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***RESEARCH ARTICLE***

# MEDIUM OPTIMIZATION FOR SOLID STATE FERMENTATIVE PRODUCTION OF XYLANASE BY *ASPERGILLUS TERREUS* USING CENTRAL COMPOSITE DESIGN

**Kanagasabai VIMALASHANMUGAM*, Thangavelu VIRUTHAGIRI**

*Department of Chemical Engineering, Annamalai University, Annamalai Nagar,Tamil Nadu, INDIA*

**Abstract**

Response surface methodology (RSM) was employed for the optimization of the composition of medium for the production of xylanase by Aspergillusterreus under solid state fermentation (SSF). The effect of twelve medium components was tested using Plackett-Burman designand the variables namely, peptone, soybean meal, $(NH_4)_2SO_4$, and $CoSO_4$ were found to be significant on the production of xylanase. These variables were selected for further optimization studies using central composite (CCD) design. The steepest ascent method was used to access the optimal region of the medium composition, followed by an application of response surface. The optimum values of the tested variables were peptone - 0.0161 g/gds or (g/gram dry substrate), soybean meal – 0.0429 g/gds, $(NH_4)_2SO_4$ – 0.0161 g/gds, and CoSO4 – 0.0031 g/gds. Under the final optimized conditions, the predicted response for xylanase production was 356.56 IU/gds (International units of xylanase activity per gram of dry substrate), and the observed validated experimental value was 355.73 IU/gds.

**Keywords:**xylanase, optimum values,Plackett-Burman design, Response Surface Methodology

## Introduction

Hemicellulose is composed mainly of xylan that constitutes about 20 to 40% of total plant biomass (Ninawe*et al.*, 2008). Xylan contains D-xylose as its monomeric unit and traces of L-arabinose. Microbial enzymes are a fast growing field in biotechnology. The global market of industrial enzymes was closed to a billion dollars in 1990 and crossed the $2.0 billion mark in 2005 (Krishna, 2005). The market has been estimated at $3.3 billion in 2010 and is expected to reach $4.4 billion by 2015 (Carter, 2011). Microbial enzymes act cooperatively to convert xylan to its constituent simple sugars. These enzymes include β-1,4-endoxylanases (xylanases; EC 3.2.1.8), which cleave internal glycosidic bonds within the xylan backbone; arabinofuranosidase (EC 3.2.1.55) which hydrolyzes arabinose side-chains; α-glucuronidase which removes glucuronic acid side-chains from the xylosyl units; xylan esterase (EC 3.1.1.6) which releases acetate group; and finally β-xylosidase (EC 3.2.1.37), which hydrolyzes xylobiose to xylose (Wong*et al.*, 1988).

*Corresponding author: vimalashanmugam@gmail.com

Xylanases (EC 3.2.1.8) have played an important role in many industrial processes and have been applied as additives to enhance the quality of baked goods, animal feeds as well as to bleach kraft pulp (Fuzi *et al.,* 2011), degradation of plant cell wall materials (Omar *et al.,* 2008), the manufacture of bread, food and drinks, textiles, bleaching of cellulose pulp, ethanol and xylitol production as well as in biomass process for example, biofuel production and future biorefineries(Keshwani *et al.,* 2009), oil obtained from plants, starch production (Mansour *et al.,* 2003). The xylan hydrolysis end product has considerable industrial applications in artificial sweetener, clarification of fruit juices and coffee extraction (Li *et al.,* 2010). Xylanases have been used industrially for xylo-oligosaccharides production from wheat bran or wheat straw. The xylo-oligosaccharides have been found to have a prebiotic effect on the selective growth of human intestinal *Bifidobacteria* and *Lactobacillus* spp. (Ai *et al.,* 2005).

Xylanolytic enzymes are produced by a wide variety of microorganisms, among which the filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeast and bacteria (Guimaraes *et al.,* 2006). Further, cellulases and hemicellulases produced under SSF are often more thermostable and pH-resistant, (Singhania *et al.,* 2010; Krishna, 2005). On the other hand, low-cost agroindustrial residues may be used as carbon sources. Moreover, the enzymes are obtained at higher concentrations, reducing downstream processing. Since purity is not a pre-requisite for various industrial applications, crude enzyme extracts may then be directly employed (Dhillon *et al.,* 2011; Singhania *et al.,* 2010; Krishna, 2005). Altogether, these advantages result in about ten times less production costs, and the optimization of culture conditions may still improve the economic viability of SSF processes (Singhania *et al.,* 2010).

RSM has been extensively utilized to optimize chemical and biochemical processes, such as production of enzymes (Bhattacharya *et al.,* 2010), composition of cultivation media (Kunamneni *et al.,* 2005). The optimal design of the culture medium is a very important aspect for successful use of microorganisms in industrial biotechnology, as medium composition can significantly affect product yield. Culture medium optimization by the traditional 'one-factor at- a-time' techniques requires a considerable amount of effort and time (Cazetta *et al.,* 2007). It is impractical to optimize all fermentation parameters in conventional methodology to establish the optimum conditions by understanding the interactions of all parameters, as this involves numerous experiments if all possible combinations are to be investigated (Prakasham *et al.,* 2005). Application of statistical design not only allows quick screen of a large experimental domain, but also helps in understanding the interaction between different independent variables to predict and calculate the optimal values of each factor and response (Box *et al.,* 1987). Statistically planned experiments effectively reduce the number of experiments by developing a specific design of experiments which also minimizes the error in determining the values for significant parameters. Response surface methodology is widely used to improve product yield and to reduce development time and overall process costs (Senthilkumar *et al.,*2005).

