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Research Article

OPTIMIZATION OF MINERAL NUTRIENT SUPPLEMENTS FOR THE PRODUCTION OF XYLANASE BY *ASPERGILLUS NIGER* UNDER SSF USING CENTRAL COMPOSITE DESIGN

K. Vimalashanmugam* and T. Viruthagiri

Department of Chemical Engineering, Annamalai University,
Annamalai Nagar, Tamilnadu, India.

ABSTRACT

Xylanase production by *Aspergillus Niger* in solid state fermentation was investigated. Sugarcane bagasse samples collected from the local agricultural field was used as the source of carbon for the miroorganism. The effect of twelve nutrient supplements in the SSF medium were investigated using Plackett-Burman design and the nutrients namely Yeast extract, $CuSO_4$ and $MgSO4.7H_2O$ were found to be significant on the production of xylanase. The respective concentrations of the selected three nutrients were also optimized using central composite design of response surface methodology to obtain the maximum enzyme yield. The optimal concentrations were found to be yeast extract – 3.47450 mg/gds; $CuSO_4$ = 0.58665 mg/gds; $MgSO_4.7H_2O$ = 0.94089 mg/gds. Under the optimum conditions the maximum xylanase yield of 399 IU/gds was obtained experimentally.

Keywords: central composite design**,** Plackett-Burman design, solid state fermentation.

INTRODUCTION

Xylanases (EC 3.2.1.8) are a class of enzymes which degrade the linear polysaccharide beta-1,4-xylan into xylose, thus breaking down hemicellulose, which is a major component of the cell wall of plants. Many bacterial and fungal species can produce a mixture of xylanase, β-xylosidase and accessory side-group cleaving enzymes in order to utilize xylan[1]. Endo-β-1, 4-xylanase plays important roles in animal feed, increasing the body weight gains of animals, in pulp and paper industry, xylanases are employed in the prebleaching process to reduce the use of toxic chlorine chemicals, in bread and bakery industry, xylanases are used to increase dough viscosity, bread volume, and shelf life , Other potential applications include the conversion of xylan in wastes from agriculture and food industries into xylose, and the production of fuel and chemical feedstocks[2]. A variety of microorganisms including bacteria[3], fungi[4], actinomycetes and yeasts[5] have been reported to produce xylanase under solid state fermentation (SSF). 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[6]. Use of abundantly available agro-residues in fermentation processes serves the dual purpose of cost effective enzyme production and environment security[7]. In large scale processes the carbon source has been estimated as the major cost factor in enzyme production. A reduction in the production costs can be achieved by the usage of inexpensive lignocellulosic materials, such as wheat bran, sugarcane bagasse, wheat straw, corn cob that are available in abundant amounts. The production of xylanase and other enzymes by fungi is known to be depending on the composition of growth medium and fermentation conditions. Therefore reducing the costs of enzyme production is by optimizing the fermentation medium and the process is the goal of basic research for industrial applications[8]. Recently, statistical designs for optimization have been successfully employed in enzyme production[9]. The response surface

methodology (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[10]. The present work was, therefore, planned for the response surface methodology optimization of medium composition for production of xylanase by *Aspergillus niger* in solid state fermentation utilizing sugarcane bagasse as substrate. Validation of the experimental model was also tested by carrying out the batch experiments in the laboratory.

MATERIALS AND METHODS

Microorganism and culture media

Aspergillus Niger (MTCC No – 16404) used in this study was purchased from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH) Chandigarh, India. The stock culture was maintained on agar slants at 5^{0}C. The culture was maintained on Potato Dextrose Agar (PDA) and subcultured at an interval of three months. The Potato Dextrose Agar (PDA) medium composition comprises of Potato - 10.0g; Dextrose - 10.0g, Agar-20.0 g; Distilled water -1 L

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^{0}C for 48 hours. The dried samples were chopped into small bits, made into 40 mesh particle size and used as substrate for xylanase production.

Solid state fermentation (SSF)

Fermentation was carried out in 250ml 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 was adjusted to 80% [11]; each flask was covered with hydrophobic cotton and autoclaved at 121^{0}C 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^{0}C and the inoculated flasks were incubated at 30^{0}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 Na-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^{0}C and filtered through a cotton cloth by squeezing. The extract was centrifuged at 15,000 rpm for 20 minutes and the supernatant was used for determination of enzyme activity.

