Enhanced Production of Xylanase enzyme from Agricultural waste using SSF

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Abstract
Nowadays, there is a growing development towards searching of cheaper carbon source for production of enzymes under SSF conditions. To achieve this utilisation of agricultural wastes such as wheat straw as a solid substrate is a good alternative. This also makes SSF process more economical owing to their nutrient content. The improved need to save valuable resources supports the usage and bioconversion of agricultural wastes into useful industrial product. It not only eliminates the existing pollution problem but also reduces the preference of costlier treatment process and lack of the places for its disposal. Wheat is the main food constituent around the world, a large mass of waste residue (wheat straw) has been generated every year and is an abundant agricultural biomass worldwide as wheat is the second major food crop in India.

Response Surface Methodology is employed to optimize the operating variables for xylanase enzyme production by Aspergillus awamori under SSF using the agricultural waste of wheat straw. The consequence of a range of operating parameters such as substrate concentration, temperature, initial pH, initial moisture content and incubation time on Xylanase activity was calculated and optimized using RSM. Using central composite design (CCD), 50 experiments were conducted for the five test variables. The statistical analysis of the experimental data reveals that the most favourable condition for maximum xylanase activity was achieved at a substrate concentration- 9.12g, temperature-32.46°C, pH-5.27, initial moisture content-75.08% and incubation time – 100.35 hrs. Under the optimized conditions, the experimental xylanase activity (703.14 IU/gds) closely coincides to that predicted by the statistical model (703.54 IU/gds).

Keywords: Agricultural waste**,** xylanase, response surface methodology, moisture content, *Aspergillus awamori*.

Introduction

Wheat is the most widely cultivated crop all over the world. Wheat straw is the rich lignocellulosic raw material for use as a cheaper carbon source and the second major food crop in India. Nowadays, concepts such as reduction, reuse and recovery of lignocellulosic biomass are being widely spread. For the production of valuable bioproducts, about 350 million tons of wheat straw is available every year throughout the world. This signifies the great potential of wheat straw as a potential renewable biomass. The composition of dry wheat straw is cellulose 35–45%, hemicellulose 20–30%, lignin 8– 15%, protein 3.1% and ash 10.1%[1]. This lignocellulosic biomass has high organic matter content.

Utilisation of agricultural waste as substrates for enzyme production using SSF conditions has offered quite a lot of reward in yield, cost reduction, time saving and cheaper medium components. Along with these advantages, the use of such lignocellulosic biomass for microbial Xylanase production is a better way not only for cost reduction but also it has given some advantage such as waste minimization to combat environmental pollution[2].

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In the plant cell wall's hemicellulose, the major portion consists of xylan which is the most important part. Xylanases (EC 3.2.1.8) belong to hydrolytic group, which are extracellular enzymes and can be produced by a number of microorganisms. β-1,4 bond present in xylan is hydrolysed by Xylanase and produces xylose which is a prime carbon source[3]. The commercial value of Xylanases is in the food industry and for juice clarification, used as a feed in poultry. Xylanases are also used in paper and pulp industries, bioconversion of agricultural wastes into useful products and textiles[4]. SSF is more favourable than SmF because of low investment, higher yield, minimizing of energy requirements, more product stability and its easy technique.

SSF makes use of agricultural wastes and of agro- industrial by-products. It involves a low investment and operating cost[5]. Based on water activity, fungi and yeast are most appropriate microorganisms for SSF, whereas bacteria is considered to be unsuitable. The filamentous fungus *Aspergillus* is found to be of great importance because of the production of metabolites such as enzymes[6].

For efficient production of enzyme during SSF, operating conditions are much more essential for microbial growth and which demand optimization of critical parameters. Optimization of conditions is required for reducing cost of enzyme production. The most favourable extent among operating variables can be attained by changing one variable at a time. It is a very laborious approach which takes lot of time and it frequently fails to guarantee the determination of optimum conditions and also it could not predict the interactive effect of all the factors.

