( i^{th} \) independent variable, \( X_0 \) the natural value of the \( i^{th} \) independent variable at the center point, and \( \Delta X_i \) is the value of step change.

Analysis of the data and generation of three dimensional surface graphs is done using Design Expert Software package (Version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA). After conducting the experiments and measuring the xylanase activity levels, a second order polynomial equation including interactions is fitted to the response data as given by equation 3.4,

\[
Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{i i} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{i j} X_i X_j \quad (3.4)
\]

Where \( Y \) is the measured response, \( \beta_0 \) is the intercept term, \( \beta_i \) are linear coefficients, \( \beta_{ii} \) are quadratic coefficient, \( \beta_{ij} \) are interaction coefficient and \( X_i \) and \( X_j \) are coded independent variables.

**ANOVA**

The significant terms in the model are found by analysis of variance (ANOVA) for each response using Design Expert software. The goodness of fit of the regression model obtained is given by the coefficient of determination \( R^2 \). The statistical significance of the model is determined by F-test. Since coding of the variables enables direct comparison of the partial regression coefficients, their significance is determined by student’s \( t \) test and associated probabilities. Lack of fit is a special diagnostic test for adequacy of a model that compares the pure error, based on the replicate measurements to the other lack of fit, based on the model performance (Noordin et al., 2004). F value calculated as the ratio between the lack of fit mean square and the pure error mean square, is the statistic parameter used to determine whether the lack of fit is significant or not, at a significance level.

**Generation of 3D graphs**

The interaction effects of variables on xylanase production are studied by plotting three dimensional surface graphs (using Design Expert software) against any two independent variables while the third and fourth variables are fixed at its central level. Three dimensional surface response graphs were obtained based on
the effect of the levels of five parameters (at five different levels each) and their optimal concentrations. From these response surfaces, the interaction of one parameter with another parameter was studied.

The optimum values of the process condition were obtained by solving the regression equations. The optimal values of the test variables are first obtained in coded units and then converted to uncoded units. The second degree polynomial equation is maximized by a constraint search procedure using the Response optimizer of the Design Expert software to obtain the optimum levels of the independent variables and the predicted maximum xylanase activity. The predicted xylanase activity is compared with the experimental values.

STEP –III

Validation of the model

Validation of the experimental model was tested by carrying out the batch experiment in triplicates under the obtained optimized conditions of the Design Expert software. If the predicted xylanase activity and the average xylanase activity found by the experiment coincides with one another. This good correlation between predicted and experimental values after optimization justifies the validity of the model.

STEP-IV

3.3.3 Optimisation using Central Composite Design of Response surface methodology (Process parameter optimization)

The factors mainly affecting xylanase production are substrate concentration, temperature, Initial pH, Initial moisture content, Incubation Time. The effects of these operating variables on xylanase production are studied by central composite design using Response surface Methodology.

For this study, the optimized values of nutrients obtained from the above CCD are fixed for a particular substrate, and the process parameters are studied with five independent variables to enhance the xylanase production. The five independent variables are studied at five different levels -2.38, -1, 0, 1, 2.38. Therefore a $2^5$ factorial design is employed with 50 experimental runs. These 50
experiments were performed with different combinations of the five independent variables.

\[ n_c = 32 : (\pm 1, \pm 1, \pm 1), \quad n_a = 10 : (\pm \alpha, 0, 0), \quad n_o = 8 : (0,0,0) \]

Where the value of \( \alpha = n_c^{1/4} = 32^{1/4} = 2.38 \) to make the design rotatable. All the variables are taken at a central coded value considered as zero. The experiments with various Substrate concentrations, Temperature, pH values, Moisture content, Incubation time are employed simultaneously covering the spectrum of combination of variables with a wide range for xylanase production in the central composite design. Batch experiments are conducted as per the central composite design for xylanase production in 250 ml Erlenmeyer flasks. All the experiments are carried out in triplicates and the average value is taken as the response. The CCD experiment is designed using the Design Expert Software package (Version 8.0.7.1, Stat-Ease, Inc., and Minneapolis, USA). Then the same procedure such as analysis of variance (ANOVA), generation of 3D graphs and validation of the model adopted for optimization of significant variables in nutrient optimization studies (STEP-II & STEP –III) is followed as such.

