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Optimization of Operating Parameters and Kinetics of Xylanase Enzyme Production under Ssf by Aspergillus Niger using Response Surface Methodology

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Abstract: The response surface methodological optimization strategy was used for xylanase production by *Aspergillus niger* MTCC-16404 under solid state fermentation using wheat bran as a substrate. The effect of various operating parameters such as Substrate concentration, temperature, initial pH, initial moisture content and incubation time on Xylanase activity was studied and optimized using response surface methodology. Using central composite design, 50 experiments were carried out for the five test variables. From the statistical analysis of the experimental data the optimum condition for maximum xylanase activity was achieved at substrate concentration-9.18 g, Temperature-32.6°C, pH-5.3, Initial moisture content-75.1% and Incubation time - 100.8 hrs. At these optimized conditions, the maximum xylanase activity was found to be 630.53 IU/gds. A high R^2 value of 0.9859 indicates the fitness of the model to predict the experimental data. The kinetics of xylanase production under solid state fermentation using *Aspergillus niger* was studied. The kinetic parameters value were found to be k_m = 0.639 mg/ml and V_{max} = 13.889 µmol/min.mg

Keywords - Response surface methodology; Central composite design; Solid state fermentation; Xylanase

Introduction

Hemicellulose is the second most abundant component in plant cell wall and xylan is the major component of hemicelluose which is found in solid agricultural and agroindustrial residues, as well as in effluents released during wood processing [1,2]. In the last decade, production of xylanase enzymes has attracted the attention of many researchers as these enzymes are essential for the degradation of xylan to xylose. Xylanases [1-4- β –D xylan xylano hydrolase E.C.3.2.1.8] catalyses the random hydrolysis of 1-4 β -D-xylosidic linkages in xylan. Xylan, the second most abundant polysaccharide constitutes from 20-40% of dry weight and forms the major component of hemicellulose in plant biomass [3]. Xylanases have potential applications in the pulp and paper, food, feed and beverage industries [4]. The potential applications of xylanase include bleaching non-cotton cellulosic fibres, melting hemp and linen fibres, clarifying fruit juice and wines [5] and also increasing the brightness of pulp, breaking up the dough, production of hydrolysates from agro industrial wastes [6], and improvement of nutritional properties in lignocellulosic feed stuff [7].

Regarding fermentation processing conditions, the current enzyme production technologies are conducted either in a liquid phase, known as submerged fermentation (SF), or using solid substrates, termed solid state fermentation (SSF). Approximately 90% of all industrial enzymes are produced by SF, often using genetically modified microorganisms [8]. However, most of these enzymes could be produced by SSF using

wild-type microorganisms. Though there are reports on xylanase through SSF, the large scale commercial processes are still using SF [9, 10]. However, growth of filamentous fungi by SSF is considered advantageous since the solid medium simulates its natural habitat [11]. This benefit is extended to the production of enzymes, yielding greater productivity when compared to SF [8]. Another advantage of SSF is the use of agro-industrial residues as a solid substrate, acting both as carbon and energy source. The choice of these lignocellulosic materials should be based on their abundance and cost, as well as their physical, chemical characteristics. Soybean and wheat bran are good sources of nitrogen and have been used in mixtures with sugarcane bagasse for the production of cellulases [12]. Solid state fermentation has gained interest from researchers in recent years and has often been employed for the production of xylanases because of economic and engineering advantages [13].

The use of waste plant materials as carbon sources in fermentation media is therefore has significance as a cost effective strategy for production of enzymes. The use of agro wastes not only helps to overcome the problem of solid waste management but allows the development of biotechnological processes from cheap natural resources [14]. A number of different sources, including bacteria [15], actinomycetes [16], and yeast [17], have been reported for xylanase production under solid state fermentation (SSF). Among the microbial sources, filamentous fungi are particularly interesting because they are non pathogenic, easily cultivated and secrete their enzymes into the medium and have high xylanase activity in contrast to yeasts and bacteria [18]. Substrate concentration, Temperature, Initial pH, Initial moisture content and Incubation time are the key operating parameters that affect the enzyme fermentation process. Mesophilic organisms can produce enzymes at temperatures from 20 to 40 ºC, with most strains capable of growing at acidic as well as alkaline pH, with moisture content ranging from 72 to 81% and substrate concentration from 5 to 15 g. It is therefore important to determine the substrate concentration, Temperature, Initial pH, % Initial Moisture content and Incubation time at which optimal microbial growth is achieved. A better understanding of these operating parameters on enzyme production will facilitate the improvement of the process. Traditional methods for optimization are "one-factor-at-a time" techniques. Unfortunately, this approach frequently fails to identify the variables that give rise to the optimum response because the effects of factor interactions are not taken into account in such procedures [19]. An alternative strategy is statistical optimization, which allows rapid screening of a number of factors and factor interactions, and reflects the role of each component. Response surface methodology (RSM), a collection of mathematical and statistical techniques for building empirical models, is gaining recognition as a powerful approach for optimizing conditions for the production of industrially important products such as chemicals and enzymes [20].

This methodology could be employed to optimize the operating parameters for enzyme production which has been widely adopted in industries such as drug and food industry, chemical and biological processes, for the purpose of either producing high quality products or operating the process in a more economical manner and ensuring the process in a more stable and reliable way [21]. Response surface methodology has also been successfully used to model and optimize biochemical and biotechnological processes related to food systems [22].