Enzyme 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 (modified Bailey method)[12]. 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[13] 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 units IU/g of 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 0.05M Na-citrate buffer, pH 5.3 and incubating at 50^{0}C for 30 min. The amount of reducing sugars released during the reaction was measured using the DNS method[13] 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[14]. Based on the Plackett–Burman design, each factor was examined at two levels, low (−1) and high (+1). To determine which variable significantly affect xylanase production by *Aspergillus Niger*, Plackett-Burman design using statistical software package MINITAB (Release 15.1, PA, USA), was used. For screening purpose a total of

twelve medium components (Table-1) 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 design matrix. Significant nutrient components viz. Yeast extract, $CuSO_4$ and $MgSO_4.7H_2O$ which increases the xylanase production was identified.

Optimization of screened components using CCD

In order to enhance the production of xylanase, central composite design of response surface methodology (RSM) 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[15]. 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 (CCD) with three independent variables [Yeast extract, $CuSO_4$, $MgSO_4.7H_2O$]. Each factor in this design was studied at three different levels (Table 4) and a set of 20 experiments was carried out. All the variables were taken at a central coded value considered as zero. The minimum and maximum ranges of variables were used. The full experimental plan with respect to their values in coded form is shown in Table 5. 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 existence of a 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[16]. A second order polynomial equation is,

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

Where Y is the measured response, β_0 is the intercept term, β_i are linear coefficients, β_{ii} are quadratic coefficient, β_{ij} are interaction coefficient and X_i 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.

RESULTS AND DISCUSSION

The twenty run design given in Table-2 indicates a wide variation in the production of xylanase from 205 to 365 IU/gds, which reflects the importance of medium optimization to attain a better yield. The estimated effects and coefficients of the Plackett-Burman design are represented in Table 3. From the Pareto chart shown in Fig 1, it was found that three components namely: Yeast extract, $CuSO_4$, $MgSO_4.7H_2O$, had positive effect on Xylanase activity. $MgSO_4$ and yeast extract were added to assist with spore germination and initial growth[17]. All the insignificant variables from the Plackett-Burman design were neglected and the optimal concentration of the significant variables was further analyzed using central composite design (CCD) of RSM. For this study, 2^3 full factorial central composite design with sixteen star points, eight axial points 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. The coded and actual values of the significant variables are shown in Table 4.

The predicted and observed responses along with design matrix are presented in Table-5, and the results were analyzed by ANOVA. The following second order polynomial equation describing the correlation between xylanase and the four variables was obtained:

$$Y = 395.97 + 2.83\,A + 1.52\,B + 4.53\,C - 3.88\,AB - 5.79\,AC + 7.6\,BC - 13.17\,A^2 - 8.39\,B^2 - 8.03\,C^2 \quad (3)$$

Where, Y is the Xylanase activity (IU/gds), A, B and C are Yeast extract, $CuSO_4$, $MgSO_4.7H_2O$, respectively.

ANOVA for the response surface is shown in Table 6. The model *F* value of 395.97 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. In this case, the coefficients A, B, C, A^2, B^2, C^2, AB, AC, and BC were found to be highly significant. Values greater than 0.1 indicates model terms are not significant. The fit of the model was checked by the coefficient of determination R^2 for xylanase activity was calculated as 0.9947, which is very close to 1, indicating that 99.47% 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[18]. The predicted R^2 value of 0.9752 was in reasonable agreement with the adjusted R^2 value of 0.9898. Usually higher the value of C.V, lower the reliability of the experiment is [19]. Here, a lower value of C.V (0.44 %) indicated a better precision and reliability of the experiments[20]. An adequate precision value greater than 4 is desirable. The adequate precision value of 38.618 indicates an adequate signal and suggests that the model can be to navigate the design space. In the present work, all the linear, interactive effects and square effects of *A*, B and C were significant for Xylanase production. The above model can be used to predict the Xylanase production within the limits of the experimental factors.