### 3.4 Artificial Neural Network (ANN) Modelling

Employing neural network models save time and cost by predicting the results of the reactions so that the most promising conditions can then be verified. In this study, a multi-layer perceptron (MLP) based feed-forward ANN which uses back-propagation learning algorithm, is applied for modeling xylanase enzyme production. In an effort to minimise the training error and avoid over training, the training process is supervised during the ANN model formulation. The network consists of an input layer, hidden layers and an output layer. Inputs for the network are concentrations of four different nutrients or various process parameters, output is xylanase activity or CMCase activity. In this study, trial and error approach is used to determine the optimum neurons in hidden layer of the network (examined from 1 to 20 neurons). Scaled data are passed into the input layer and then is propagated from input layer to hidden layer and finally to the output layer of the network. Every node in hidden or output layer firstly acts as a
summing junction which combines and modifies the inputs from the previous layer using the following equation 3.5,

\[ y_i = \sum_{j=1}^{i} x_i w_{ij} + b_j \quad (3.5) \]

where \( y_i \) is the net input to node \( j \) in hidden or output layer, \( x_i \) are the inputs to node \( j \) (or outputs of previous layer), \( w_{ij} \) are the weights representing the strength of the connection between the \( i^\text{th} \) node and \( j^\text{th} \) node, \( i \) is the number of nodes and \( b_j \) is the bias associated with node \( j \). Output from a neuron is determined by transforming its input using a suitable transfer function (Razavi et al. 2003).

Generally, the transfer functions for function approximation (regression) are sigmoidal function, hyperbolic tangent sigmoidal and linear function (Jorjani et al. 2008). The transfer function used here is the hyperbolic tangent sigmoidal function. The MATLAB software version 7.0 is used to construct ANN.

### 3.4.1 Evaluation of predictability of the model

It is required to calculate ANN output error between the actual and the predicted output to evaluate model predictability. A popular measure is the mean squared error (MSE) or root mean squared error (RMSE) (Ghaffari et al. 2006). The coefficient of determination, \( R^2 \) reflects the degree of fit for the mathematical model (Nath and Chattopadhyay, 2007). The closer the \( R^2 \) value is to 1, the better the model fits to the actual data (Sin et al. 2006). Absolute average deviation (AAD) is another important index to evaluate the ANN output error between the actual and the predicted output (Bas and Boyaci, 2007). The network having minimum RMSE, minimum AAD and maximum \( R^2 \) is considered as the best neural network model (Basri et al. 2007, Izadifar and Zolghadri Jahromi 2007, Wang et al. 2008).

The following formula's are used to calculate AAD, RMSE, \( R^2 \)

(i) **Absolute Average Deviation**

\[
AAD = \frac{\sum_{i=1}^{n} \left| \frac{\text{ANN predicted Value} - \text{Experimental value}}{\text{Experimental Value}} \right| \text{number of points}}{n} \times 100
\]

Model calculation, nutrient optimization – wheat bran – *A.niger*
AAD = \left[ \frac{\sum_{i=1}^{30} \left( \frac{457.38 - 458}{458} \right)}{30} \right] \times 100

Solving the above equation using MS office Excel 2007

= 0.001946

Therefore, AAD = 0.1946

(i)  Root Mean square Error

\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n} (\text{ANN predicted value} - \text{Experimental value})^2}{\text{number of points}}}

= \sqrt{\frac{\sum_{i=1}^{30} (457.38 - 458)^2}{30}}

Solving the above equation using MS office Excel 2007

= 0.9604

(ii)  Coefficient of Determination (R^2)

R^2 = 1 - \left[ \frac{\sum_{i=1}^{n} (\text{ANN predicted value}_i - \text{Experimental value}_i)^2}{\sum_{i=1}^{n} (\text{Average Experimental value}_i - \text{Experimental value}_i)^2} \right]