The present study reports the use of low cost agro-industrial residue wheat bran as carbon source for the production of xylanase by *Aspergillus niger (MTCC No* - 16404*)*. The optimization of process parameters such as substrate concentration, temperature, Initial pH, initial moisture content, and incubation time are carried out by applying central composite design (CCD) of response surface methodology (RSM) and experimental validation of the model is validated. The kinetics of the xylanase production under SSF is also studied.

Materials & Methods

M*icroorganism and culture media*

Aspergillus niger (MTCC No - 16404*)* used in this study was purchased from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology (IMTECH) Chandigarh, India. The stock culture was maintained on agar slants at 5^0C. The culture was maintained on Potato Dextrose Agar (PDA) and sub cultured at an interval of three months. The Potato Dextrose Agar (PDA) medium composition comprises of Potato - 10.0 g; Dextrose - 10.0g, Agar -20.0 g, Distilled water -1.0 L.

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, 0.1% (v/v) of Tween-80, 0.1% (w/v) of oat spelt xylan, supplemented with nutrient concentration (% w/w) : yeast extract - 26.0 mg/gds, $ZnSO_4.7H_2O$ – 281.2 mg/gds, K_2HPO_4 - 54.7 mg/gds and soyabean meal – 581.8 mg/gds, adjusted with process parameters such as Substrate (wheat bran) concentration, Temperature, Intial pH, Initial moisture content (%) [23], Incubation time and as defined by experimental design. 0.1 % of oat spelt xylan serves as an inducer for xylanase production. Each flask was covered with hydrophobic cotton and autoclaved at 121^0C, 15 psi pressure 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 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 4^{th} 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 flask 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.

Enzyme Assay

Endoxylanase activity was measured by incubating 0.5ml of 1% (w/v) oat spelt xylan in 0.05 M Na-citrate buffer (pH 5.3). And 0.5 ml of suitably diluted enzyme extract at 50°C for 30 min [24]. The release of reducing sugar was measured by dinitro-salicylic acid (DNS) method [25] and xylose was used as the standard. One International unit (IU) of xylanase activity is defined as the amount of enzyme releasing 1 µmol of xylose per minute under the assay conditions. Xylanase production in SSF was expressed as IU/g dry substrate (IU/gds). Cellulase activity was assayed by adding 0.5 ml of appropriately diluted enzyme to 0.5 ml of 1 % (w/v) of carboxy methyl 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 [25] and D-glucose was used as the standard. One International unit of carboxy methyl cellulase activity was defined as the amount of enzyme that liberated 1 µmol of glucose equivalent under the assay conditions.

Michaelis–Menten kinetics

In this study, the kinetics of the xylanase production was carried out using Michaelis–Menten equation. For carrying out kinetics, experiments were carried out at various substrate concentrations viz. 0.8, 0.9, 1.0 and 1.2 mg/ml. The Michaelis–Menten equation is,

$$V_o = \frac{V_{\max}\ [S]}{K_m\ + [S]} \qquad (1)$$

Where S is substrate concentration, V_o is rate of xylanase formation, V_{max} is maximum rate of xylanase formation, K_m is inverse of enzyme affinity. A plot of time versus production was drawn. From the plot, the values of dP/dt was found for various points. Using the different dP/dt values the constants V_{max} and K_m were evaluated using Lineweaver Burk Plot.

Optimization of Process Parameters

Response surface methodology (RSM) is an empirical statistical technique employed for multiple regression analysis by using quantitative data obtained from designed experiments to solve multivariate equations simultaneously. A central composite experimental design with 10 star points, (2^5 = 32) axial points and eight replicates at the center point (n_0 = 8), resulting in a total of 50 experiments covers the entire range of variables was used for fitting a second order response surface. These 50 experiments were performed with different combinations of the five independent variables. The five independent variables were studied at five different levels -2.38, -1, 0, 1 and 2.38. The range and levels of independent variables with coded values are shown in Table.1 and a set of 50 experiments was carried out as shown in Table.2.

Table 1: Range and Levels of Independent Variables

Variables	Code	Levels				
		-2.38	-1	0	1	2.38
Substrate Concentration (g)	A	5.2	8.0	10.0	12.0	14.8
Temperature (^{0}C)	B	27.2	30.0	32.0	34.0	36.8
Initial pH	C	2.7	4.0	5.0	6.0	7.3
Initial Moisture content (%)	D	70.2	73.0	75.0	77.0	79.8
Incubation Time (hrs)	E	88.1	95	100	105	110.9

Table 2: Central Composite Design (CCD) Matrix of factors in Orthogonal and Real Values along with enzyme activity as Response