One alternative to prevail over this problem is the use of response surface methodology (RSM),[7] which is a collection of statistical techniques for design of experiments, model building, estimating the effect of factors involved and searching for best possible conditions for advantageous responses. RSM can identify and calculate a range of interactions between different parameters and it has been applied for medium and process optimisation in bioprocesses. In addition, the entire process can be finished in a reasonable time scale[8]. In the present study, the RSM was employed to optimize various process parameters for maximum xylanase production using low cost agricultural waste (Wheat straw) as a substrate during SSF by *Aspergillus awamori* [MTCC No: 6652].

Material and Methods

Substrate and Microorganism: Wheat straw was obtained from the local agricultural field near Chidambaram. It was powdered, sieved (40 mesh size), treated with 0.2 N NaOH alkali extraction and used as a substrate. The collected substrate was placed in an oven at 80°C for 12 hours to remove the moisture content and stored for further use. The microorganism employed in the present study was *A.awamori MTCC-6652* which was obtained from MTCC, IMTECH, Chandigarh, India.

Inoculum preparation: The cultures were maintained on PDA and sub cultured at an interval of three months and incubated for 72 hrs at 28°C. After incubation 10ml of sterile water was added to the slants and the spores were removed using a sterile inoculation needle. Transfer spores from slant to PDA broth and incubate for 5 days at 28^{0}C. This spore suspension after filtration through a muslin cloth was used as the inoculum (2 - 3ml).

Xylanase production using SSF: SSF was carried out in Erlenmeyer flasks (250 ml) by varying the process parameters such as substrate concentration, pH, initial moisture content and incubation time according to the experimental design. The contents were thoroughly mixed and autoclaved at 121°C and 15 psi pressure for 15 minutes. After cooling, the inoculation was carried out using 5% (v/v) of the filtered inoculum.

All the experiments were done in duplicate and the samples were collected after 100 hrs. The flasks were removed and by adding 50.0 ml of 0.05 M Na-citrate buffer (pH 5.3), the contents were agitated at 200 rpm for 30 minutes in an orbital shaker at 30°C. The enzyme extraction was done by squeezing the fermented contents using a cotton cloth. It was then centrifuged at 15,000 rpm for 20 minutes and the supernatant was investigated for determination of xylanase activity.

Enzyme Assay: Xylanase activity was carried out by measuring the amount of reducing sugar according to the DNS method[9]. Xylanase production was expressed as IU/g of dry substrate (IU/gds).

Optimization of Process Parameters: A CCD experimental design with 10 star points, (2^5 = 32) axial points and eight replicates at the center point (n_0 = 8), which results in a total of 50 experiments covering the entire spectrum of combination of variables, was used for fitting a response surface. These 50 experiments were performed with different combinations of the five independent variables. At five different levels -2.38, -1, 0, 1 and 2.38, the five independent variables were tested. The levels and range of independent variables are presented in table 1 and 50 experiments were carried out as indicated in table 2.

The experiments with various substrate concentrations, temperature, pH values, moisture content, incubation time were carried out at the same time covering the whole spectrum of combination of variables with a wide range for xylanase production in the CCD. 50 experiments were performed out as a batch process as given in CCD from table 2. All the 50 experiments were performed thrice and the response is taken from the average of these values.

The coded values of the process parameters were determined by the following equation:

$$z_i = \frac{Z_i - Z_0}{\Delta Z_i} \quad (1)$$

where x_i is coded value of the ith variable, Z_i is uncoded value of the ith test variable and Z_0 is uncoded value of the ith test variable at center point. The levels and range of independent variables are shown in table 1.

Table 1
Levels and Range of Independent Variables

Variables		Levels				
	Code	**-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	103.1	110	115	120	126.9

