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Statistical Optimization of Media Components for Xylanase Production *by Aspergillus fumigatus* using SSF

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ABSTRACT

Aspergillus fumigatus is one of the thermophilic fungal species known to produce extracellular xylanases which are capable of solubilising lignocellulosic wastes. The production of Xylanase from Aspergillus fumigatus MTCC NO - 343, using wheat bran as an inexpensive substrate in solid state fermentation(SSF) was enhanced by medium optimization from twenty nutrients using Plackett-Burman design. The optimization process was further analyzed using central composite design and response surface methodology. Three significant factors influencing Xylanase production was identified as $NaNO_3$, $(NH_4)_2SO_4$ and KH_2PO_4 using Plackett-Burman design study. The effects of these nutrients on production of Xylanase were studied using central composite design. The optimized conditions by response surface analysis were $NaNO_3$ = 0.29066 (g/gds), $(NH_4)_2SO_4$ = 0.14448 (g/gds) and KH_2PO_4 0.2369 (g/gds). Under these conditions, the maximum production of Xylanase was found to be 525.76 U/gds. The experimental verification reveals an enhanced xylanase production 531 U/gds.

Keywords: Central composite design, nutrients, Plackett-Burman design, Response surface methodology, Xylanase

1. Introduction

Discovered in the later half of the 19th century as an effective biocatalyst especially in the field of protein engineering & genetics, enzymes found their way in several new industrial processes [1]. The biotechnological applications of xylanases began in 1980's in the preparation of animal feed and later expanded to food, textile and paper industries. It has important applications in industry due to their enormous potential to modify & transform the lignocellulose & cell wall materials abundant in vegetable biomass [2]. Substrate xylan, a biopolymer comprising of D-xylose monomers linked through β-1,4-glycosyl bond, is found abundantly in lignocellulosic biomass. Microbial xylanases have important applications in the degradation of xylan **[**3**]**. Microbial xylanases represent one of the largest groups of industrial enzymes and have attracted a great deal of attention during the past few decades. Their potential biotechnological applications in various industries include the food, feed, fuel, textile, detergents, paper and pulp industries and in waste treatment [4-5]. In bread and bakery industry, xylanases are used to increase the dough viscosity, bread volume and shelf life [6]. The need of the xylanase enzyme has dramatically increased recently, particularly in pharmaceutical, food and beverages, and paper industries [7].

Production of xylanases by solid state fermentation (SSF) using varying lignocellulosic substances has been reported using different fungal [8-12] and bacterial strains [13]. Among microbial sources, filamentous fungi are especially interesting as they secrete these enzymes into the medium and their xylanase activities are much higher than those found in yeasts and bacteria [14-15]. The Xylanase enzyme can be produced by a number of microorganisms including bacteria, yeast and filamentous fungi like Trichoderma, Aspergillus, Penicillium, Fusarium, Chaetomium, Humicola, Taloromyces, and many others [16]**.** Solid-state fermentation (SSF) is defined as a fermentation process occurring in absence (or near absence) of free water, with enough moisture content to support growth and metabolism of microorganisms [17] widely used for fungal cultivations and few reports are available for bacterial cultivation [18], also an attractive method for xylanase production, and a large number of different *Aspergillus* species have been reported as good xylanases producers [19-20]. Cellulase free xylanases active at high temperature and pH are gaining importance in pulp and paper technology as alternatives to the use of toxic chlorinated compounds [21-22]. For commercial production, optimization of medium composition is one of the essential steps to minimize the amount of unutilized components for a cost-effective yield [23]. 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. 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 [24].

The solid-state medium was optimized based on the 'change-one-factor-at-atime' approach and detailed studies were carried out using response surface methodology (RSM) to optimize the recovery of xylanase from optimized medium. The RSM is an empirical statistical technique used to find the optimum conditions of a process response variable when the mechanism underlying the process is either not well understood or is too complicated to allow the exact model to be formulated from theory. It evaluates the relation existing between a group of controlled experimental factors and the observed results of one or more selected variables [25]. In the present study, the Xylanase enzyme was produced by *Aspergillus fumigatus (MTCC NO – 343)* in batch process, using wheat bran as an inexpensive substrate, enhanced by medium optimization. In medium optimization, screening of nutrients was done by Plackett-Burman design (PBD), the screened nutrients were further analysed by Central composite design (CCD) and a regression model was established and the experimental verification of the model was validated.