In the present study, the xylanase was produced by *Aspergillusterreus* (MTCC NO – 1782) in batch process, using sugarcane bagasse as an inexpensive substrate, by medium optimization. The medium optimization was carried out through a stepwise optimization strategy including, i) screening of nutrients was done by Plackett- Burman design (PBD), ii) the screened nutrients were further analyzed by Central Composite Design (CCD) and a regression model was established iii) the experimental verification of the model.

## Materials and Methods

### *Microorganism and culture media*

*Aspergillusterreus* (MTCC No - 1782)used in this study was purchased from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The stock culture was maintained on agar slants at 5°C. The medium composition comprises of : *Czapekconcentrate, 10.0 ml; $K_2HPO_4$ 1.0g,

Yeast extract, 5.0 g; Sucrose, 30.0 g; Agar, 15.0 g; Distilled water, 1.0 L. *Czapek concentrate: $NaNO_3$, 30.0g; KCl, 5.0g; $MgSO_4.7H_2O$, 5.0g; $FeSO_4.7H_2O$, 0.1g; and distilled water, 100.0 ml.

***Substrate Preparation***

Sugarcane bagasse samples were obtained from the agricultural field, Cuddalore District, Tamil Nadu. The samples were sun-dried for a period of three weeks and subsequently oven-dried slowly at 50°C for 48 hours. The dried samples were chopped into small bits, made into 100 mesh particle size and used as substrate for xylanase production.

***Solid state fermentation (SSF)***

Fermentation was carried out in 250 ml Erlenmeyer flasks (plugged with cotton) with 10 g of sugarcane bagasse, 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 (Adhinarayana *et al.*, 2004) was adjusted to 80%, each flask was covered with hydrophobic cotton and autoclaved at 121°C for 20 min. After cooling, each flask was inoculated with 2 ml of the spore suspension containing 1x106 spores/ml prepared from 6 day old slants of the culture grown at 30°C and the inoculated flasks were incubated at 30°C for 4 days in an incubator. During preliminary screening process, the experiments are carried out for 6 days and it was found that the maximum xylanase production occurs at the 4th day. Hence experiments are carried out for 4 days.

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

***Enzymes Assay***

Xylanase activity was determined by mixing 0.5 ml of 1% (w/v) oat spelt xylan (prepared in 0.05M Na-citrate buffer, pH 5.3) with 0.5 ml of suitably diluted enzyme and the mixture was incubated at 50°C for 30 min (Bailey *et al.*, 1992). The reaction was stopped by the addition of 3 ml of 3, 5-dinitrosalicylic acid (DNS) and the contents were boiled for 30 min. After cooling, the absorbance was read at 540 nm. The amount of reducing sugar liberated was quantified (Miller, 1959) using D-xylose as standard. One International unit (IU) of xylanase activity was defined as the amount of enzyme that liberates 1 µmol of xylose equivalents per minute under the assay conditions. Xylanase production was expressed as IU/gds (International units of xylanase activity per gram of dry substrate)

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 0.05M 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 International unit of cellulase activity was defined as the amount of enzyme that liberated 1 µmol of glucose equivalent under the assay conditions.

***Screening of nutrients using Plackett-Burman design***

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 (Plackett*et al.*,1998). Based on the Plackett–Burman design, each factor was examined at two levels, low (−1) and high (+1).

This design assumes that there are no interactions between the different media constituents, xi in the range of variable under consideration (Kammoun *et al.*, 2008).

To determine which variable significantly affect xylanase production by *Aspergillus terreus*, Plackett-Burman design using statistical software package MINITAB (Release 15.1, PA, USA), was used.

***Table 1.*** *Nutrient screening using a PlackettBurman design*

| Variables | | Levels (g/gds)or (gram/gramdry substrate) | |
|---|---|---|---|
| Nutrient code | Nutrient | Low (-1) | High (+1) |
| A | $KH_2PO_4$ | 0.01 | 0.03 |
| B | $FeSO_4$. $7H_2O$ | 0.01 | 0.02 |
| C | $MnSO_4.7H_2O$ | 0.01 | 0.05 |
| D | Soybean meal | 0.03 | 0.06 |
| E | Peptone | 0.01 | 0.02 |
| F | $CuSO_4$ | 0.002 | 0.008 |
| G | $NaNO_3$ | 0.002 | 0.02 |
| H | Urea | 0.01 | 0.03 |
| J | $CoSO_4$ | 0.002 | 0.004 |
| K | $ZnSO_4.7H_2O$ | 0.01 | 0.05 |
| L | $(NH_4)_2SO_4$ | 0.01 | 0.02 |
| M | $MgSO_4.7H_2O$ | 0.003 | 0.012 |

***Table 2.****Plackett–Burman experimental design matrix for screening of important variables for xylanase production*

| Run No | A | B | C | D | E | F | G | H | J | K | L | M |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 |
| 2 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 |
| 3 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 |
| 4 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 |
| 5 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 |
| 6 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 |
| 7 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 |
| 8 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 |
| 9 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 |
| 10 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 |
| 11 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 |
| 12 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 |
| 13 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 |
| 14 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 |
| 15 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 |
| 16 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 |
| 17 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 |
| 18 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 |
| 19 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 |
| 20 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 |

For screening purpose a total of twelve medium components (Table1) were tested for their significance in 20 experimental runs (Table 2) and insignificant ones were eliminated.