Run No	Coded Values					Xylanase Activity (IU/gds)		CMCase Activity (IU/gds)
	A	B	C	D	E	Exptl.	Pred.	Exptl.
1	1	1	1	-1	-1	603.21	603.09	146.30
2	1	1	1	-1	1	575.45	574.78	139.57
3	-1	1	-1	-1	-1	510.00	513.99	123.70
4	1	-1	1	-1	-1	584.34	582.05	141.73
5	1	-1	-1	-1	-1	526.54	524.22	127.71
6	0	0	0	0	2.38	548.78	555.43	133.10
7	-1	1	1	1	-1	565.12	568.30	137.07
8	-1	1	1	-1	-1	603.65	601.45	146.41
9	-1	-1	-1	1	1	599.32	602.02	145.36
10	1	-1	1	1	-1	567.32	572.71	137.60
11	1	1	-1	-1	1	549.34	543.86	133.24
12	-2.38	0	0	0	0	593.00	596.20	143.83
13	1	1	-1	-1	-1	516.32	521.30	125.23
14	0	0	0	0	-2.38	537.00	534.99	130.24
15	-1	-1	1	-1	1	571.21	565.99	138.54
16	-1	-1	1	1	1	574.21	568.04	139.27
17	-1	1	-1	1	1	601.21	598.66	145.82
18	-1	1	1	-1	1	600.21	602.26	145.58
19	1	-1	1	1	1	540.23	538.23	131.03
20	0	0	0	0	0	627.23	625.60	152.13
21	0	0	-2.38	0	0	521.12	516.89	126.39
22	0	0	0	0	0	627.33	625.60	152.15
23	-1	1	1	1	1	589.43	588.64	142.96
24	0	0	0	0	0	605.43	625.60	146.84
25	0	0	0	0	0	627.53	625.60	152.20
26	0	0	0	2.78	0	541.09	548.44	131.24
27	1	-1	-1	-1	1	515.12	521.07	124.94
28	0	0	0	0	0	627.67	625.60	152.24
29	1	-1	-1	1	-1	566.20	561.51	137.33

30	0	0	0	2.38	0	523.00	520.29	126.85
31	1	-1	1	-1	1	527.61	528.04	127.97
32	0	0	0	0	0	627.97	625.60	152.31
33	-1	1	-1	-1	1	567.02	565.66	137.53
34	1	1	1	1	-1	585.00	578.09	141.89
35	0	0	0	0	0	627.75	625.60	152.26
36	0	2.38	0	0	0	578.00	578.83	140.19
37	0	0	2.38	0	0	564.87	573.74	137.00
38	0	-2.38	0	0	0	554.00	557.81	134.37
39	1	1	-1	1	-1	537.34	542.93	130.33
40	-1	-1	-1	1	-1	552.43	556.54	133.99
41	1	1	-1	1	1	579.03	585.01	140.44
42	0	0	0	0	0	627.67	625.60	152.24
43	2.38	0	0	0	0	568.00	569.44	137.76
44	-1	-1	1	-1	-1	591.23	590.89	143.40
45	1	1	1	1	1	576.43	569.31	139.81
46	-1	1	-1	1	-1	535.64	527.47	129.92
47	-1	-1	-1	-1	1	554.76	553.35	134.55
48	1	-1	-1	1	1	585.63	577.89	142.04
49	-1	-1	-1	-1	-1	524.53	527.39	127.22
50	-1	-1	1	1	-1	579.32	573.41	140.51

The experiments with substrate concentration, temperature, initial pH, initial moisture content, and incubation time were employed, simultaneously for the production of xylanase in the central composite design, batch experiments were conducted. The coded values of the process parameters were determined by the following equation:

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

Where x_i-coded value of the i^{th} variable, X_i-uncoded value of the i^{th} test variable and X_0-uncoded value of the i^{th} test variable at center point. The range and levels of independent variables with coded values are shown in Table 1.

The experimental design is shown in Table 2. A mathematical model, relating the relationships among the process dependent variable and the independent variables in a second-order equation, was developed (Equation 2). The regression analysis was performed to estimate the response function as a second order polynomial.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i=1,i<j}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad (3)$$

Where Y is the predicted response, β_i, β_j, β_{ij} are coefficients estimated from regression. They represent the linear, quadratic and interactive effects of A, B, C, D and E on response.

A statistical software package Design Expert 8.0.7.1.5, was used for regression analysis of the data obtained and to estimate the coefficient of the regression equation. The equations were validated by the statistical tests called the ANOVA analysis. Design-based experimental data were matched according to the second order polynomial equation. The independent variables were fitted to the second order model equation and examined for the goodness of fit. The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by *F*-test. The significance of each term in the equation is to estimate the goodness of fit in each case. To establish the individual and interactive effects of the test variable on the xylanase production response surfaces were drawn. The optimal values of the test variables were obtained in coded values and transformed to uncoded values.

Results and Discussion

By using multiple regression analysis, the response xylanase activity was correlated with the five independent variables studied using the second-order polynomial as represented by Equation 2. The coefficients of the model equation and their statistical significance were evaluated using Design-Expert 8.0.7.1.5. The following second order polynomial equation describing the correlation between xylanase activity and the five dependent variables was obtained:

Xylanase activity, $Y = 627.918 - 5.625A + 4.421B + 11.950C + 5.918D + 4.295E + 2.621AB - 1.419AC + 2.037AD - 7.278AE + 5.989BC - 3.916BD + 6.427BE - 11.657CD - 12.714CE + 4.882DE - 8.011A^2 - 10.574B^2 - 14.641C^2 - 16.576D^2 - 14.659E^2$ ..... **(4)**

Where *A, B, C , D, E* were coded values of substrate concentration, Temperature, Initial pH, Initial moisture content and Incubation time respectively.