2. Materials and Methods

2.1. Microorganism and culture media

Aspergillus fumigatus (MTCC No - 343) 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 5^0C. The medium composition comprises of : *Czapek Concentrate, 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.

2.2. Solid state fermentation (SSF)

Wheat bran was used as substrate for xylanase production. Wheat bran was oven dried at 70^0C for 48 hrs, ground to 40 mesh particle size and used as a substrate. Fermentation was carried out in Erlenmeyer flasks (250 ml) 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. Moisture was adjusted to 80%, each flask was covered with hydrophobic cotton and autoclaved at 121^0C for 20 min. After cooling, each flask was inoculated with 2 ml of the spore suspension containing $1x10^6$ spores/ml prepared from 6 day old slants of the culture grown at 30^0C and the inoculated flasks were incubated at 30^0C for 5 days in an incubator. During Preliminary screening process, the experiments are carried out for 7 days and it was found that the maximum Xylanase production occurs at the 5th day. Hence experiments are carried out for 5 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 minutes at 200 rpm in an orbital shaker at 30^0C and filtered through a wet muslin cloth by squeezing. The extract was centrifuged at 15,000 rpm for 20 minutes and the supernatant was used for determination of enzyme activity.

2.3 Enzyme Assay

Xylanase activity was 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 was measured by dinitro salicylic acid (DNS) method [26] and xylose was used as the standard. One unit (U) of xylanase activity is defined as the amount of enzyme releasing 1µmol of xylose per minute under the assay conditions. Xylanase production in SSF was expressed as U/g dry substrate (U/gds).

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 50^0C for 30 min. The amount of reducing sugars released during the reaction was measured using the DNS method [26] and D-glucose was used as the standard. One unit of cellulase activity was defined as the amount of enzyme that liberated 1 µmol of glucose equivalent under the assay conditions.

2.4 Experimental Design and Statistical Analysis

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. Response surface methodology (RSM) was employed to optimize the selected three variables. RSM consist of a group of empirical techniques used for evaluation of relationship between cluster of controlled experimental factors and measured response. A prior knowledge with understanding of the related bioprocesses is necessary for a realistic modeling approach. To determine which variable significantly affect xylanase production by *Aspergillus fumigatus*, Plackett-Burman design using statistical software package MINITAB (Release 15.1, PA, USA), was used. 18 variables (Table-1) were screened in 20 experimental runs (Table-2) and insignificant ones were eliminated in order to obtain a smaller, manageable set of factors. The low level (-1) and high level (+1) of each factor are listed in Table-1.

Significant nutrient components viz. $NaNO_3$, $(NH_4)_2SO_4$, and KH_2PO_4 which increases

the xylanase production were identified. The three independent variables were studied at five different levels (Table-3) and sets of 20 experiments (batch experiments) were carried out (Table-4). The statistical software package 'Design Expert 8.0.7.1' was used to analyse the experimental data. All variables were taken at a central coded value of zero. The minimum and maximum ranges of variables investigated are listed in Table-3.