Table 2
Central Composite Design Matrix with Xylanase activity as Response

Run No	Coded Values					Xylanase Activity (IU/gds)	
	A	B	C	D	E	Exp	Pred
1	1	1	1	-1	-1	680.21	676.09
2	1	1	1	-1	1	650.45	649.23
3	-1	1	-1	-1	-1	585.00	586.99
4	1	-1	1	-1	-1	659.34	658.45
5	1	-1	-1	-1	-1	602.54	599.26
6	0	0	0	0	2.38	624.78	623.45
7	-1	1	1	1	-1	641.12	641.3
8	-1	1	1	-1	-1	678.65	676.87
9	-1	-1	-1	1	1	673.32	675.02
10	1	-1	1	1	-1	643.32	645.71
11	1	1	-1	-1	1	625.34	621.52
12	-2.38	0	0	0	0	675.13	673.41
13	1	1	-1	-1	-1	591.32	594.3
14	0	0	0	0	-2.38	613.02	610.21
15	-1	-1	1	-1	1	646.21	643.54
16	-1	-1	1	1	1	649.21	646.21
17	-1	1	-1	1	1	676.21	674.56
18	-1	1	1	-1	1	676.21	675.26
19	1	-1	1	1	1	612.23	611.23
20	0	0	0	0	0	700.23	700.71
21	0	0	-2.38	0	0	595.12	592.14
22	0	0	0	0	0	700.33	699.48
23	-1	1	1	1	1	666.43	661.64
24	0	0	0	0	0	690.43	698.60
25	0	0	0	0	0	700.53	700.42
26	0	0	0	2.78	0	616.09	621.44
27	1	-1	-1	-1	1	588.12	594.07
28	0	0	0	0	0	700.67	700.61
29	1	-1	-1	1	-1	642.20	634.51
30	0	0	0	-2.38	0	599.12	596.21
31	1	-1	1	-1	1	603.61	601.04
32	0	0	0	0	0	700.21	700.12
33	-1	1	-1	-1	1	645.02	642.12
34	1	1	1	1	-1	657.12	651.09
35	0	0	0	0	0	700.35	701.56
36	0	2.38	0	0	0	655.23	651.83
37	0	0	2.38	0	0	639.21	646.74
38	0	-2.38	0	0	0	631.59	630.81
39	1	1	-1	1	-1	615.34	615.93
40	-1	-1	-1	1	-1	629.43	629.54
41	1	1	-1	1	1	656.03	658.01
42	0	0	0	0	0	700.23	702.35
43	2.38	0	0	0	0	645.69	642.44
44	-1	-1	1	-1	-1	666.23	663.89
45	1	1	1	1	1	652.43	649.12
46	-1	1	-1	1	-1	612.64	600.47
47	-1	-1	-1	-1	1	635.76	626.35
48	1	-1	-1	1	1	662.63	652.14
49	-1	-1	-1	-1	-1	612.53	600.39
50	-1	-1	1	1	-1	655.32	646.41

Std. Dev.-5.57, R^2-0.9840, Mean-647.98, Adj R^2-0.9730, C.V. %-0.86, Pred R^2- 0.9456, Adeq Precision-30.08

The experimental design is shown in table 2. The data were subjected to variance analysis and fitted using the following second order polynomial equation:

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

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.

The data obtained were subjected to regression analysis using a statistical software package Design Expert 8.0.7.1.5 and the coefficient of the regression equation was estimated. The statistical tests called the ANOVA analysis were employed for validation of the regression equation.

Results and Discussion

From the second-order polynomial equation 2, the response xylanase activity was related with the five independent variables by using multiple regression analysis and the statistical significance of the coefficients of the model equation was evaluated using Design-Expert 8.0.7.1.5. The correlation between the five dependent variables and xylanase activity was explained by the following second order polynomial equation:

$$\begin{aligned} Y = {} & 698.996 - 6.396A + 4.242\,B + 11.3\,C + 6.949D \\ & + 4.036\mathrm{E} + 3.156\,A*B - 1.017\,A*C \\ & + 2.571X_1\,X_4\;A*D - 7.125\,A*E \\ & + 6.462\,B*C - 3.381B*D + 7.079\,\mathrm{B} \\ & *\mathrm{E} - 11.434\,\mathrm{C}*\mathrm{D} - 12.374\,\mathrm{C}*\mathrm{E} \\ & + 4.909\,\mathrm{D}*\mathrm{E} - 6.764A^2 - 9.765\,B^2 \\ & - 14.399\,C^2 - 13.713\,D^2 \\ & - 14.092E^2 \end{aligned} \quad (3)$$

where Y is the Xylanse Activity and *A, B, C, D, E* were coded values of substrate concentration, temperature, initial pH, initial moisture content and incubation time respectively.

The experimental and predicted xylanase activity values were given in table 2. Analysis of Variance (ANOVA) was performed to analyse the results and are given in table 3. The significance of the model is indicated by the ANOVA of the quadratic regression model. The model *F*-value of 89.33 inferred the model to be significant.