To study the interaction effects of variables on xylanase production Contour plot & Three-dimensional surface graphs were generated for pair wise combinations of the three factors, with the third & fourth variable in each case fixed at its central (0) level for Xylanase production. The three dimensional curves of the calculated response (Xylanase production) and contour plots from the interactions between the variables are shown in Figures.2 to 4. 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.2 shows the dependency of Xylanase activity on Yeast extract and $CuSO_4$. Yeast extracts contain a lot of vitamins, minerals and amino acids, which are usually used as growth stimulants or growth factors for microbes. As can be seen from Figure 2, an increase in Yeast extract resulted in an increase in xylanase activity to about 3.47450 mg/gds and thereafter Xylanase activity decreased with further increase Yeast extract. The same trend was observed in Fig 3. Increase in $CuSO_4$ resulted in increase of Xylanase activity up to 0.58665 mg/gds. This is evident from Fig. 2 and 4. Fig.3 and 4 shows the dependency of Xylanase activity on $MgSO_4.7H_2O$. Mg^+ ions are known to be metal activators which protect the enzymes against thermal denaturation and play a vital role in maintaining the active configuration of the enzyme at high temperatures[21]. The effect of $MgSO_4.7H_2O$ on Xylanase observed was similar to other variables. The maximum xylanase activity was observed at 0.94089 gds of $MgSO_4.7H_2O$.

The maximum predicted yield is indicated by the confined surface in the plot and the optimum values were obtained by solving the second order polynomial equation[22-24]. The second degree polynomial equation was maximized by a constraint search procedure using the MINITAB software to obtain the optimal levels of the independent variables and the predicted maximum xylanase activity. The optimum conditions are: Yeast extract – 3.47450 mg/gds; $CuSO_4$ = 0.58665 mg/gds; $MgSO_4.7H_2O$ = 0.94089 mg/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. Fig.5 shows that the experimental xylanase activity values agree well with the predicted response values.

Model Adequacy Fitting

It is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely give poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy[25]. By plotting a normal probability plot of the residuals, a check was made for the normality assumption[19] as given in Fig.6. The normality assumption was satisfied as the residual plot approximated along a straight line. Fig.7 shows a plot of residuals versus the predicted response. The residuals scatter randomly on the display, suggesting that the variance of the orginal observation is constant for all values of Y. Both the graphs are satisfactory, we conclude that the empirical model is adequate to describe the xylanase activity by response surface.

Validation of the Model

Validation of the experimental model was tested by carrying out the batch experiments under optimal operation conditions. Three repeated experiments were performed, and the results arecompared. The xylanase activity 399 IU/gds obtained from experiments was very close to the actual response 396.10 IU/gds, predicted by the regression model, which proved the validity of the model.

CONCLUSION

The results showed the potential use of solid state fermentation for the production of xylanase using *Aspergillus niger*. In this study, a statistical methodology, a combination of Plackett–Burman design and central composite design, was shown to be efficient and reliable in selecting the statistically significant factors and finding the optimal concentrations of those factors in SSF using sugarcane bagasse for xylanase production. Among twelve nutrients investigated in the Plackett–Burman design yeast extract, $CuSO_4$ and $MgSO4.7H_2O$ were selected. Thereafter a central composite design was employed to find the optimal concentrations of these four nutrients in the fermentation medium in order to maximize xylanase production. In conclusion, a higher xylanase activity of 399 IU/gds was obtained with the optimal mineral nutrients concentration of yeast extract – 3.47450 mg/gds; $CuSO_4$ = 0.58665 mg/gds ; $MgSO_4.7H_2O$ = 0.94089 mg/gds. Determination of the optimal concentrations of nutrient medium components leads to decrease the cost of raw materials and achievement of maximal xylanase activity.

Table 1: Nutrient screening using a Plackett Burman design

Variables		Levels mg/10 g dry substrate	
Nutrient code	Nutrient	Low (-1)	High (+1)
A	KH_2PO_4	100.0	300.0
B	CuSO4	2.0	8.0
C	MgSO4.7H2O	3.0	12.0
D	MnSO4.7H2O	50.0	150.0
E	Yeast Extract	20.0	50.0
F	$FeSO_4.7H_2O$	5.0	20.0
G	$NaNO_3$	20.0	40.0
H	Urea	100.0	300.0
J	CaCl2	20.0	30.0
K	$(NH_4)_2SO_4$	100.0	200.0
L	$CoSO_4$	2.0	4.0
M	$ZnSO_4.7H_2O$	100.0	500.0