Table 1 illustrates the factors under investigation as well as the levels of each factor used in the experimental design.

Table 2 shows the Plackett–Burman experimental design matrix.

***Optimization of screened components using CCD***

In order to enhance the production of xylanase, central composite design was employed to optimize the three most significant factors, identified by the Plackett–Burman design. 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 (Shrama*et al.*,2006). 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 statistical model was obtained using the Central Composite Design with three independent variables [peptone, soybean meal, $(NH_4)_2SO_4$, and $CoSO_4$]. Each factor in this design was studied at five different levels (Table 4) and a set of 30 experiments was carried out. All the variables were taken at a central coded value considered as zero.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1, i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where Y is the measured response, β0 is the intercept term, βi are linear coefficients, βiiare quadratic coefficient, βij are interaction coefficient and Xi and Xj are coded independent variables.

The following equation was used for coding the actual experimental values of the factors in the range of (−1 to +1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad (2)$$

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.

The minimum and maximum ranges of variables were used. All the experiments were carried out in triplicates and the average value was taken as the response.

***Statistical analysis and Modeling***

This method is suitable for fitting a quadratic surface and it helps to optimize the effective parameters with minimum number of experiments as well as to analyze the interaction between the parameters. In order to determine the relationship between the factors and response variables, the collected data were analyzed in a statistical manner, using regression. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. The quadratic regression models are one of the most widely used in practice. They allow description of the object in a comparatively wide area of the input variables change (Vuchkov*et al.*,1980). A second order polynomial equation is,

**Results and Discussion**

***Placket-Burman design***

12 assigned variables (Table 2) were screened in 20 experimental designs. All experiments were carried out in triplicate and the average of the xylanase activity was taken as response (Table 2.1). A low amount of carboxy methyl cellulase activity was also obtained in all the experimental runs. Plackett-Burman experiment showed a wide variation in xylanase activity. This variation reflected the importance of optimization to attain higher productivity.The variables, which were significant at 95% level ($P < 0.05$) were considered to have greater impact on xylanase production. Using the statistical software package MINITAB (Release 15.1, PA, USA), Pareto chart was drawn to find the significant variables for xylanase production.From the Pareto chart (Fig 1), the variables namely peptone, soybean meal, $(NH_4)_2SO_4$, and $CoSO_4$ had significant effects on xylanase production.

***Table 2.1.****Plackett–Burman experimental design matrix for screening of important variables for Xylanase production along with enzyme activities*

| Run No | A | B | C | D | E | F | G | H | J | K | L | M | Xylanase activity (IU/gds) | CMCellulase activity (IU/gds) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 195.00 | 35.45 |
| 2 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | 214.45 | 44.38 |
| 3 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 174.54 | 23.23 |
| 4 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 206.43 | 34.12 |
| 5 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | 206.43 | 29.34 |
| 6 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 185.90 | 38.04 |
| 7 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | 165.34 | 21.23 |
| 8 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 174.32 | 32.54 |
| 9 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | 258.23 | 34.21 |
| 10 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 180.43 | 21.20 |
| 11 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | 197.65 | 32.12 |
| 12 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | 182.34 | 22.10 |
| 13 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | 170.32 | 36.90 |
| 14 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 234.23 | 25.31 |
| 15 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | 167.43 | 26.09 |
| 16 | -1 | 1 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 173.43 | 38.01 |
| 17 | -1 | 1 | -1 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 170.43 | 27.31 |
| 18 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 1 | -1 | 1 | 184.09 | 44.03 |
| 19 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | -1 | -1 | -1 | 1 | -1 | 195.45 | 28.14 |
| 20 | 1 | 1 | 1 | 1 | -1 | -1 | 1 | 1 | -1 | 1 | 1 | -1 | 145.32 | 30.11 |

The levels of factors peptone, soybean meal, $(NH_4)_2SO_4$, and $CoSO_4$ and the effect of their interactions on xylanase production was determined by central composite design of RSM.

The selected four independent variables were studied at five different levels (Table 3).Thirty experiments were performed at different combinations of the factors shown in Table 4.