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

Source	Coeff estimate	Sum of squares	Df	Mean squares	F Value	p-value Prob>F
Model	698.9968	55427.91	20	2771.396	89.33416	< 0.0001
A	-6.39591	1772.481	1	1772.481	57.1348	< 0.0001
B	4.241595	779.5338	1	779.5338	25.12777	< 0.0001
C	11.29951	5532.174	1	5532.174	178.3261	< 0.0001
D	6.948631	2156.904	1	2156.904	69.52641	< 0.0001
E	4.036318	705.9067	1	705.9067	22.75445	< 0.0001
AB	3.155625	318.655	1	318.655	10.27164	0.0033
AC	-1.07125	36.72245	1	36.72245	1.183725	0.2856
AD	2.571875	211.6653	1	211.6653	6.822896	0.0141
AE	-7.125	1624.5	1	1624.5	52.36472	< 0.0001
BC	6.461875	1336.187	1	1336.187	43.07112	< 0.0001
BD	-3.38125	365.8513	1	365.8513	11.79298	0.0018
BE	7.079375	1603.762	1	1603.762	51.69623	< 0.0001
CD	-11.4344	4183.838	1	4183.838	134.8633	< 0.0001
CE	-12.3738	4899.51	1	4899.51	157.9326	< 0.0001
DE	4.909375	771.2628	1	771.2628	24.86116	< 0.0001
A^2	-6.76411	2536.416	1	2536.416	81.75973	< 0.0001
B^2	-9.76531	5286.534	1	5286.534	170.408	< 0.0001
C^2	-14.3986	11493.21	1	11493.21	370.4764	< 0.0001
D^2	-13.7127	14341.44	1	14341.44	462.2871	< 0.0001
E^2	-14.0923	11009.43	1	11009.43	354.8819	< 0.0001
Residual		899.6611	29	31.0228		
Lack of Fit		813.1252	22	36.96023	2.989759	0.0705
Pure Error		86.53595	7	12.36228		
Cor Total		56327.57	49			

Such a large F value could occur due to noise and the chance of getting such a high value is 0.01 % only. 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 is the magnitude of the P, the more significant is the corresponding coefficient. Model *P* value (Prob > *F*) is very low [< 0.0001] which restates that the model is significant.

Values of *P* less than 0.05 state that the model terms are significant. The coefficient estimates and the corresponding *P* values imply that the linear effects *C, D, E,* the interactive effects *AD, BC, BE, CD, DE* and squared effects A^2, B^2, C^2, D^2, E^2 are found to be highly significant model terms for Xylanase production.

The fit of the model is checked by the coefficient of determination R^2. The nearer is the values of R^2 to 1, the model would explain better the response namely the xylanase activity under various experimental process conditions and are closer to the predicted values by CCD. The coefficient of determination (R^2) for xylanase activity is calculated as R^2=0.9840 which is nearly equal to 1, indicating that 98.40% variability of the response could be explained by the model.

This shows that the model is very much suitable for xylanase production using *A.awamori* by SSF. The predicted R^2 values of xylanase activity are found to be 94.56% which are in reasonable agreement with the adjusted R^2 values of 97.30%.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Adeq Precision of 30.08 for xylanase indicates an adequate signal. The coefficient of variation (C.V) is the ratio of standard error of estimate to the mean value of the observed response expressed as a percentage. Here a lower value of C.V (0.86) for xylanase indicates a greater reliability of the experiments performed.

For understanding both the interactive and the main effects of two factors, response surface curves as a function of two factors at a time are more helpful while holding all other factors at fixed levels.

The interaction effects of variables and optimal levels of the each variable on production are investigated by plotting the 3D response surfaces with the vertical axis representing enzyme activity (response) and two horizontal axes representing the coded levels of two explanatory factors while maintaining other variables at their median levels shown in figures 1 to 10.

The surface confined in the smallest ellipse in the contour indicates the maximum predicted yield. An elliptical or saddle nature of the contour plot in the response surfaces signifies significant interaction between the corresponding variables whereas circular contour plot signifies no interaction.

Fig. 1 represents the interactive effect of substrate concentration and temperature on xylanase activity. The nature of the response surface curves confirms excellent interaction between these tested variables. The xylanase activity increases as the substrate concentration increases and reaches maximum activity at 9.12 g of substrate. Thereafter the xylanase activity decreases.