Table 3 : Analysis of Variance (ANOVA) for response surface quadratic model for the production of Xylanase

Source	Coeff estimate	Sum of squares	df	Mean squares	F Value	p-value Prob>F	
Model	627.918	59450.795	20	2972.540	101.539	< 0.0001	significant
A	-5.625	1370.481	1	1370.481	46.814	< 0.0001	significant
B	4.421	846.506	1	846.506	28.916	< 0.0001	significant
C	11.950	6185.230	1	6185.230	211.282	< 0.0001	significant
D	5.918	1517.141	1	1517.141	51.824	< 0.0001	significant
E	4.295	799.055	1	799.055	27.295	< 0.0001	significant
AB	2.621	219.766	1	219.766	7.507	0.0104	significant
AC	-1.419	64.411	1	64.411	2.200	0.1488	insignificant
AD	2.037	132.764	1	132.764	4.535	0.0418	insignificant
AE	-7.278	1694.784	1	1694.784	57.892	< 0.0001	significant
BC	5.989	1147.924	1	1147.924	39.212	< 0.0001	significant
BD	-3.916	490.784	1	490.784	16.765	0.0003	significant
BE	6.427	1321.751	1	1321.751	45.150	< 0.0001	significant
CD	-11.657	4348.248	1	4348.248	148.532	< 0.0001	significant
CE	-12.714	5172.462	1	5172.462	176.687	< 0.0001	significant
DE	4.882	762.647	1	762.647	26.051	< 0.0001	significant
A^2	-8.011	3565.928	1	3565.928	121.809	< 0.0001	significant
B^2	-10.574	6213.084	1	6213.084	212.233	< 0.0001	significant
C^2	-14.641	11911.215	1	11911.215	406.877	< 0.0001	significant
D^2	-16.576	15269.085	1	15269.085	521.578	< 0.0001	significant
E^2	-14.659	11941.436	1	11941.436	407.909	< 0.0001	significant
Residual		848.968	29	29.275			
Lack of Fit		848.561	22	38.571	663.791	0.0561	insignificant
Pure Error		0.407	7	0.058			
Cor Total		60299.763	49				

Std. Dev.-5.41, R^2-0.9859, Mean-572.08, Adj R^2-0.9762, C.V. %-0.95, Pred R^2- 0.9465, Adeq Precision-32.468

The xylanase activity, both experimental values and predicted values along with Carboxy methyl cellulase (CMCase) activity were given in Table 2. The results were analyzed by Analysis of Variance (ANOVA) and were given in Table 3. The ANOVA of the quadratic regression model indicates the model to be significant. The Model *F*-value of 101.539 implied the model to be significant. Model *F*-value is calculated as a ratio of mean square regression and mean square residual. Model *P* value (Prob > *F*) is very low [< 0.0001]. This reiterates that the model is 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. The smaller the magnitude of the *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.05 indicates that the model terms are significant. The coefficient estimates and the corresponding *P* values suggests that, among the test variables used in the study *A, B, C, D, E, AE, BC, BE, CD, CE, DE,* A^2, B^2, C^2, D^2, E^2 were highly significant model terms for xylanase enzyme production. 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. *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. The fit of the model was also expressed by the coefficient of regression R^2, which was found to be 0.9859, indicating that 98.59 % of the variability in the response could be explained by the model. This implies that the prediction of experimental data is quite satisfactory. The predicted R^2 value of 0.9465 is in reasonable agreement with the adjusted R^2 value of 0.9762. Adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Here the ratio of 32.468 indicates an adequate signal. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. Usually, the higher the value of the CV is, the lower the reliability of the experiment. Here a lower value of CV (0.95) indicates better precision and greater reliability of the experiments performed.

To investigate the interactive effects of variables on the Xylanase activity, 3D plots were drawn. Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels are more helpful in understanding both the main and the interactive effects of two factors. The 3D response surface curves for Xylanase production was shown in Fig.1 – Fig.10**.**

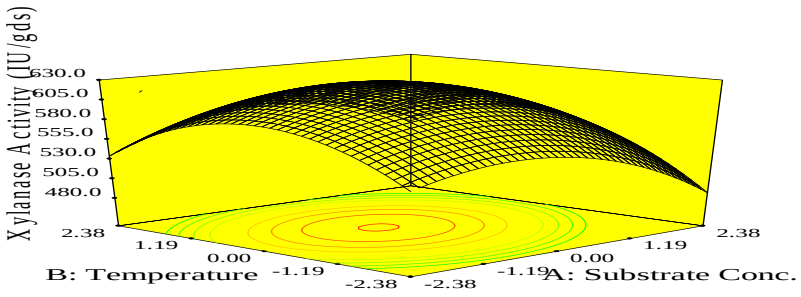

Fig.1 3D Response surface plot showing interactive effect of Substrate concentration and Temperature on Xylanase activity

Fig. 1 represents the interactive effect of substrate concentration and temperature on xylanase activity. The shape of the response surface curves show good interaction between these tested variables. The xylanase activity increases as the substrate concentration increases, and reaches maximum activity at 10.24 g of substrate. Thereafter the xylanase activity decreases. This may be due to the fact that high concentration of substrate led to increase in medium viscosity, which influenced the medium components and oxygen transfer [26]**.** This is evident from the Figs 2, 3 and 4.

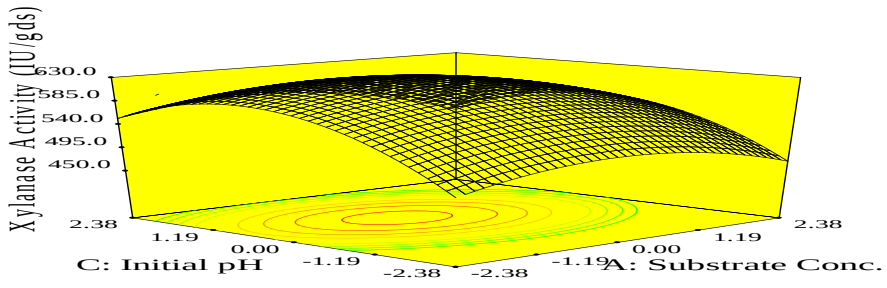

Fig.2 3D Response surface plot showing interactive effect of Substrate concentration and Initial pH on Xylanase activity