R^2 = 1 - \left[ \frac{\sum_{i=1}^{30} (457.38_i - 458)^2}{\sum_{i=1}^{30} (376.46_i - 458_i)^2} \right]

Solving the above equation using MS office Excel 2007

= 1 - 0.0008

R^2 = 0.9992
3.5 Materials

3.5.1 Chemicals and Equipments used in the Research work

All the reagents used in this study are of analytical grade. Solutions are prepared using deionised distilled water. The chemicals and equipment used in this study along with their specifications are given in the Table 3.6 and Table 3.7.

Table 3.6 Specifications of Chemicals used

<table>
<thead>
<tr>
<th>NAME</th>
<th>GRADE</th>
<th>MANUFACTURER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Soyabean meal</td>
<td>AR</td>
<td>Sigma chemicals</td>
</tr>
<tr>
<td>Ferrous sulphate hepta hydrate</td>
<td>AR</td>
<td>Fischer</td>
</tr>
<tr>
<td>Manganese sulphate hepta hydrate</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Magnesium sulphate hepta hydrate</td>
<td>AR</td>
<td>Fischer</td>
</tr>
<tr>
<td>Urea</td>
<td>AR</td>
<td>Nice</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Di ammonium sulphate</td>
<td>AR</td>
<td>Nice</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
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<tr>
<td>Zinc sulphate hepta hydrate</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>AR</td>
<td>Nice</td>
</tr>
<tr>
<td>Peptone</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Diammonium hydrogen phosphate</td>
<td>AR</td>
<td>Sigma chemicals</td>
</tr>
<tr>
<td>Beef Extract</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Meat Extract</td>
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<td>Hi-media Laboratories, India</td>
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<tr>
<td>Yeast extract</td>
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<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Cobalt sulphate</td>
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<td>Nice</td>
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<tr>
<td>Cupric sulphate</td>
<td>AR</td>
<td>Nice</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>AR</td>
<td>Nice</td>
</tr>
<tr>
<td>Calcium chloride dihydrate</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Tween- 80</td>
<td>AR</td>
<td>S.D fine chemicals</td>
</tr>
<tr>
<td>3, 5 dinitro salicyclic acid</td>
<td>AR</td>
<td>Merck, India</td>
</tr>
<tr>
<td>Oat spelt xylan</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>D(+) Xylose</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Carboxy methyl cellulose</td>
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<td>Sodium hydroxide</td>
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<td>Sodium sulphite</td>
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<td>Hi-media Laboratories, India</td>
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<tr>
<td>Phenol</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Sodium potassium tartarate</td>
<td>AR</td>
<td>Sigma Aldrich</td>
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<td>Citric acid (anhydrous)</td>
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<tr>
<td>Sodium citrate di hydrate</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>Sucrose</td>
<td>AR</td>
<td>Hi-media Laboratories, India</td>
</tr>
<tr>
<td>EQUIPMENTS</td>
<td>MAKE</td>
<td>PURPOSE</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Biospectrophotometer</td>
<td>ELICO Instruments (India)</td>
<td>Measuring Absorbance</td>
</tr>
<tr>
<td>Orbital shaker</td>
<td>Hi-Tech Equipment (India)</td>
<td>Agitating the fermented matter</td>
</tr>
<tr>
<td>High Speed centrifuge</td>
<td>Remi Scientific Instruments</td>
<td>Centrifugation of samples</td>
</tr>
<tr>
<td></td>
<td>(India)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Model: C-24 BL</td>
<td></td>
</tr>
<tr>
<td>Hot air oven</td>
<td>Hi-Tech Equipment (India)</td>
<td>Drying of substrate</td>
</tr>
<tr>
<td>Temperature bath</td>
<td>Remi Scientific Instruments</td>
<td>To maintain constant temperature</td>
</tr>
<tr>
<td></td>
<td>(India)</td>
<td></td>
</tr>
<tr>
<td>BOD Incubator</td>
<td>ELICO Instruments (India)</td>
<td>Culture growth and fermentation</td>
</tr>
<tr>
<td></td>
<td>Model – OSI-200</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Temperature range -10 to 60°C</td>
<td></td>
</tr>
<tr>
<td>Refrigerator</td>
<td>Whirlpool (India)</td>
<td>Preservation of vial cultures</td>
</tr>
<tr>
<td>Weighing microbalance</td>
<td>Shimadzu (Japan)</td>
<td>Weighing of chemicals</td>
</tr>
<tr>
<td>Autoclave</td>
<td>Hi-Tech Equipment (India)</td>
<td>Sterilization of culture medium</td>
</tr>
<tr>
<td>UV Laminar Flow Chamber</td>
<td>Clean air system, India</td>
<td>UV Sterilization</td>
</tr>
</tbody>
</table>