Upon the completion of experiments, the average maximum xylanase were taken as the response (Y). A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second order polynomial equation is,

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

Where Y is the measured response, β_0 is the intercept term, β_i are linear coefficients, β_{ii} are quadratic

Table 1: Nutrient screening using a Plackett Burman design

Variables		**Levels g/10 gds**	
Nutrient code	**Nutrient**	**Low (-1)**	**High (+1)**
A	K_2HPO_4	0.05	0.20
B	Soyabean meal	0.3	0.6
C	$FeSO_4.7H_2O$	0.005	0.01
D	$MnSO_4.7H_2O$	0.05	1.5
E	$MgSO_4.7H_2O$	0.003	0.005
F	Urea	0.02	0.04
G	Kcl	0.04	0.08
H	$(NH_4)_2SO_4$	0.10	0.20
J	CaCl2	0.02	0.03
K	K_2HPO_4	0.02	0.12
L	$ZnSO_4.7\ H_2O$	0.01	0.02
M	NaCl	0.10	0.30
N	KH_2PO_4	0.10	0.30
O	$NaNO_3$	0.20	0.40
P	Peptone	0.005	0.015
Q	$(NH_4)_2HPO_4$	0.015	0.030
R	Beef Extract	0.005	0.015
S	Meat Extract	0.001	0.005

coefficient, β_{ij} 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):

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

where x_i is the dimensionless value of an independent variable, X_i is the real value of an

independent variable, X_0 is the value of X_i at the center point, and ΔX_i is the step change.

Statistical analysis of the data was performed by design package Design Expert 8.0.7.1 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 fitted polynomial equation was then expressed in the form of three-dimensional response surface plots to illustrate the main and interactive effects of the independent variables on the dependent ones. The combination of different optimized variables, which yielded the maximum response, was determined to verify the validity of the model. In order to verify the accuracy of the predicted model an experiment was conducted with initial and optimized media. The optimal concentrations of the critical variables were obtained by analyzing 3D plots. The statistical analysis of the model was represented in the form of analysis of variance (ANOVA).

3. Results and Discussion

Plackett-Burman experiments (Table-2) showed a wide variation in Xylanase activity. This variation reflected the importance of optimization to attain higher productivity. From the Pareto chart (Fig.1), the variables, namely, $NaNO_3$, $(NH_4)_2SO_4$, and KH_2PO_4 were selected for further optimization to attain a maximum production of Xylanase. The levels of factors $NaNO_3$, $(NH_4)_2SO_4$, and KH_2PO_4 and the effect of their interactions on Xylanase production were determined by central composite design using RSM. For this study, 2^3 full factorial central composite design with six star points, six axial and six replicates at the centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure shown in Table-3. The predicted and observed responses along with design matrix are presented in Table-4, and the results were analyzed by ANOVA. The second-order

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	N	O	P	Q	R	S	Xylanase Activity (U/gds)	Cellulase Activity (U/gds)
1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	274.0	72
2	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	394.4	51
3	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	415.0	70
4	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	254.1	93
5	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	229.5	104
6	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	276.6	65
7	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	256.1	44
8	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	188.9	94
9	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	262.4	90
10	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	148.9	87
11	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	211.6	82
12	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	309.6	97
13	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	402.0	107
14	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	304.3	76
15	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	219.3	50
16	1	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	312.3	74
17	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1	1	-1	217.1	33
18	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1	285.2	64
19	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	213.8	26
20	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	355.0	59