This may be due to the fact that high concentration of substrate led to increase in viscosity of the medium, which influenced the medium components and transportation of oxygen to the cells through substrate because of poor aeration[10]**.** This is evident from the figures 2, 3 and 4.

The effect of temperature on enzyme activity is studied by performing experiments at various temperatures ranging from 27.2°C to 36.8°C. The results are shown in figures 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 32.8°C. The xylanase activity decreased considerably even for slight increase in the temperature from 32.46°C. The reason for decrease in enzyme activity on increase in temperature is due to the fact that the thermal denaturation of enzymes of the metabolic pathway is high because of the maintenance energy requirement needed for cellular growth which results in minimum amount of product formation.

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 figures 2, 5, 8, 9. The maximum xylanase activity was obtained at pH 5.5. At this pH value, high xylanase activities were obtained when compared to that for other pH values. However, the xylanase activity decreases when the pH is raised above 5.27. This indicates that *Aspergillus awamori* was resistant to acidic operating conditions**.** An acidic condition is also found to be more favourable condition for maximum xylanase production[11].

The effect of initial moisture content on enzyme activity is studied by performing experiments with the initial moisture content ranging from 71.2 % to 79.8 %. The results are shown in figures 3, 6, 8, 10. Xylanase activity was found to increase as initial moisture content is raised from 71.2 % to 76.1 % and is found to decrease with further increase with initial moisture content of the medium.

The maximum xylanase activity of 620.45 IU/gds is obtained with the initial moisture content of 75.08% respectively. The decrease in the enzyme activity for initial moisture content greater than 75.08 % might be due to the fact that higher moisture level levels frequently leads to substrate particles sticking either together, or to the wall of the reactor which reduced the porosity of substrate preventing the transfer of oxygen into the substrate as a barrier.[12]

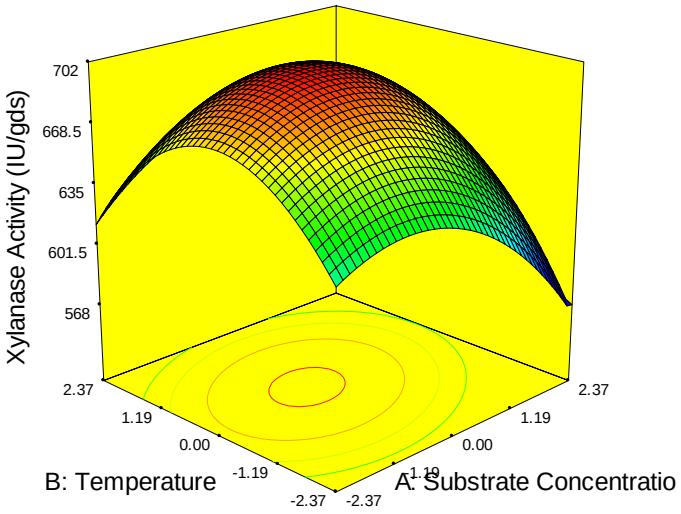

Fig. 1: 3D Response surface interactive effect plot of Substrate concentration and Temperature

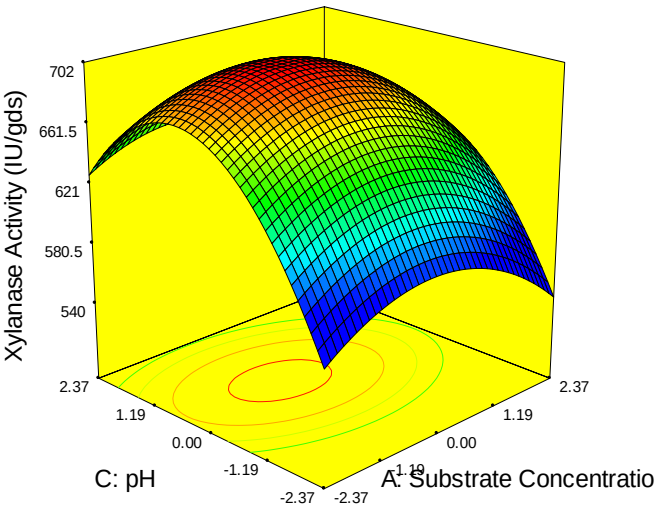

Fig. 2: 3D Response surface interactive effect plot of Substrate concentration and pH