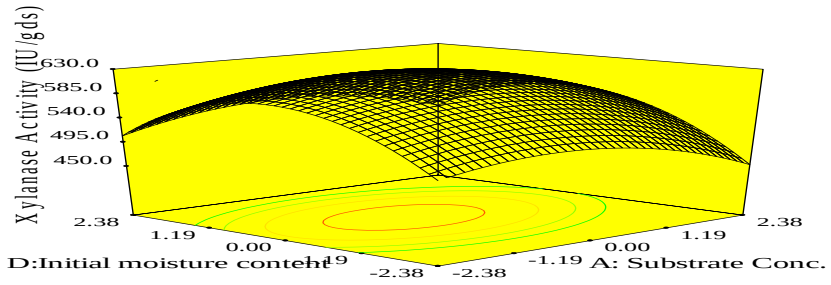

Fig.3 3D Response surface plot showing interactive effect of Substrate concentration and Initial moisture content on Xylanase activity

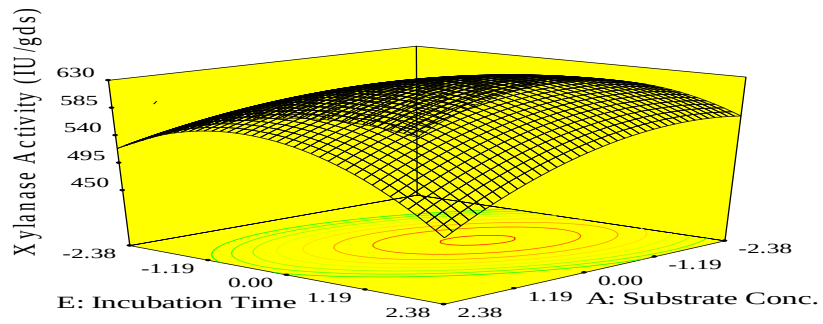

Fig.4 3D Response surface plot showing interactive effect of Substrate concentration and ncubation Time on Xylanase activity

The effect of temperature on enzyme activity is studied by conducting experiments at various temperatures ranging from 27.2°C to 36.8°C. The results are shown in Fig**.** 1, 5**,** 6 and 7. An increase in xylanase activity could be achieved when the value of temperature is increased from 27.2 °C to 32.6°C. The xylanase activity decreased considerably even for slight increase in the temperature from 32.6 °C. The reason for decrease in enzyme activity on increase in temperature is due to the fact that the maintenance energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway resulting in minimum amount of product formation as reported by [27]. The optimum temperature for maximum is found to be 30.81℃, which is in coincidence with the results obtained by [28, 6] *as Aspergillus niger* USMA1 produces maximum of xylanase at 28℃. These results are well supported in Fig 1, 5, 6 and 7**.**

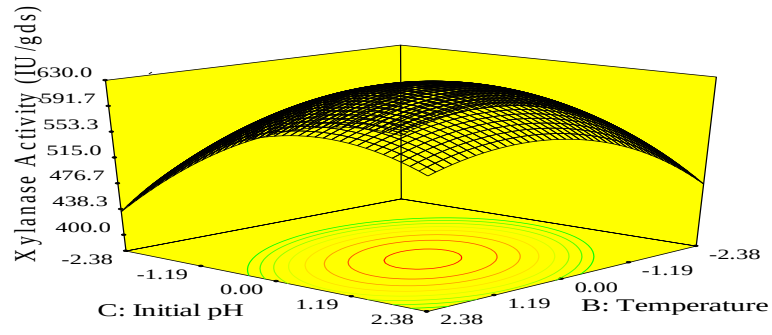

Fig.5 3D Response surface plot showing interactive effect of Temperature and Initial pH on Xylanase activity

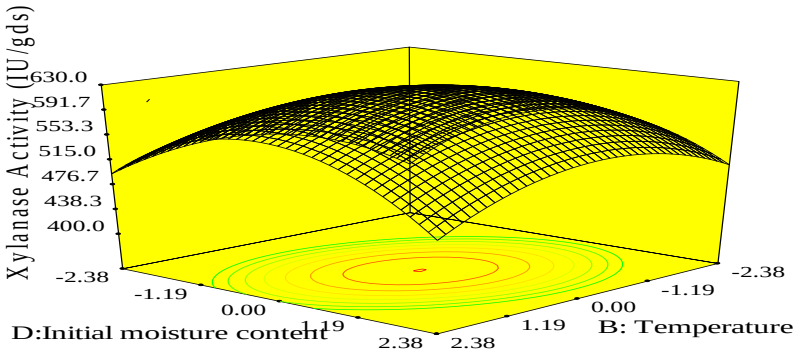

Fig.6 3D Response surface plot showing interactive effect of Temperature and Initial moisture content on Xylanase activity

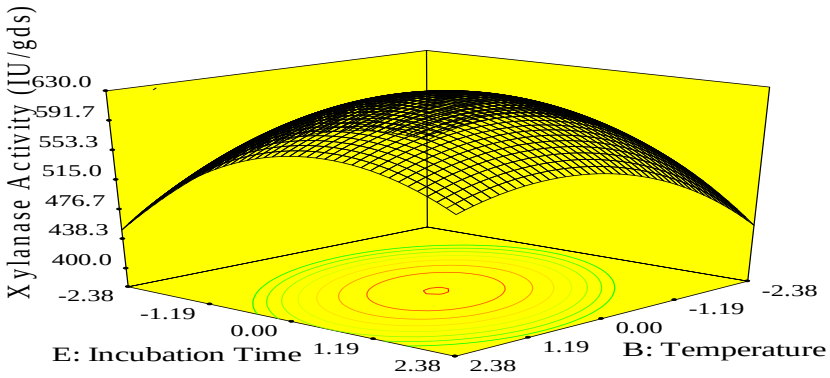

Fig.7 3D Response surface plot showing interactive effect of Temperature and Incubation Time on Xylanase activity