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	Xylanase Activity (IU/gds)		CMCellulase Activity (IU/gds)
													Exp	Pred	
1	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	365	357.9	36.41
2	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	290	283.5	40.21
3	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	320	328.3	48.46
4	1	-1	1	1	1	1	-1	-1	1	1	-1	1	280	275.9	65.91
5	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	325	318.7	50.67
6	1	1	1	1	-1	-1	1	1	-1	1	1	-1	255	251.7	58.61

7	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	205	212.1	34.71
8	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	235	225.1	65.75
9	-1	1	1	1	1	-1	-1	1	1	-1	1	1	320	317.9	50.71
10	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	259	257.1	39.23
11	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	260	262.7	38.31
12	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	320	318.9	35.42
13	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	349	350.3	58.42
14	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	298	301.7	64.62
15	1	-1	1	-1	1	1	1	1	-1	-1	1	1	280	290.1	82.37
16	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	320	317.3	44.32
17	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	245	244.1	33.21
18	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	255	251.9	42.76
19	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	245	249.9	35.70
20	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	290	300.9	58.56

Table 3: Estimated effects and coefficients of the Plackett–Burman design

Terms	Effects	Coeffi.	SE Coeffi.	T	P
constant		285.8	2.191	130.47	0.000
A	-6.6	-3.3	2.191	-1.51	0.176
B	33.2	16.6	2.191	7.58	0.000
C	-30	-15	2.191	-6.85	0.000
D	-4	-2	2.191	-0.91	0.392
E	63.8	31.9	2.191	14.56	0.000
F	5	2.5	2.191	1.14	0.291
G	8.8	4.4	2.191	2.01	0.085
H	6.8	3.4	2.191	1.55	0.165
J	5.8	2.9	2.191	1.32	0.227
K	-3.4	-1.7	2.191	-0.78	0.463
L	-3	-1.5	2.191	-0.68	0.516
M	-4.6	-2.3	2.191	-1.05	0.329

Table 4: Ranges of the independent variables used in RSM

Variables		Levels (mg/10g dry substrate)				
	Code	-1.68	-1	0	1	1.68
Yeast extract	A	9.8	20.0	35.0	50.0	60.2
CuSO4	B	0.0	2.0	5.0	8.0	10.4
$MgSO_4.7H_2O$	C	0.0	3.0	7.5	12.0	15.0

Table 5: Central composite design (CCD) of factors in coded levels with Enzyme activity as response

Run. No	Type	Coded Values			Xylanase Activity(IU/gds)		Carboxy methyl Cellulase Activity (IU/gds)
		A	B	C	Exp.	Pred.	
1	Center	0	0	0	394.14	396.10	101.32
2	Center	0	0	0	394.45	396.10	98.54
3	Factorial	-1	1	-1	350.06	351.30	104.87
4	Factorial	-1	1	1	387.18	387.40	130.42
5	Axial	-1.68	0	0	355.93	354.44	126.7
6	Axial	0	0	1.68	382.46	381.15	115.34
7	Factorial	1	1	-1	362.43	360.89	92.7
8	Axial	0	-1.68	0	370.08	369.76	98.56
9	Axial	0	0	-1.68	365.7	366.38	120.51
10	Factorial	-1	-1	-1	356.25	355.64	79.46
11	Factorial	1	-1	1	363.44	362.68	65.01
12	Axial	0	1.68	0	375.28	374.89	64.05
13	Center	0	0	0	398.02	396.10	82.19
14	Center	0	0	0	397.67	396.10	66.26
15	Center	0	0	0	396.19	396.10	78.67
16	Factorial	1	1	1	372.05	373.14	76.43
17	Axial	1.68	0	0	363.06	363.85	73.76
18	Factorial	1	-1	-1	380.85	381.11	75.36
19	Center	0	0	0	396.04	396.10	101.32
20	Factorial	-1	-1	1	359.06	361.08	98.54

Table 6: 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	395.97	5039.36	9	206.67	< 0.0001	Significant
A-Yeast Extract	2.83	*109.31*	*1*	*40.35*	*< 0.0001*	Significant
B-CuSO4	1.52	*31.4*	*1*	*11.59*	*0.0067*	
C-MgSO4.7H2O	4.53	*280.45*	*1*	*103.51*	*< 0.0001*	
AB	-3.88	*120.75*	*1*	*44.57*	*< 0.0001*	
AC	-5.79	*268.19*	*1*	*98.99*	*< 0.0001*	
BC	7.6	*462.08*	*1*	*170.55*	*< 0.0001*	
A^2	-13.17	*2492.38*	*1*	*919.93*	*< 0.0001*	
B^2	-8.39	*1010.9*	*1*	*373.12*	*< 0.0001*	Significant
C^2	-8.03	*927.31*	*1*	*342.27*	*< 0.0001*	
Residual	395.97	27.09	10			Significant
Lack of Fit	2.83	*12.23*	*5*	*0.82*	*0.5818*	Significant
Pure Error	1.52	*14.86*	*5*			Significant
Cor Total	4.53	5066.45	19			Significant