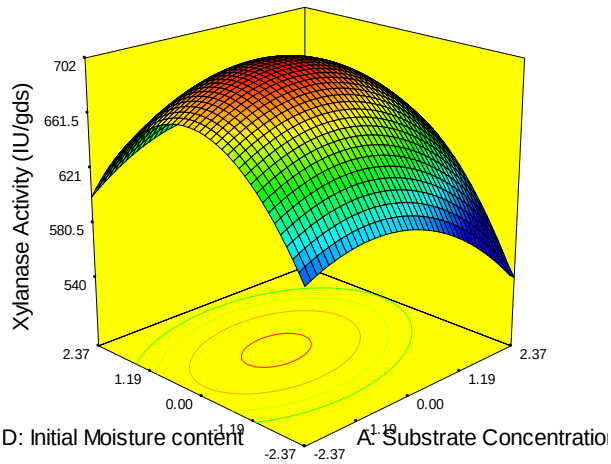

Fig. 3: 3D Response surface interactive effect plot of Substrate concentration and Initial moisture content

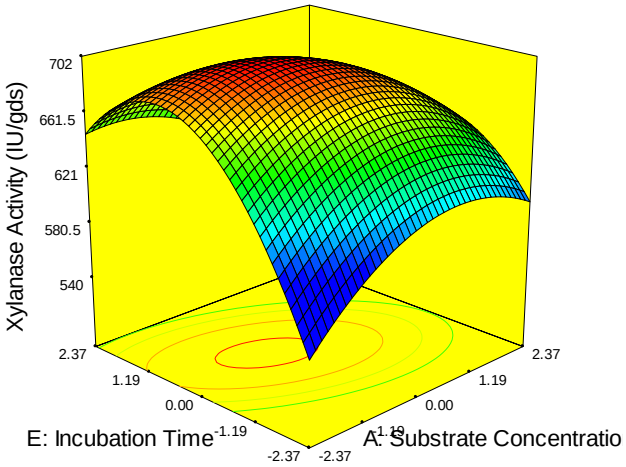

Fig. 4: 3D Response surface interactive effect plot of Substrate concentration and Incubation Time

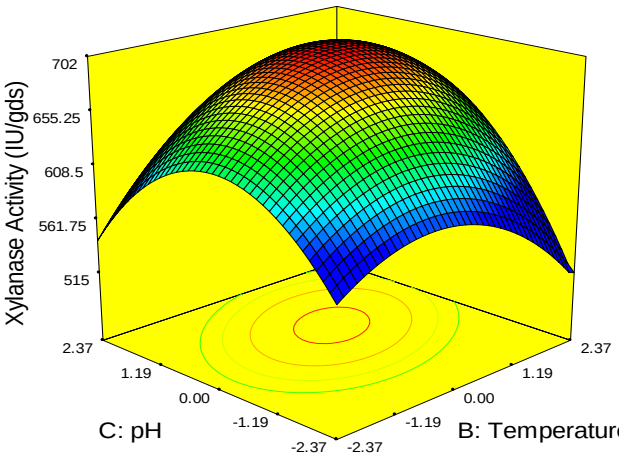

Fig. 5: 3D Response surface interactive effect plot of Temperature and pH

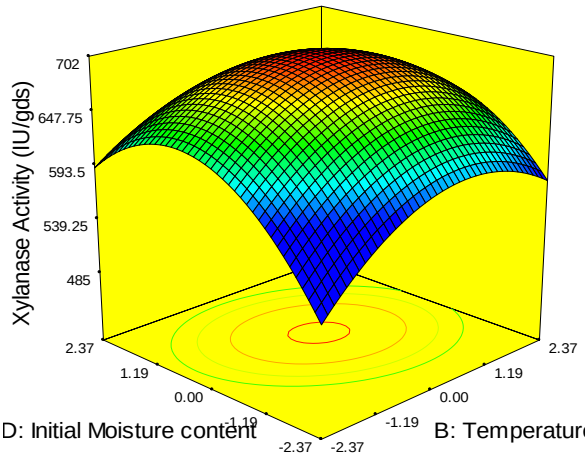

Fig. 6: 3D Response surface interactive effect plot of Temperature and Initial moisture content