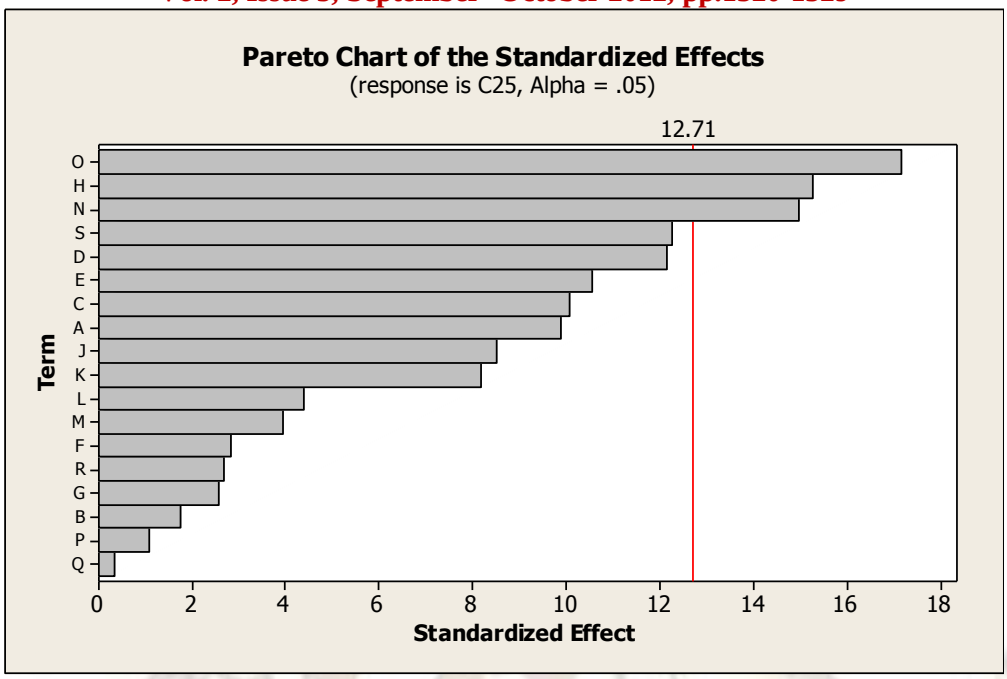

Fig. 1. Pareto chart showing the effect of media components on Xylanase activity

Table 3: Ranges of the independent variables used in RSM

Variable		Levels (g/10gds)				
	Code	**-1.68**	**-1**	**0**	**1**	**1.68**
$NaNO_3$	X1	0.20	0.25	0.30	0.35	0.40
$(NH4)_2SO_4$	X2	0.10	0.125	0.15	0.175	0.20
KH_2PO_4	X3	0.10	0.15	0.20	0.25	0.30

Table 4: Central composite design (CCD) of factors in coded levels with Enzyme activity as response

Run. No	**Type**	**Coded Values**			**Inulinase Activity(U/gds)**		**Cellulase Activity (U/gds)**
		X1	**X2**	**X3**	**Experimental**	**Predicted**	
1	Axial	0.00	0.00	1.68	337.28	342.83	110.13
2	Factorial	-1.00	-1.00	1.00	396.80	406.51	120.20
3	Centre	0.00	0.00	0.00	520.80	519.03	135.65
4	Axial	0.00	-1.68	0.00	430.00	396.08	107.78
5	Factorial	1.00	-1.00	-1.00	322.40	332.79	101.89
6	Factorial	-1.00	1.00	-1.00	215.70	222.59	97.45
7	Axial	-1.68	0.00	0.00	349.68	319.92	121.09
8	Axial	0.00	0.00	1.68	297.60	267.03	101.32
9	Factorial	1.00	-1.00	1.00	255.44	266.23	98.54
10	Axial	1.68	0.00	0.00	223.20	227.94	96.47
11	Factorial	-1.00	1.00	1.00	372.00	379.29	104.87
12	Centre	0.00	0.00	0.00	513.36	519.03	130.42
13	Centre	0.00	0.00	0.00	525.76	519.03	126.70

14	Centre	0.00	0.00	0.00	520.80	519.03	115.34
15	Factorial	1.00	1.00	1.00	250.40	227.60	92.76
16	Factorial	-1.00	-1.00	-1.00	250.00	290.48	91.48
17	Factorial	1.00	1.00	-1.00	245.50	253.48	98.56
18	Axial	0.00	1.68	0.00	297.60	306.50	103.12
19	Centre	0.00	0.00	0.00	508.40	519.03	120.54
20	Centre	0.00	0.00	0.00	520.80	519.03	110.43

regression equation provided the levels of Xylanase activity as the function of $NaNO_3$, $(NH_4)_2SO_4$ and KH_2PO_4which can be presented in terms of coded factors as in the following equation:

$$Y = 518.43 - 27.35X1 - 26.62X2 + 30.91X3 - 82.86X1^2 - 55.45X2^2 - 90.27X3^2 - 2.85X1X2 - 45.63X1X3 + 10.17 X2X3 \quad (3)$$