Std. Dev.- 1.65; R^2 – 99.47 %; Mean – 375.78; Adj R^2 – 98.98; C.V – 0.44 %;
Pred R^2 – 97.52 %; Adeq Precision – 38.618

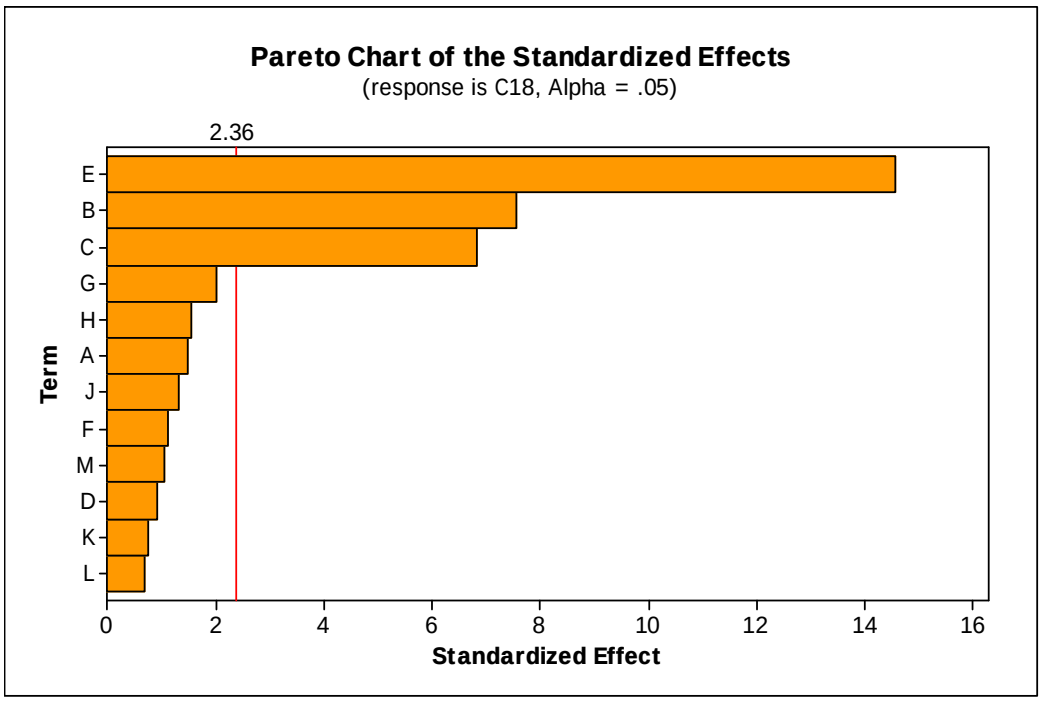

Fig. 1: Pareto chart showing the effect of media components on Xylanase activity

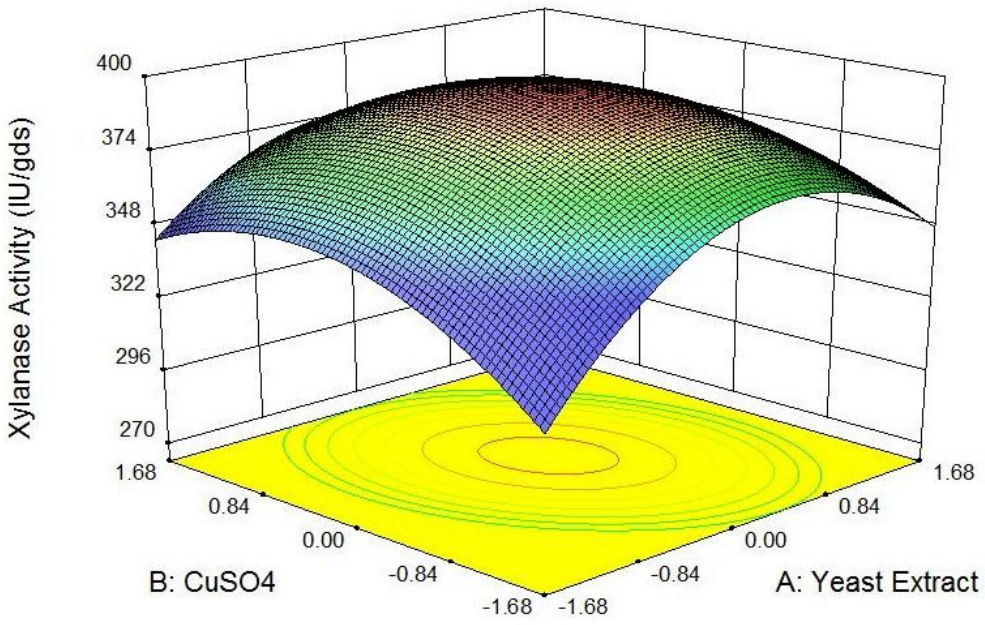

Fig. 2: Contour plot and Three-dimensional Surface plot showing the interactive effect of CuSO4 and MgSO4.7H2O on Xylanase activity

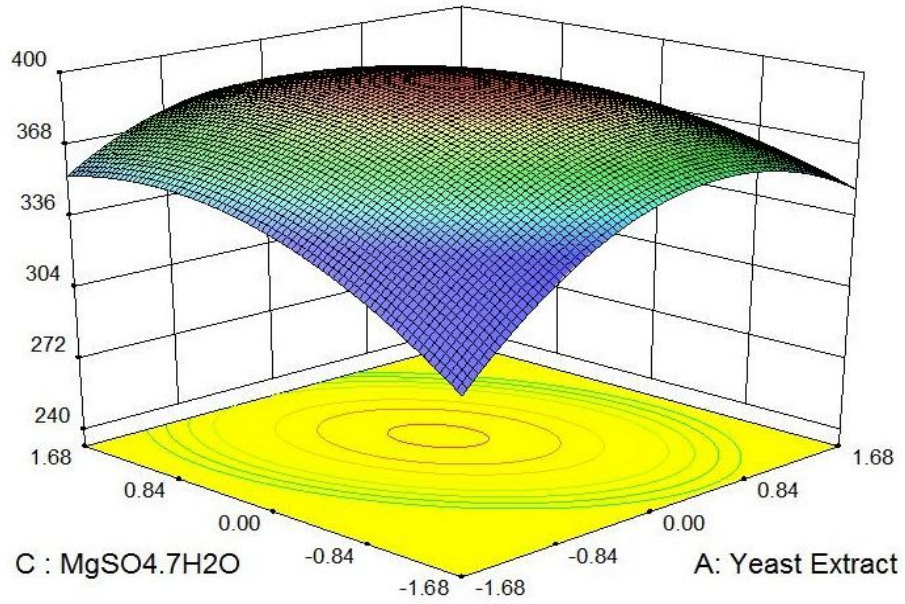

Fig. 3: Contour plot and Three-dimensional Surface plot showing the interactive effect of Yeast Extract and MgSO4.7H2O on Xylanase activity