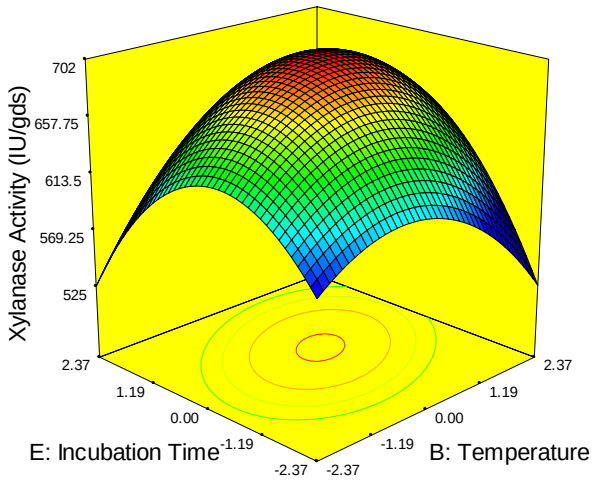

Fig. 7: 3D Response surface interactive effect plot of Temperature and Incubation Time

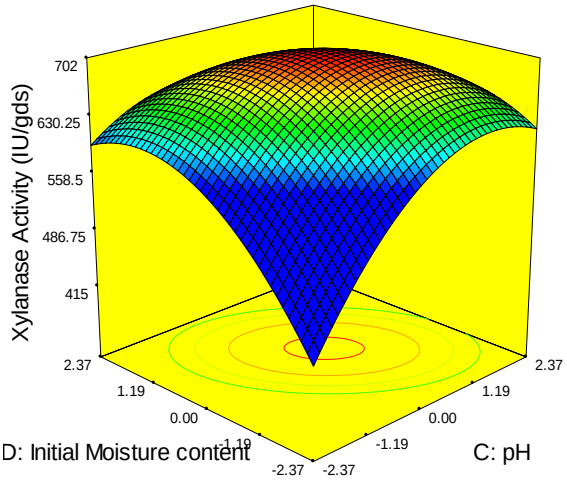

Fig. 8: 3D Response surface interactive effect plot of pH and Initial moisture content

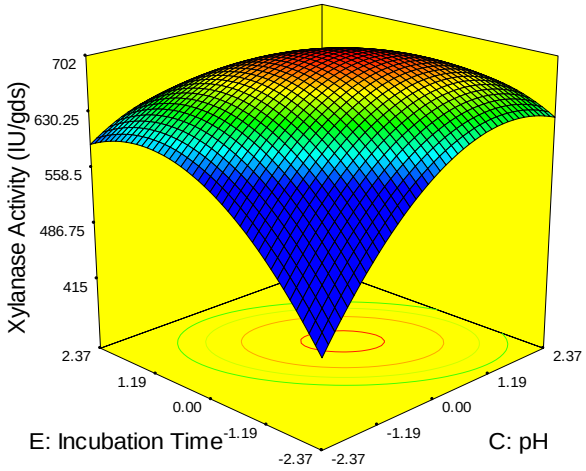

Fig. 9: 3D Response surface interactive effect plot of pH and Incubation Time

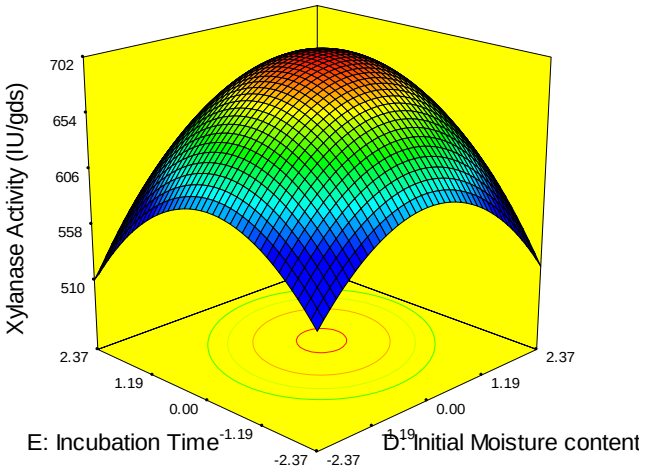

Fig. 10: 3D Response surface interactive effect plot of Initial moisture content and Incubation Time