The effect of initial pH on enzyme activity is studied by conducting experiments from pH 2.7 to 7.3 and the results are shown in Fig 2, 5, 8, 9. The maximum xylanase activity was obtained at pH 5.3. At this pH value, xylanase activities were high when compared to the xylanase activity obtained for other pH values. However, the xylanase activity and CMCase activity decrease when the pH is raised above 5.3. This indicates that *Aspergillus niger* was acid resistant as reported by [28, 29].

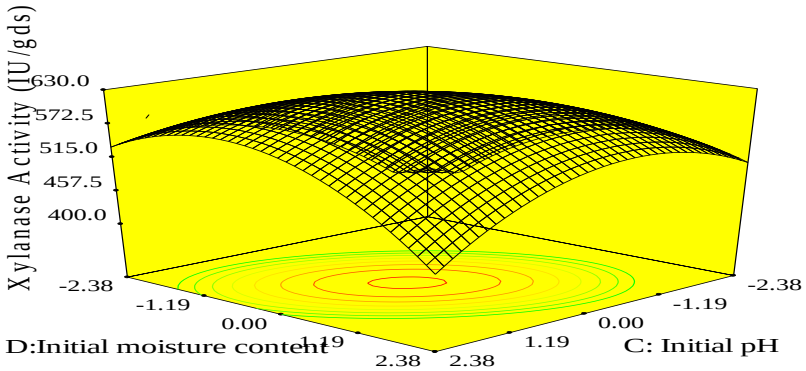

Fig.8 3D Response surface plot showing interactive effect of Initial pH and Initial moisture content on Xylanase activity

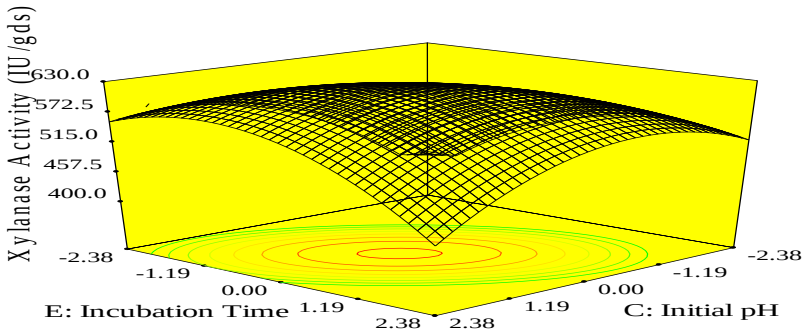

Fig.9 3D Response surface plot showing interactive effect of Initial pH and Incubation Time on Xylanase activity

The effect of initial moisture content on enzyme activity is studied by conducting experiments with the initial moisture content ranging from 71.2 % to 79.8 %. The results are shown in Fig 3, 6, 8,10. The Xylanase activity was found to increase as initial moisture content is raised from 71.2 % to 75.1 %, and is found to decrease with further increase with initial moisture content of the medium. The maximum xylanase activity of 627.75 IU/gds is obtained with the initial moisture content of 75.1% respectively. The decrease in the enzyme activity for initial moisture content greater than 75.1 % might be due to the fact that higher moisture level would reduce the porosity of substrate, and limiting the oxygen transfer into the substrate as reported by [30].

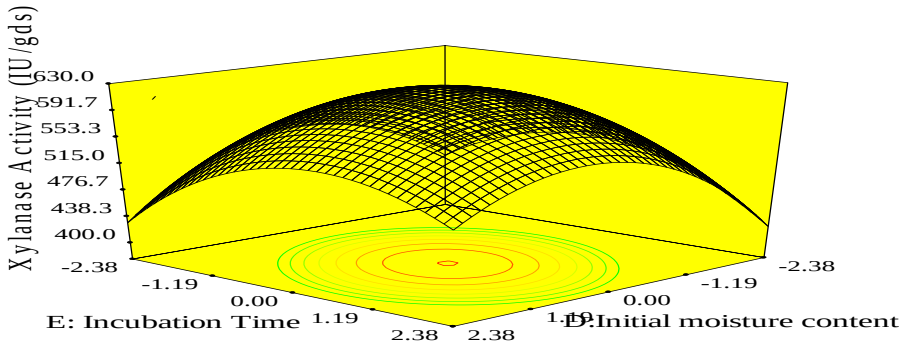

Fig.10 3D Response surface plot showing interactive effect of Initial moisture content and Incubation Time on Xylanase activity

The effect of Incubation time on enzyme activity is studied by conducting experiments with incubation period ranging from 88.1 hours to 110.9 hours. The results are shown in the Fig 4, 7, 9 and 10. The maximum xylanase of 627.75 IU/gds is obtained with the incubation period of 100.8 hours respectively. Further increase in the incubation time results in the reduction of xylanase activity. This may be due to decrease in the depletion of the nutrients in the medium which leads to decreased growth as well as enzyme production. With prolonged incubation, enzyme activity decreased sharply suggesting that the end-point of fermentation should be carefully controlled because synthesized xylanase could be degraded by non-specific proteases secreted by the fungus. This is concurrent with the results obtained by [31].