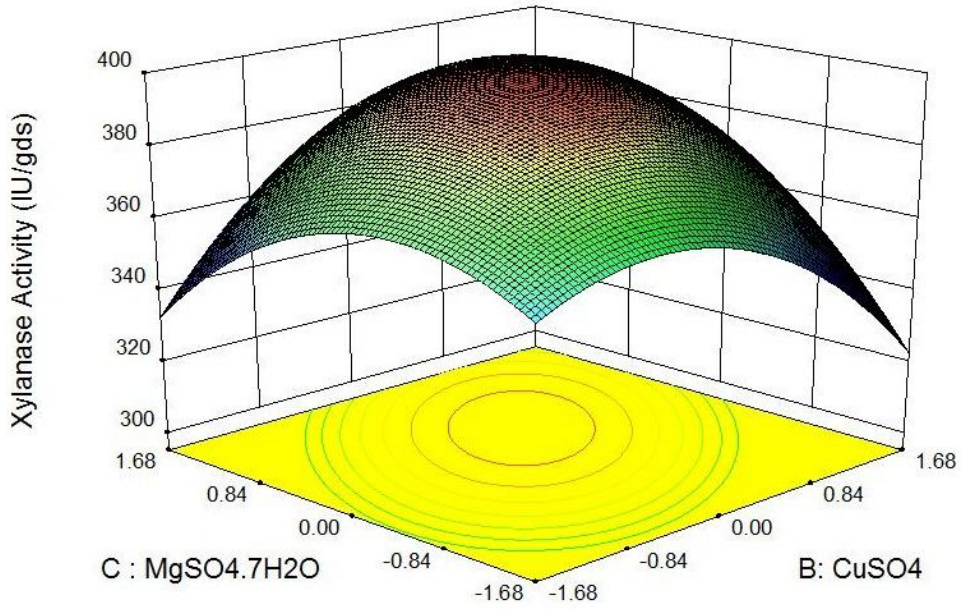

Fig. 4: Contour plot and Three-dimensional Surface plot showing the interactive effect of Yeast Extract and CuSO4 on Xylanase activity