Where, Y is the Xylanase activity (U/gds), X1, X2 and X3 are $NaNO_3$, $(NH4)_2SO_4$, KH_2PO_4 respectively. ANOVA for the response surface is shown in Table 5. The Model F-value of 67.46 implies the model is significant. Values of "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. In the present work, all the linear, interactive effects of *X*1*X*3 and square effects of *X*1, *X*2, and *X*3 were significant for Xylanase production. The coefficient of determination (R^2) for Xylanase activity was calculated as 0.9838, which is very close to 1 and can explain up to 98.38% variability of the response. The predicted R^2 value of 0.8651 was in reasonable agreement with the adjusted R^2 value of 0.9692. "Adeq Precision" measures the signal to noise ratio. An adequate precision value greater than 4

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	518.43	*2.492E+005*	9	67.46	< 0.0001	Significant
X1	-27.35	*10209.96*	1	24.88	0.0005	
X2	-26.62	*9671.56*	1	23.57	0.0007	
X3	30.911	*8719.94*	1	21.25	0.0010	
X1 * X2	-2.851	*64.75*	1	0.16	0.6996	
X1 * X3	-45.63	*16660.43*	1	40.59	< 0.0001	Significant
X2 * X3	10.17	*828.24*	1	2.02	0.1859	
X1 * X1	-82.86	*96214.39*	1	234.43	< 0.0001	Significant
X2 * X2	-55.45	*43086.61*	1	104.98	< 0.0001	Significant
X3 * X3	-90.27	*86938.65*	1	211.83	< 0.0001	Significant
Residual		*4104.14*	10			
Lack of Fit		*3120.08*	4	4.76	0.0452	
Pure Error		*984.06*	6			
Cor Total		*2.533E+005*	19			

Std. Dev. - 20.26; R^2 -98.38%; Mean - 367.68; Adj R^2- 96.92%; C.V - 5.51%;
Pred R^2-86.51%; Adeq Precision – 21.760

is desirable. The adequate precision value of 21.76 indicates an adequate signal and suggests that the model can be used to navigate the design space. The above model can be used to predict the Xylanase production within the limits of the experimental factors. Fig.2 shows that the actual response values agree well with the predicted response values. The interaction effects of variables on xylanase production were studied by plotting Contour plot & 3D surface curves against any two independent variables, while keeping another variable at its central (0) level. The 3D curves of

the calculated response (Xylanaseproduction) and contour plots from the interactions between the variables are shown in Fig.3- 5. A circular contour plot indicates that the interactions between the corresponding variables are negligible, while an elliptical contour plot indicates that the interactions between them are significant. Fig.3 shows the dependency of $NaNO_3$ and $(NH_4)_2SO_4$. The xylanase activity increased with increase in $NaNO_3$ to about 0.29066 g/gds and thereafter Xylanase activity decreased with further increase in $NaNO_3$. The same trend was observed in Figure 4. Increase in $(NH_4)_2SO_4$ resulted increase in Xylanase activity up to 0.14448 g/gds. This is evident from Fig. 3 and 5. Fig.4 and 5 shows the dependency of Xylanase activity on KH_2PO_4. The effect of KH_2PO_4 on Xylanase observed was similar to other variables. The maximum xylanase activity was observed at 0.23690 g/gds of KH_2PO_4. The optimum conditions for the maximum production of Xylanase were determined by response surface analysis and also estimated by regression equation. The optimum conditions are: $NaNO_3$ - 0.29066 (g/gds), $(NH_4)_2SO_4$ – 0.14448 (g/gds) and KH_2PO_4 – 0.23690 g/gds). The predicted results are shown in (Table-4). The predicted values from the regression equation closely agreed with that obtained from experimental values. Along with nutrient optimized xylanase production, a small amount of cellulase activity also found in each experimental runs (Table-4).

Validation of the experimental model was tested by carrying out the batch experiment under optimal operation conditions. Three repeated experiments were performed, and the results are compared. The xylanase activity obtained from experiments was very close to the actual response predicted by the regression model, which proved the validity of the model. At these optimized conditions, the maximum xylanase activity was found to be 525.76 U/gds.