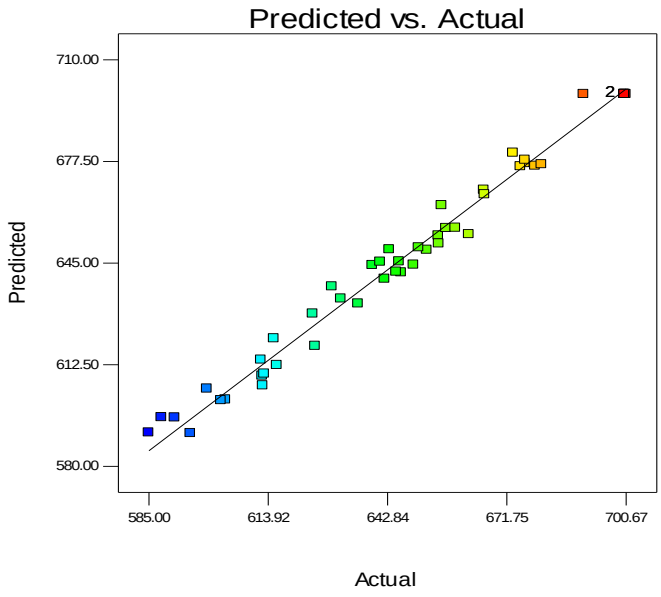

Fig. 11: Predicted vs Experimental xylanase activity

The effect of Incubation time on enzyme activity is studied by conducting experiments with incubation period ranging from 103.1 hours to 126.9 hours, which are shown in figures 4, 7, 9 and 10. The maximum xylanase of 700.67 IU/gds is obtained with the incubation period of 100.03 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 of the cells as well as enzyme production. With prolonged incubation, the decrease in enzyme production may be due to the fact that susceptible portion of xylan molecules is rapidly digested and only the crystalline portion remains[13].

Optimum conditions are the one at which the maximum xylanase production is obtained. By solving the second order polynomial equation using RSM, the most favourable condition for Xylanase production can be obtained. The position at which the slope of the contour is zero in all directions is called as central point. The optimum values drawn from these figures are in close agreement with those obtained by optimizing the regression model equation 2. The optimum values of the tested variables for maximum xylanase production are: substrate concentration - 9.12 g, temperature - 32.46°C, pH - 5.27, initial moisture content - 75.08% and incubation time - 100.03 hrs. The most favourable values for the variables as predicted are within the design region. Fig. 11 shows that the experimental xylanase activity values agree well with the predicted response values.

Using *Aspergillus sp.* under SSF, the optimum values for substrate concentration, temperature, initial pH, initial moisture content and Incubation time for maximum xylanase activity[14-16] were 10g, 32ºC - 35ºC, 4.2 - 5.5, 77% and 72h - 96h respectively which are in excellent agreement with the present research data also.

Experimental Validation of the model: Experimental validation of the model is checked by conducting the batch experiment under more favourable operating conditions. After conducting three repeated experiments, the results are compared. The experimental xylanase activity is found to be 703.14 IU/gds which is very close to that of 703.54 IU/gds predicted by the regression model. The experimental and predicted values of enzyme activity show excellent agreement with one another with high extent of precision of the model proving the model validation under the experimental conditions.

Conclusion

The optimisation of xylanase production using inexpensive agricultural waste under cheaper solid-state fermentation technology by RSM was investigated in the current research. The usage of wheat straw as low-cost agro-wastes brought down the xylanase enzyme production cost and also reduces environmental pollution. To formulate enzyme production cost effective, optimization of various process parameters was done by response surface methodology.

Enhanced xylanase production of 703.14 IU/gds was obtained with the following optimum process parameters: substrate concentration – 9.12, temperature - 32.46°C, initial pH - 5.247, initial moisture content – 75.08.1% and incubation time – 100.03 hrs. It is concluded that solid state fermentation technique using inexpensive agro-waste as substrate was more effective as well as economical for production of xylanase by *A. awamori.*

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