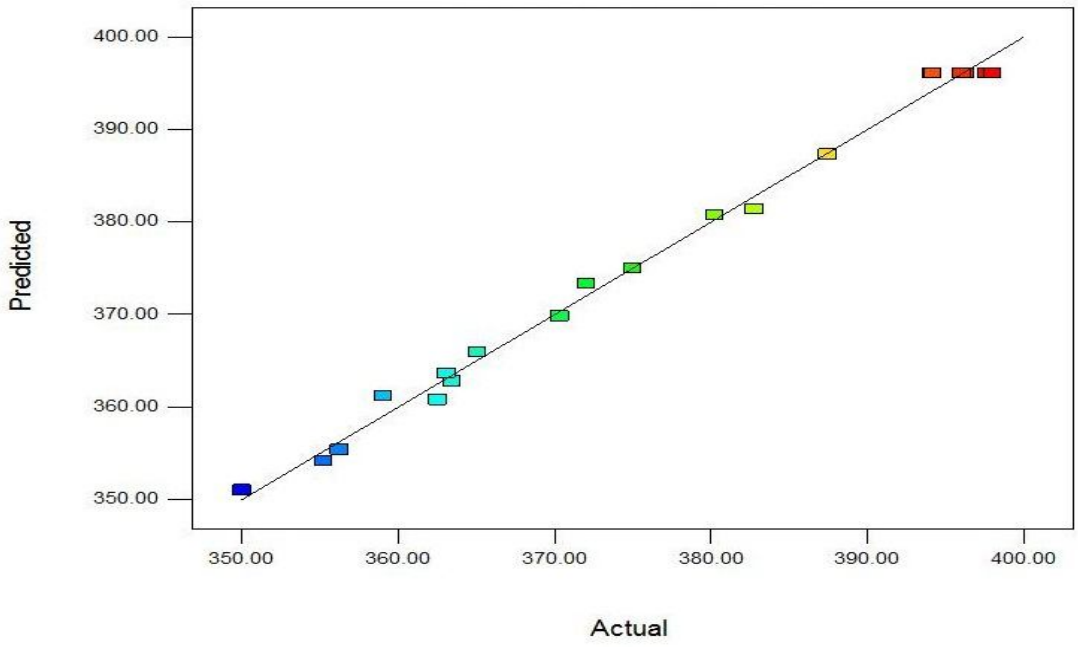

Fig. 5: Predicted Response Vs Actual Value

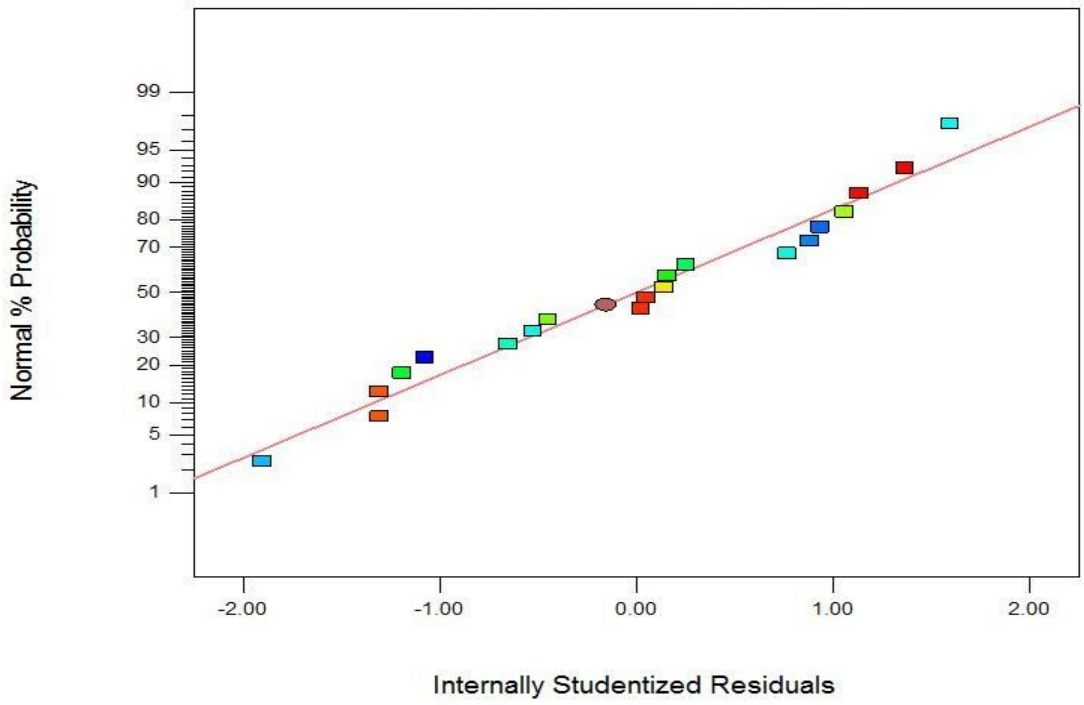

Fig. 6: Normal Probability plot of Internally Studentized Residuals