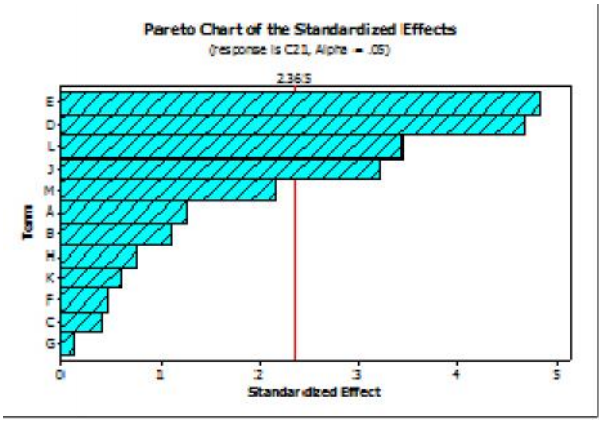


***Figure 1.*** *Pareto chart showing the effect of media components on xylanase activity*

***Table 3.*** *Ranges of the independent variables used in RSM*

| **Variables** | | **Levels (g/gds) or (gram / gram dry substrate)** | | | | |
|---|---|---|---|---|---|---|
| | **Code** | **-2** | **-1** | **0** | **1** | **2** |
| Peptone | A | 0.005 | 0.01 | 0.015 | 0.02 | 0.025 |
| Soybean meal | B | 0.015 | 0.03 | 0.045 | 0.06 | 0.075 |
| $(NH_4)_2SO_4$ | C | 0.005 | 0.01 | 0.015 | 0.02 | 0.025 |
| $CoSO_4$ | D | 0.001 | 0.002 | 0.003 | 0.004 | 0.005 |

***Table 4.*** *Central composite design (CCD) of factors in coded levels with enzyme activity as response*

| **Run No** | **Coded Values** | | | | **Xylanase Activity (IU/gds)** | | **Carboxy Methyl Cellulase (CMCase) Activity (IU/gds)** |
|---|---|---|---|---|---|---|---|
| | **A** | **B** | **C** | **D** | **Exp.** | **Pred.** | |
| 1 | 0 | 0 | 2 | 0 | 298.34 | 297.55 | 44.02 |
| 2 | 0 | 0 | 0 | 2 | 315.76 | 315.96 | 65.17 |
| 3 | 0 | 0 | -2 | 0 | 274.43 | 283.69 | 90.36 |
| 4 | 2 | 0 | 0 | 0 | 301.12 | 308.37 | 94.03 |
| 5 | -2 | 0 | 0 | 0 | 275.23 | 279.66 | 82.36 |
| 6 | 0 | -2 | 0 | 0 | 302.56 | 310.65 | 87.20 |
| 7 | 0 | 2 | 0 | 0 | 285.00 | 288.58 | 92.34 |
| 8 | 0 | 0 | 0 | 0 | 353.56 | 353.56 | 118.76 |
| 9 | 0 | 0 | 0 | -2 | 288.12 | 299.60 | 98.48 |
| 10 | 0 | 0 | 0 | 0 | 353.56 | 353.56 | 91.56 |
| 11 | -1 | 1 | 1 | 1 | 285.54 | 288.78 | 120.12 |
| 12 | 1 | 1 | -1 | -1 | 307.43 | 306.91 | 103.54 |
| 13 | -1 | 1 | 1 | -1 | 245.23 | 242.73 | 73.43 |
| 14 | -1 | -1 | -1 | -1 | 307.23 | 305.67 | 110.96 |
| 15 | 0 | 0 | 0 | 0 | 353.56 | 353.56 | 65.37 |
| 16 | 1 | 1 | -1 | 1 | 301.12 | 297.55 | 94.54 |
| 17 | -1 | 1 | -1 | 1 | 298.78 | 296.20 | 70.13 |
| 18 | -1 | -1 | 1 | 1 | 320.65 | 318.70 | 68.75 |
| 19 | 1 | -1 | -1 | -1 | 311.54 | 299.06 | 71.83 |
| 20 | 1 | 1 | 1 | -1 | 310.24 | 304.37 | 76.65 |
| 21 | -1 | 1 | -1 | -1 | 285.45 | 279.25 | 63.37 |
| 22 | 1 | -1 | -1 | 1 | 269.34 | 269.37 | 72.84 |
| 23 | -1 | -1 | -1 | 1 | 305.67 | 302.30 | 87.57 |
| 24 | 0 | 0 | 0 | 0 | 353.56 | 353.56 | 61.37 |
| 25 | 1 | -1 | 1 | -1 | 320.23 | 320.34 | 73.85 |
| 26 | 1 | 1 | 1 | 1 | 325.00 | 324.10 | 67.27 |
| 27 | 0 | 0 | 0 | 0 | 353.56 | 353.56 | 79.36 |
| 28 | 1 | -1 | 1 | 1 | 322.78 | 319.75 | 87.46 |
| 29 | -1 | -1 | 1 | -1 | 298.65 | 292.98 | 76.26 |
| 30 | 0 | 0 | 2 | 0 | 294.34 | 297.55 | 65.76 |