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: substrate concentration - 9.18 g, Temperature - 32.6°C, pH - 5.3, initial moisture content - 75.1% and Incubation time - 100.8 hrs. The optimal values for the variables as predicted are found to be within the design region. This shows that the model correctly explains the influence of the chosen variables on the xylanase production. 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.11 shows that the experimental xylanase activity values agree well with the predicted response values.

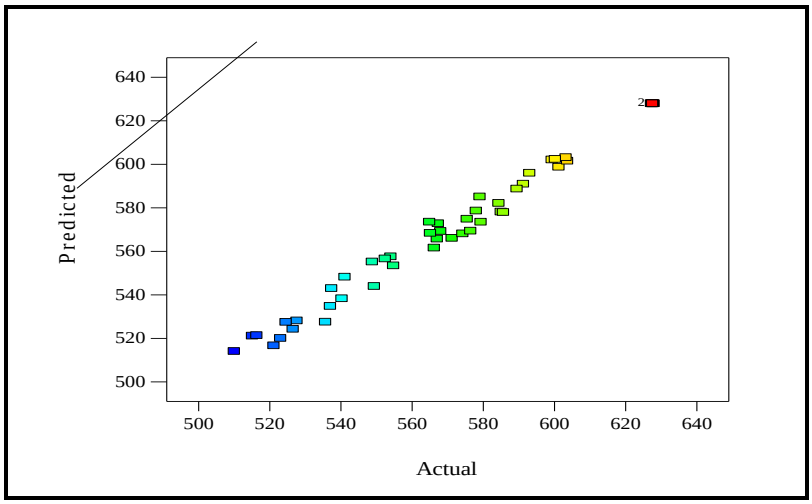

Fig.11. Predicted Response Vs Actual (Experimental) Value.

The optimum values for substrate concentration, temperature, initial pH, initial moisture content and Incubation time for maximum xylanase activity using *A. niger* in SSF as reported by [32, 33, 34] were 10g, 32 ^{o}C - 35^{o}C, 4.2 - 5.5, 77% a nd 72h -96h respectively, which are in good agreement with the present research also.

Experimental Validation of the model:

Experimental Validation of the model is tested by carrying out the batch experiment under optimal operation conditions. Experiments are repeated thrice, and the results are compared. The xylanase activity obtained from experiments is found to be 630.01 IU/, which is very close to the response of 630.53 IU/gds, predicted by the regression model. The experimental and predicted values of enzyme activity show good agreement with one another with high degree of accuracy of the model substantiating the model validation under the experimental conditions.

Kinetics of Xylanase Production

The kinetics of xylanase production using *A.niger* with wheat bran under optimum conditions of temperature and pH was studied using Michaelis–Menten equation.

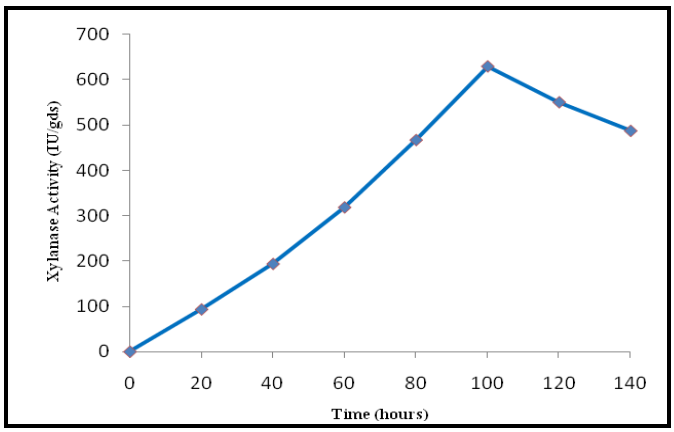

Fig.12 Effect of fermentation time on xylanase production using *A.niger*

The xylanase activity was found to increase with respect to fermentation time as shown in Fig.12 and reaches a maximum of 630.01 IU/gds at the end of 100 hours of fermentation time and later on it was found to decrease till the end of the fermentation. The rate of product formation (dp/dt) was found to increase gradually and was maximum at the end of 100 hours and later on it was found to decrease due to depletion of substrates (as indicated in **Table**. 4). The results showed that the maximum xylanase production rate (dp/dt) of 8.15 IU/gds is obtained at the end of the 100 hours and was found to be optimum fermentation period.

TABLE 4. Effect of Fermentation Time on Xylanase Production

Time (hrs)	Xylanase activity (IU/gds)	Rate of xylanase formation (IU/gds.hr)
0	0	0
20	93.44	4.672
40	193.78	5.017
60	318.50	6.236
80	467.05	7.427
100	630.01	8.148
120	550.32	3.984
140	487.23	2.654