4. Conclusions

In this work, the applied Statistical tools proved to be efficient for optimizing xylanase enzyme production in solid-state fermentation. Plackett-Burman design was used to test the relative importance of medium components on Xylanase production. Among the variables, $NaNO_3$, $(NH_4)_2SO_4$ and KH_2PO_4 were found to be the most significant variables. From further optimization studies, using RSM the optimized values of the variables for Xylanase production were as follows: $NaNO_3$ - 0.29066 g/gds, $(NH_4)_2SO_4$ - 0.14448 g/gds and KH_2PO_4 - 0.2369 g/gds. Using the optimized conditions, the experimentally obtained Xylanase activity reaches 531.0 U/gds. This study showed that Xylanase production along with small amount of cellulase by *Aspergillus fumigatus* strain could be enhanced by statistical optimization of the medium components using the cheaper substrate wheat bran. The results show a close concordance between the expected and obtained activity level. Further work on statistical optimization of process parameters such as temperature, pH, substrate conconcentration, moisture content is currently underway.

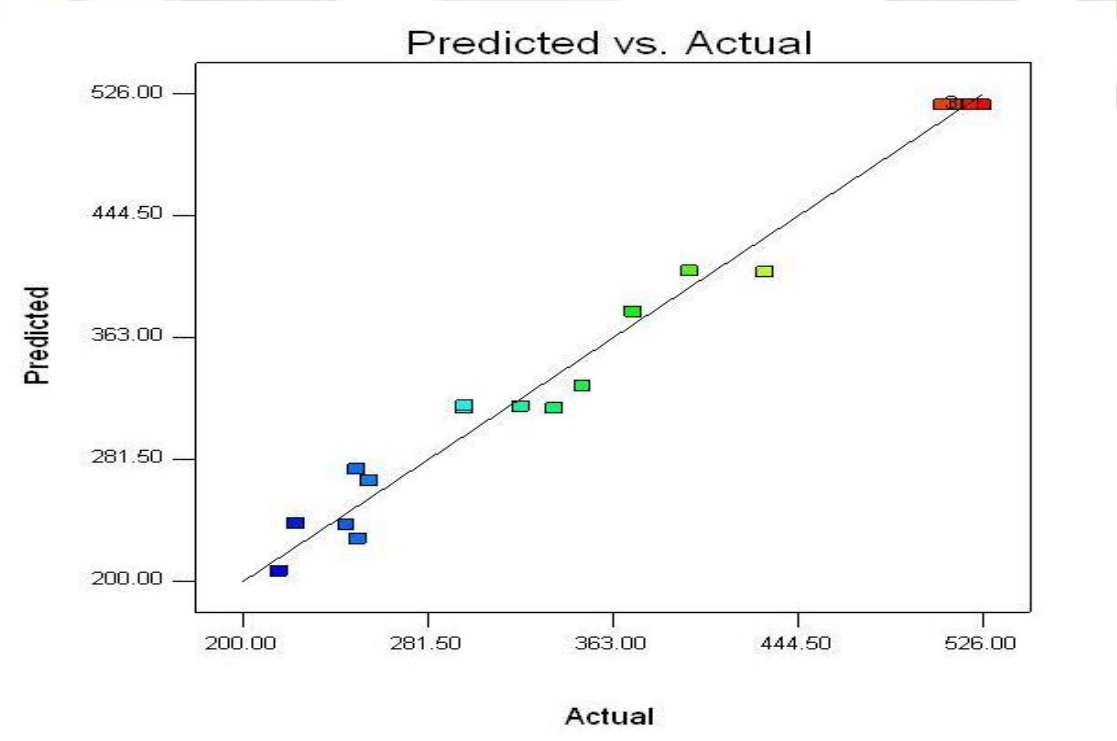

Fig.2. Predicted response versus Actual value

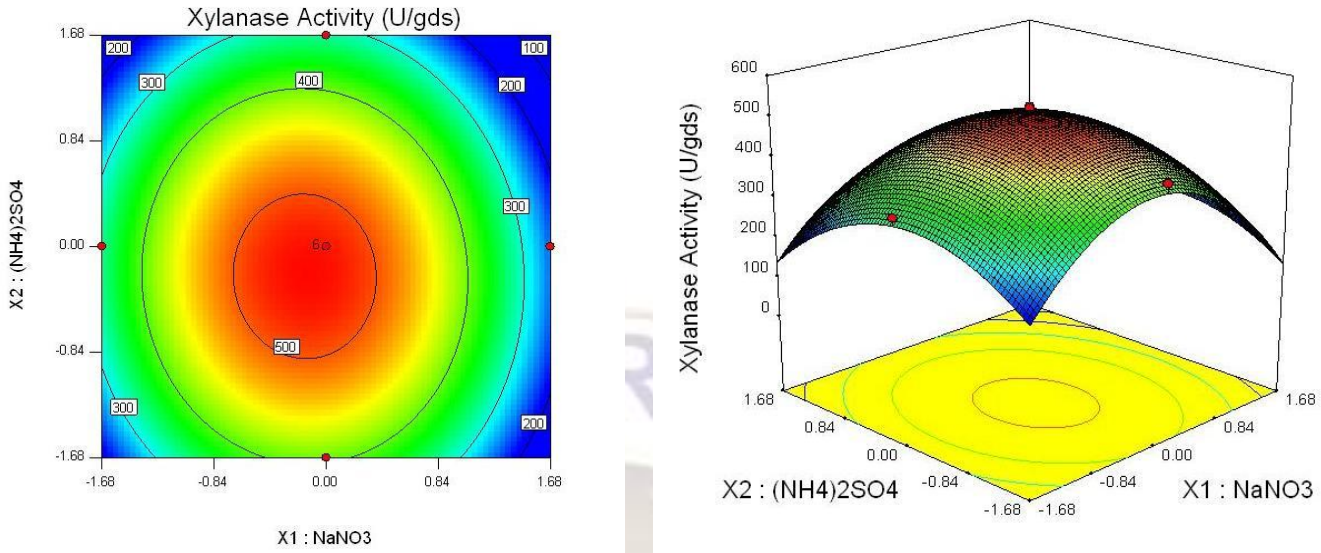

Fig. 3. Contour plot and 3D Surface plot showing the effect of $NaNO_3$ and $(NH_4)_2SO_4$ on Xylanase activity