***Table 5.****Analysis of Variance (ANOVA) for response surface quadratic model for the production of xylanase*

| **Source** | **Coefficient factor** | **Sum of squares** | **DF** | **F** | **P > F** | |
|---|---|---|---|---|---|---|
| Model | 353.56 | 20708.7 | 14 | 31.54 | < 0.0001 | Significant |
| A-Peptone | 7.18 | 1236.4 | 1 | 26.36 | <0.0001 | Significant |
| B-Soybean meal | -5.52 | 730.63 | 1 | 15.58 | 0.0013 | |
| C-(NH4)2SO4 | 3.46 | 322.04 | 1 | 6.87 | 0.0193 | |
| D-CoSO4 | 4.09 | 401.47 | 1 | 8.56 | 0.0104 | |
| AB | 8.57 | 1174.78 | 1 | 25.05 | 0.0002 | |
| AC | 8.49 | 1153.96 | 1 | 24.61 | 0.0002 | |
| AD | -6.58 | 692.74 | 1 | 14.77 | 0.0016 | |
| BC | -5.96 | 567.63 | 1 | 12.1 | 0.0034 | |
| BD | 5.08 | 413.11 | 1 | 8.81 | 0.0096 | |
| CD | 7.27 | 846.23 | 1 | 18.04 | 0.0007 | |
| A2 | -14.89 | 5790.65 | 1 | 123.47 | < 0.0001 | Significant |
| B2 | -13.48 | 4751.79 | 1 | 101.32 | < 0.0001 | Significant |
| C2 | -15.73 | 7813.08 | 1 | 166.59 | < 0.0001 | Significant |
| D2 | -11.44 | 3422.82 | 1 | 72.98 | < 0.0001 | Significant |
| Residual | | 703.49 | 15 | | | |
| Lack of Fit | | 695.49 | 10 | 43.47 | 0.0003 | |
| Pure Error | | 8 | 5 | | | |
| Cor Total | | 21412.2 | 29 | | | |

Std. Dev.- 6.85; R2 – 0.9671; Mean – 307.25; Adj R2 – 0.9365; C.V – 2.23%; Pred R2 – 0.8193; Adeq Precision – 22.885

The predicted and observed responses along with design matrix are also presented in Table 4, and the results were analyzed by ANOVA.

The following second order polynomial equation describing the correlation between xylanase and the four variables was obtained:

Y = 353.56 + 7.18A - 5.52B + 3.46C + 4.09D + 8.57AB +8.49AC - 6.58AD - 5.96BC + 5.08BD + 7.27CD -14.89A2 – 13.48B2 – 15.73C2 – 11.44D2 (3)

Where, Y is the xylanase activity (IU/gds), A, B, C and D are peptone, soybean meal, $(NH_4)_2SO_4$, and $CoSO_4$ respectively.

ANOVA for the response surface is shown in Table 5. The model F value of 31.54 implies the model is significant. There is only a 0.01% chance that a "Model F value" this large could occur due to noise. Values of "prob> F" less than 0.05 indicate model terms are significant. Values greater than 0.1 indicate that the model terms are not significant.

In the present work, the linear effect of A, B, C, D, the interactive effects of AB, AC, AD, BC, BD, CD and square effects of A2, B2, C2, D2 are significant model terms for xylanase production. To test the fit of the model equation, the regression - based determination coefficient $R^2$ was evaluated. The nearer the values of $R^2$to 1, the model would explain better for variability of experimental values to the predicted values. The coefficient of determination, $R^2$ for Xylanase activity was calculated as ($R^2$=0.9671) which is nearly equal to 1, indicating that 96.71% of variability in the response could be explained by the model.Normally, a regression model having an $R^2$ value higher than 0.90 is considered to have a very

high correlation (Haaland, 1989). The predicted $R^2$value of xylanase activity was 0.8193 was in reasonable agreement with the adjusted $R^2$value of 0.9365. Usually higher the value of C.V, lower the reliability of the experiment is (Yin Li *et al.*, 2007). Here, a lower value of C.V (2.23%) indicated a better precision and reliability of the experiments. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Adeq Precision of 22.885 indicates an adequate signal. This model can be used to navigate the design space. The above models can be used to predict the xylanase production within the limits of the experimental factors.

The interaction effects of variables on xylanase was studied by plotting three dimensional surface curves against any two independent variables, while keeping another variable at its central (0) level. The plotted three dimensional surface curves of the calculated responses (xylanase activity) from the interactions between the variables are shown in Figures 2-7.

Fig 2, 3, 4 shows the dependency of xylanase activity on peptone. The xylanase activity increases with increase in peptone about 0.0161 g/gdsand thereafter xylanase activity decreases with further increase in peptone. The same trend was observed in Fig 2, 5, 6 which shows the dependency of xylanase activity on soybean meal. The xylanase activity increases with increase in soybean meal about 0.0429 g/gds and thereafter xylanase activity decreases with further increase in soybean meal. Haltrich *et al.* (1996) reported that for fungal organisms when peptone or yeast extract, cheaper complex nitrogen supplement such as soybean meal were used in the production medium higher xylanase activities could be obtained. Fig 3, 5, 7 shows the dependency of xylanase activity on $(NH_4)_2SO_4$. Increased concentrations of ammonium sulphate had positive effect on the formation of enzyme activities (Haltrich *et al.*, 1996). The xylanase activity increases with increase in $(NH_4)_2SO_4$ about 0.0161 g/gds and thereafter xylanase activity decreases with further increase in $(NH_4)_2SO_4$. Ammonium salts have enhanced the growth rate as well as improved the protein expression by mediating ammonium assimilating enzymes (Wang *et al.*, 2009). The dependency of xylanase activity on $CoSO_4$ is shown in Fig 4, 6, 7. The xylanase activity increases with increase in $CoSO_4$ to about 0.0031 mg/gds and thereafter xylanase activity decreases with further increase in $CoSO_4$. The xylanase activity was greatly elevated by the addition of CO+2, Zn+2, Fe+2, Cu+2, Mg+2 and Ca+2 ions (Khandeparkar *et al.*, 2006).