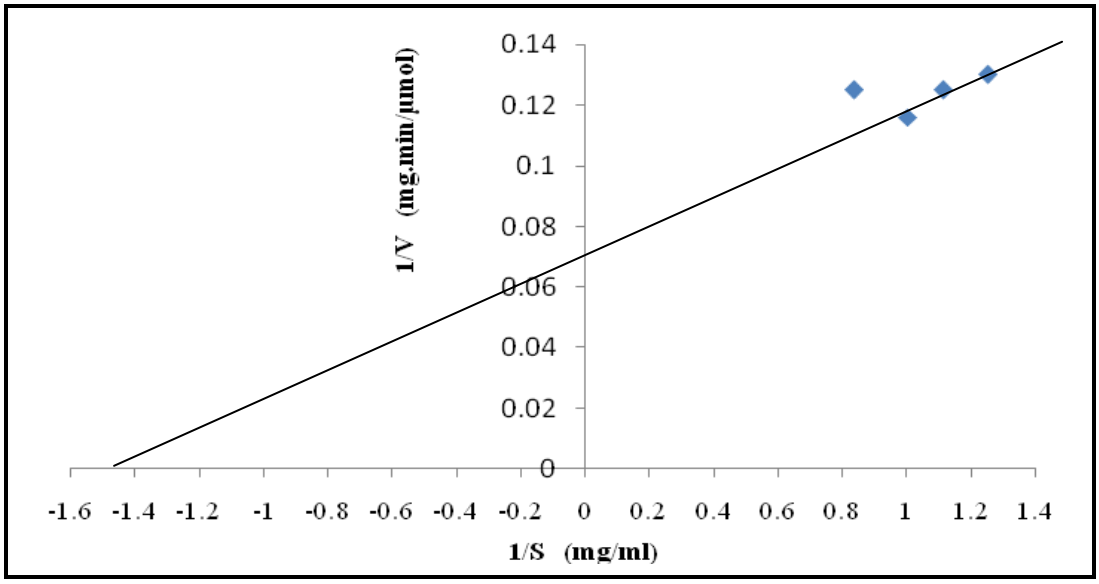

Fig.13 Line weaver-Burk Plot for xylanase production using A.niger

The kinetic parameters were evaluated using Lineweaver-Burk plot shown in **Fig**.13. The kinetic parameters were found to be V_{max} = 13.889 µmol/min.mg and K_m =0.639 mg/ml.

[35] reported that for fungal endoxylanases K_m values vary within 0.09-40.9 mg/ml and V_{max} values within 0.106 - 6300 µmol/min.mg. [36] also reported that K_m values of xylanases lie in the range from 0.5 to 19.6 mg/ml.

The K_m and V_{max} values found in this study are well within the range of 0.09-40.9 mg/ml and 0.106-6300 µmol/min mg. This is in accordance with the literature.

Therefore Michaelis-Menten Kinetic model for Xylanase production was found to be,

$$V = \frac{13.889 \times S}{0.639 + S} \qquad (5)$$

Where V is rate of xylanase formation, S is substrate concentration. The Comparison between experimental and Predicted values for rate of xylanase formation is shown in Table 5. Fig.14 showed that our kinetic model (predicted values) was well fitted with the experimental values with R^2 value of 0.999.

Table 5. Comparison between Experimental and Predicted Values

Substrate Concentration (mg/ml)	Rate of xylanase formation (Experimental) µmol/min.mg	Rate of Xylanase formation (Predicted) µmol/min.mg
1.2	8.993	9.063
1.0	8.023	8.124
0.9	7.905	8.022
0.8	7.613	7.722

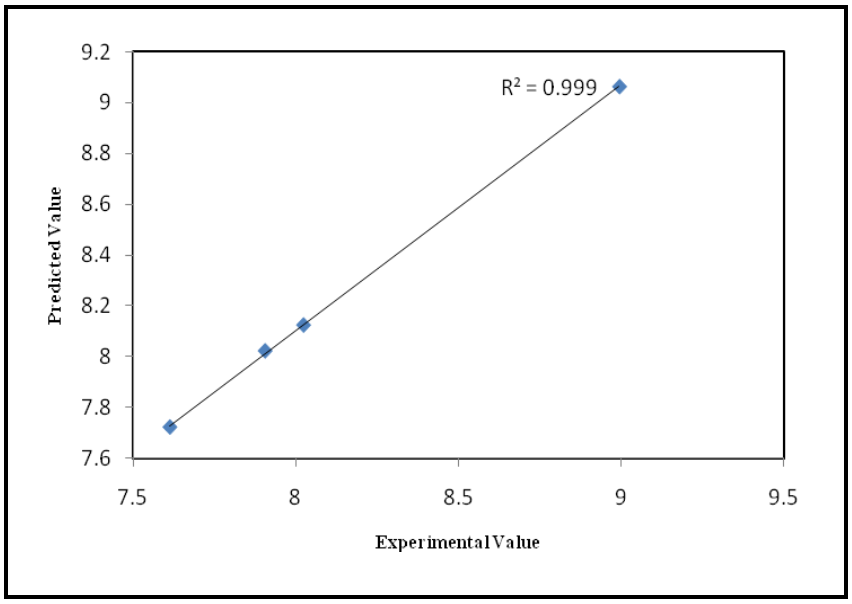

Fig.14 Comparison between Experimental values and predicted values for rate of xylanase production using *A.niger*

Conclusions

The response surface methodology is an efficient and feasible technique for operating parameters optimization and development of a polynomial model for the production of xylanase from *A.niger*. A maximum xylanase production of 630.53 IU/gds was achieved with the following optimal parameters: substrate concentration -9.18 g/10gds, Temperature-32.6°C, Initial pH-5.3, Initial moisture content-75.1% and Incubation

time - 100.8 hrs using the response surface methodology. Validations of the experiments were also carried out to verify the adequacy and the accuracy of the model, and the results showed that the predicted value agreed well with the experimental values. The kinetic parameters were determined using Lineweaver – Burk Plot and their values are given below: k_m = 0.639 mg/ml and V_{max} = 13.889 µmol/min.mg.

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