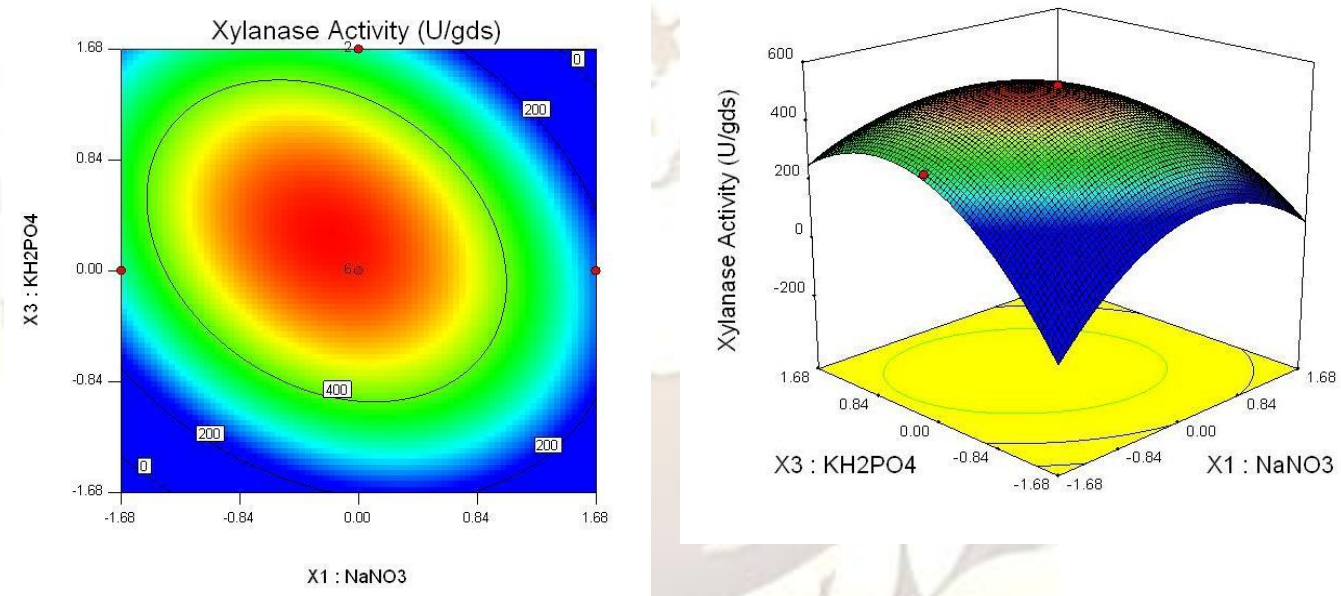

Fig. 4. Contour plot and 3D Surface plot showing the effect of $NaNO_3$ and KH_2PO_4 on Xylanase activity

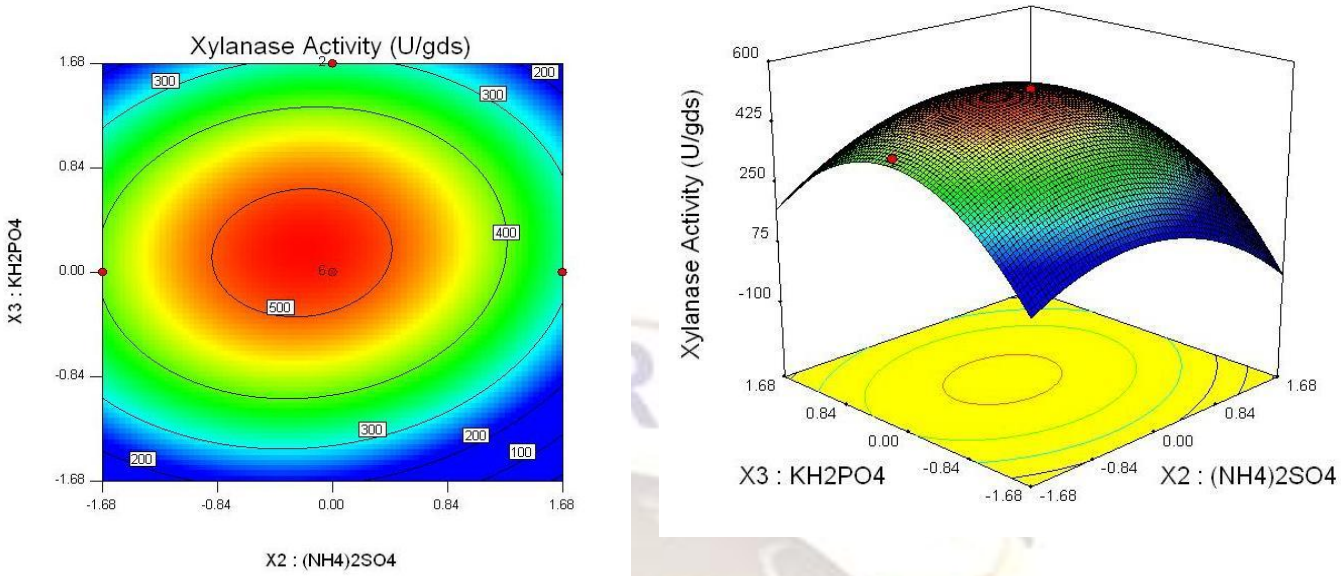

Fig. 5. Contour plot and 3D Surface plot showing the effect of $(NH_4)_2SO_4$ and KH_2PO_4 on Xylanase activity

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