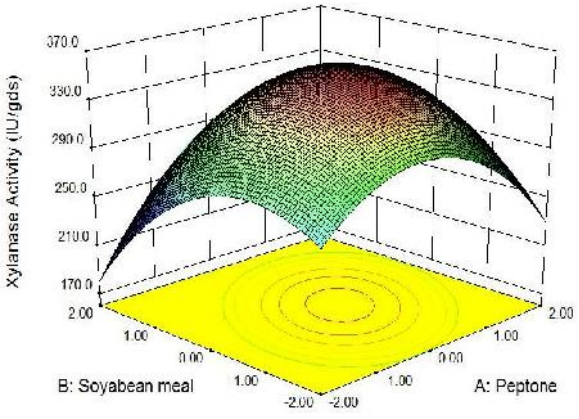


***Figure 2.*** *Three-dimensional surface plot showing the interactiveeffect of peptone and soybean meal on xylanase activity*

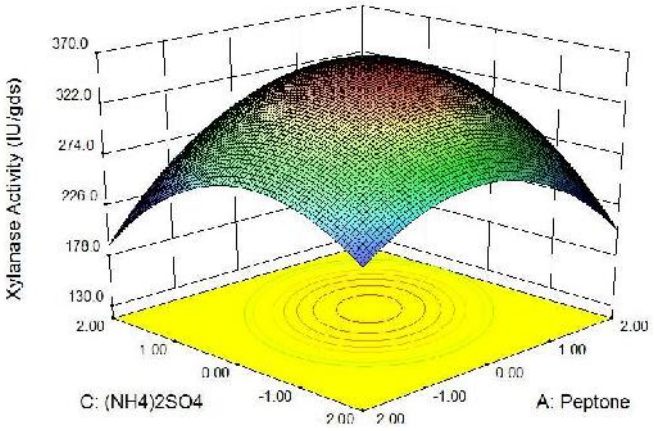


***Figure3.****Three-dimensional surface plot showing the interactive effect of peptone and $(NH_4)_2SO_4$ on xylanase activity*

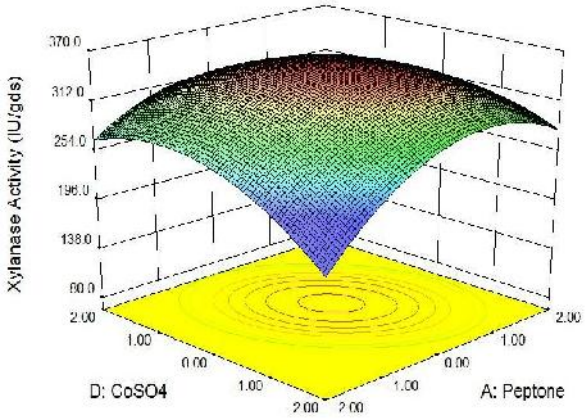


***Figure 4.*** *Three-dimensional surface plot showing the interactive effect of peptone and $CoSO_4$ on xylanase activity*

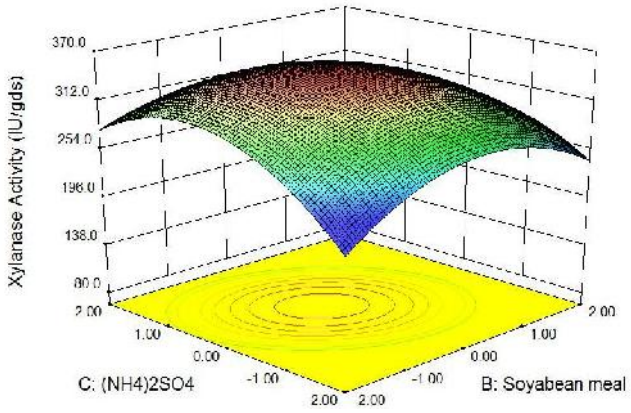


***Figure 5.*** *Three-dimensional surface plot showing the interactive effect of soybean meal and $(NH_4)_2SO_4$ on xylanase activity*

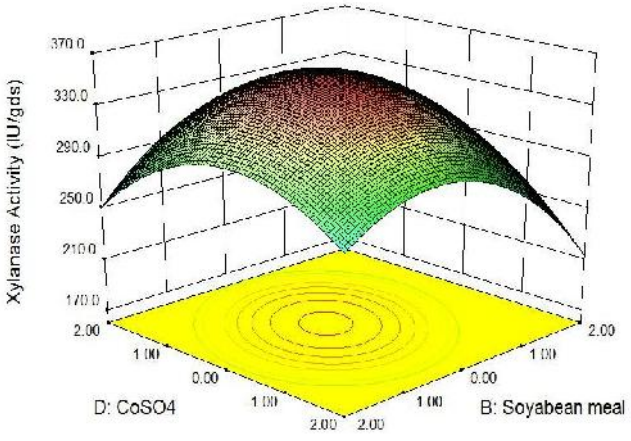


***Figure 6.*** *Three-dimensional surface plot showing the interactive effect of soybean meal and $CoSO_4$ on xylanase activity*