Table 3.7 Equipments used
Step by Step of the methods utilized for the results of the Tables and figures of PB design presented

Step 1
The PB experimental design and the results obtained for the twenty experiments along with the twelve components were shown in the Table.

Step 2
From the above table, the maximum xylanase activity obtained for the medium having the respective composition described by the particular run number was identified.

Step 3
The analyses of the results of PB design were also shown in the Table. It shows t-values and P values and % confidence levels for twelve nutrient components. The variables with P value < 0.05 and confidence levels > 95% are considered as most significant components affecting xylanase production.

Step 4
The results of the PB design were also shown in the Pareto chart. From the Pareto chart, the most significant components affecting the xylanase production were identified and were considered for further optimization using CCD.

The remaining components with confidence levels < 95% are considered to be insignificant.

Step 5
All the insignificant variables from the PB design were neglected and the optimal concentrations of the most significant variables were used for further optimization using central composite design (CCD) of RSM to obtain maximum xylanase production.

Step by Step of the methods utilized for the results of the Tables (CCD) presented along with comments

Step 1
Statistical analysis of the data was performed by design package Design-Expert software (Version 8.0.7.1, Stat-Ease, Inc., Minneapolis, USA) to evaluate the
analysis of variance (ANOVA), to determine the significance of each term in the equations fitted and to estimate the goodness of fit in each case.

**Step 2**
The results were analyzed by using ANOVA (analysis of variance).

**Step 3**
Values of the ANOVA of the quadratic regression model indicates the model to be significant. The P values are used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. “Prob > F” less than 0.05 indicate that the model terms are significant, values greater than 0.1 indicate that the model terms are not significant. The smaller the magnitude of the P, the corresponding coefficient is more significant. From the P values the linear, interactive effects and squared effects of the variables significant for Xylanase production can be identified.

**Step 4**
The fit of the model was checked by the coefficient of determination $R^2$ for xylanase activity, if $R^2$ is very close to 1, indicating that (% $R^2$) variability in the response could be explained by the model. Normally, a regression model having an $R^2$ value higher than 0.80 is considered to have a very high correlation.

**Step 5**
Adequate precision measures the signal to noise ratio. An adequate precision value greater than 4 is desirable. If the adequate precision value of is < 4, it indicates an adequate signal and suggests that the model can be to navigate the design space

**Step 6**
The Coefficient of Variation (CV) indicates the degree of precision with which the experiments are compared. Generally, the higher the value of the CV is, the lower the reliability of the experiment
Step by Step of the methods utilized for the results of the figures (3D graphs) presented

Step 1
The elliptical shape of the contour in the 3D graphs indicates good interaction between the variables while the circular shape indicates no interaction between the variables.

Step 2
The maximum predicted yield is indicated by the surface confined in the smallest ellipse in the plot and the optimum values were obtained by solving the second order polynomial equation.

Step 3
The optimal values of the test variables are first obtained in coded units and then converted to uncoded units.

Step 4
The second order polynomial equation is maximized by a constraint search procedure using the Response optimizer of the Design Expert software to obtain the optimum levels of the independent variables and the predicted maximum xylanase activity.

Step 5
The predicted xylanase activity is compared with the experimental values.