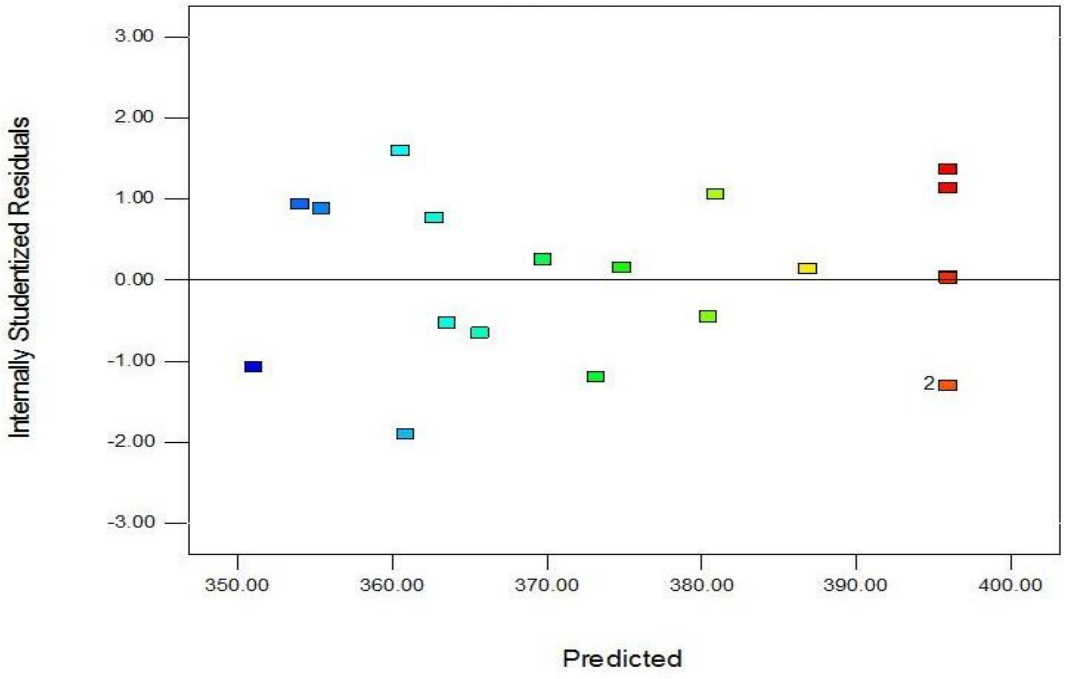

Fig. 7: Plot of Internally studentized residuals vs. Predicted response

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