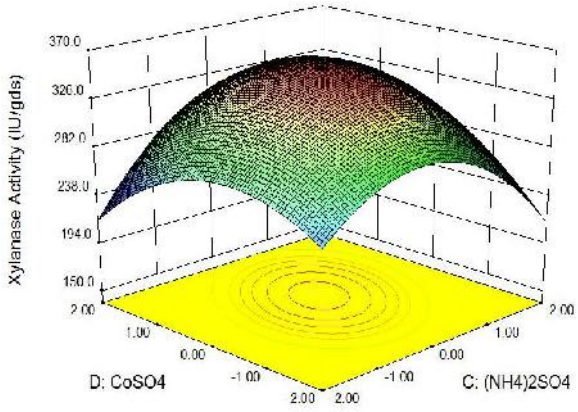


***Figure 7.*** *Three-dimensional surface plot showing the interactive effect of $(NH_4)_2SO_4$ and $CoSO_4$ on xylanase activity*

The optimum conditions for the maximum production of xylanase were determined by response surface analysis and also estimated by response optimizer tool of MINITAB (Release 15.1, PA, USA) software. The optimum conditions are: peptone – 0.0161 g/gds, soybean meal – 0.0429 g/gds, $(NH_4)_2SO_4$ – 0.0161 g/gds, and CoSO4 – 0.0031 g/gds. The predicted values from the regression equation closely agreed with that obtained from experimental values. Along with nutrient optimized xylanase production, very poor carboxy methyl cellulase activity was detected in all the 30 experimental runs. The predicted results are shown in Table 4.

Fig.8 shows that the experimental xylanase activity values agree well with the predicted response values.

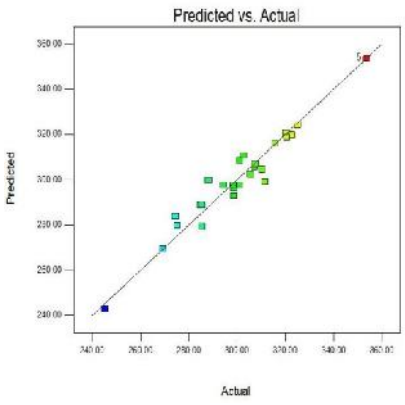


*Figure 8. Predicted Response Vs Actual Value response values*

### *Validation of the Model*

Validation of the experimental model was tested by carrying out the batch experiment in triplicates under the calculated optimized conditions: peptone – 0.0161 g/gds, soybean meal – 0.0429 g/gds, $(NH_4)_2SO_4$ – 0.0161 g/gds, and $CoSO_4$ – 0.0031 g/gds. The predicted xylanase activity was 356.56 IU/gds and the average xylanase activity found by experiment was 355.73 IU/gds. The experimental and predicted xylanase activity coincides with one another. The good correlation between predicted and experimental values after optimization justified the validity of the model.

## Conclusion

In this work, the applicability of statistical methodology, a combination of Plackett–Burman design and central composite designof response surface methodology proved to be efficient in determining the significant variables and optimum condition for the production of xylanase enzyme in solid-state fermentation. Among the twelvevariables peptone, soybean meal $(NH_4)_2SO_4$ and $CoSO_4$ were found to be most significant variables. From further optimization Studies, using RSM the optimized values of the variables for xylanase production were: peptone – 0.0161 g/gds, soybean meal – 0.0429 g/gds, $(NH_4)_2SO_4$ – 0.0161 g/gds, and $CoSO_4$ – 0.0031 g/gds. Under the optimized conditions, the experimentally obtained xylanase activity reaches 355.73 IU/gds. The results show a close concordance between the predicted (356.56 IU/gds) and experimental activity level. This study showed sugarcane bagasse as a cheaper and better substrate for xylanase production in solid state fermentation. Further work on optimization of process parameters such as temperature, pH, substrate concentration, innoculum size is currently underway.

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## References

Adhinarayana K., BapiRaju K. V. V. S. N., IqbalZargar M., Bhavani Devi R., Jhansi Lakshmi P., Ellaiah P. (2004) Optimization of process parameters for production of lipase in SSF by newly isolated A. species, *Indian Journal of Biotechnology*, 3, 65-69.

Ai Z., Jiang Z., Li L., Deng W., Kusakabe I., Li H. (2005) Immobilization of Streptomyces olivaceoviridis E-86 xylanase on Eudragit S-100 for xylo-oligosaccharide production. *Process Biochem*, 40, 2707-2714.

Bailey M. J., Biely P., Poutanen K. (1992) Interlaboratory testing of methods for assay of xylanase activity, *J Biotechnol*, 23, 257-270.

Bhattacharya S. S., Garlapati V. K., Banerjee R. (2010) Optimization of laccase production using response surface methodology coupled with differential evolution. *New Biotechnol*, 28(1), 31-39.

Box G. E. P., Draper N. R. (1987) Emperical model building and response surfaces, Wiley, New York.

Carter L.(2011) Global Industrial Enzymes Market is Expected to Reach $4.4 Billion by 2015, PRNewswire.com.

Cazetta M. L., Celligoi M. A. P. C., Buzato J. B., Scarmino I .S. (2007) Fermentation of molasses by *Zymomonasmobilis*: effects of temperature and sugar concentration on ethanol production, *Bioresour. Technol*., 98(15), 2824–2828.

Dhillon G. S., Oberoi H. S., Kaur S., Bansal S., Brar S. K.(2011) Value-addition of agricultural wastes for augmented cellulase and xylanase production through solid-state tray fermentation employing mixed-culture of fungi., *Ind. Crop Prod*, 34, 1160–1167.

Fuzi F. Z. M., Mahadi N. M., Jahim J. M., Murad A. M. A., Bakar F. D. A., Jusoh M., Rahman R. A., Illias R. M. (2011) Development and validation of a medium for.recombinantendo-β-1,4-xylanase production by *Kluyveromyceslactis* using a statistical experimental design. *Ann Microbiol*, 62(1), 283–292.

Guimaraes L. H. S., Nogueira P. S., Michelin M., Rizzatti A. C. S., Sandrim V. C., Zanoela F. F., Aquino A. C. M., Junior A. B., POLIZELI M. L. T. M. (2006) Screening of filamentous fungi for production of enzymes of biotechnological interest. *Braz. J. Microbiol*, 37, 474–480.

Haaland P. D. (1989) Separating signals from the noise In: Experimental design in biotechnology, New York, Michael Deckker, 61-83.

Haltrich D., Nidetzky B., Kulbe K. D., Steiner W., Zupancic S. (1996) Production of Fungal Xylanases, *Bioresoure Techno*, 58, 137–161.

Kammoun R., Naili B., Bejar S. (2008) Application of a statistical design to the optimization of parameters and culture medium for 8-amylase production by Aspergillusoryzae CBS 819.72 grown on gruel (wheat grinding by-product), *Bioresour. Technol*., 99(13), 5602–5609.

Keshwani D. R., Cheng J. J. (2009) Switchgrass for Bioethanol and Other Value Added Applications: A Review. *Bioresourc.Technol*, 100(4), 1515-1523.

Khandeparkar R. D. S., Bhosle N. B. (2006) Isolation, purification and characterization of the xylanase produced by Arthrobactersp MTCC 5214 when grown insolid-state fermentation,Enzyme*Microbiol. Tech*, 39, 732-767.

Krishna C. (2005) Solid-state fermentation systems-an overview, *Crit Rev Biotechnol*, 25, 1-30.

Kunamneni A., Singh S. (2005) Response surface optimization of enzymatic hydrolysis of maize starch for higher glucose production. *Biochem. Eng. J*, 27, 179–190.

Li X., She Y., Sun B., Song H., Zhu Y., Lv Y., Song H. (2010) Purification and characterization of a cellulase-free, thermostablexylanase from Streptomyces rameus L2001 and its biobleaching effect on wheat straw pulp, *Bioch. Engg J*, 52, 71-78.

Mansour F. A., Shereif M. M., Nour el-dein M. M., Abou-dobara M. I., Ball A. S. (2003) Purification and characterization of xylanase from a thermophilic*Streptomyces*sp K.37,*ActaMicrobiologicaPolonica*, 32(2, 159,172.

Miller G. L., (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar,*Anal.Chem*, 31, 426–428.

Ninawe S., Kapoor M., Kuhad R. C. (2008) Purification and characterization of extracellular xylanase from Streptomyces cyaneus SN32.*BioresourTechnol*,99, 1252-1258.

Omar A.W., Khataibeh M. H., Abu-alruz K. (2008) The use of xylanases from different microbial origin in bread making and their effects on bread quality. *J. Appl. Sci*, 8(4), 672-676.

Plackett R L., Burman J. P. (1998) The design of optimum multifactorial experiments, *Biometrika*, 33, 305-325.

Prakasham R. S., SubbaRao CH., SreenivasRao R., Sarma P. N. (2005) Alkaline protease production by an isolated Bacillus circulans under solid-state fermentation using agroindustrial waste: process parameters optimization, *Biotechnol. Prog*, 21, 1380–1388.

Senthilkumar S. R., Ashokkumar B., Chandra Raj K., Gunasekaran P. (2005) Optimization of medium composition for alkali-stable xylanase production by *Aspergillusfischeri*Fxn 1 in solid-state fermentation using central composite rotary design, *Bioresour. Technol*., 96, 1380–1386.

Shrama D. C., Satyanarayana T. (2006) A marked enhancement in the production of a highly alkaline and thermostable pectinase by *Bacillus pumilus*dcsr 1 in submerged fermentation by using statistical methods, *Bioresour. Technol*, 97, 727–733.

Singhania R. R., Sukumaran R. K., Patel A. K., Larroche C., Pandey A. (2010) Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases, *Enzyme Microb. Technol*, 46, 541–549.

SreenivasRao R., Kumar C. G., Prakasham R. S., Hobbs P. J. (2008) The Taguchi methodology as a statistical tool for biotechnological applications: a critical appraisal, *Biotechnol J*, 3, 510–523.

Vuchkov I., Stoyanov S. (1980) Mathematical modelling and optimization of technological objects, *Technics*. Sofia, 135–151.

Wang H., Wang F., Wei D. (2009) Impact of oxygen supply on rtPA expression in *E.coli*BL21 (DE3): ammonia effect, *In Applied Microbiology Biotechnology*, 82, 249-259.

Wong K. K., Tan L. U., Saddler J. N. (1988) Multiplicity of beta-1,4-xylanase in mircroorganisms: functions and applications. *Microbiol Rev*, 52, 305-17.

Yin Li., Fengjie.,Zhiqiang Liu., Ying YingXu., Hui Zhao. (2007) Improvement of xylanase production by *Pencilliumoxalicum* ZH-30 using response surface methodology, *Enzyme and Microbial Technology*, 40, 1381-1388.