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Optimization of process and conditions for enhanced xylanase production under SSF using inexpensive agro-industrial waste

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ABSTRACT

The usage of agricultural wastes for enzymes production is considered an essential part in any approach to accomplish goals to reduce environmental pollution and disposal of waste. In the present investigation, xylanase enzyme production by *Aspergillus fumigatus* using agro-industrial waste sugarcane bagasse with solid state fermentation was studied by keeping the best possible values of process variables, substrate concentration, temperature, incubation time, initial moisture content, and initial pH of the medium. The above-mentioned variables affecting the fermentation conditions were optimized using response surface methodology (RSM). To estimate individual and interaction effects, the central composite design was used. The most favorable process variables attained were substrate concentration = 9.88 g, temperature = 35.73°C, incubation time = 120.05 hours, initial moisture content = 71.30%, and initial pH = 4.98. From analysis of variance, an R^2-value of 0.9848 signifies a good agreement between the experimental and predicted values for sugarcane bagasse. Also, the fitness of the model is confirmed by a high R^2-value. The RSM shows that xylanase activity of 417.521 IU/gds was achieved for the optimized process environment. In addition to xylanase activity, a poor quantity of carboxy methyl cellulase activity was also recorded. This study is carried out for cost-effective xylanase production by using agro-industrial waste as cheaper carbon source. It can reduce environment pollution and also minimizes the cost for disposal of industrial waste.

1. INTRODUCTION

Industrial enzymes have expanding markets as they have the potential to be employed as biocatalysts in different industrialized segments for fresh functions which create requirements for enzymes with new performance and enhanced consistency. Commercially, the production of enzymes has significantly developed all through the past century. Among the currently known 4,000 enzymes, around 200 enzymes are used for commercial production [1]. The enzyme market had a value of $4.9 billion for the production of commercial enzymes in 2015 and 4.7% annual growth rate was expected for the years 2016–2021 [2]. The approximate sharing of xylanase enzyme is expected to attain $500 million by 2023 [3]. In recent years, great attention is attained for xylanase because of its extensive use in industrial biotechnological applications of this enzyme, mostly for the conversion of hemicellulose to xylose, pharmaceuticals, textile and leather, agricultural waste treatment, ethanol, and clarification of juices and wines [4].

The enzyme xylanase degrades β-1,4 xylan by cleaving β-1,4 glycosidic linkages and shapes utilizable products like xylose and xylobiose [5]. The joint action of enzymes, xylanases, and β-xylosidize for hydrolyzing main chain and additional enzymes like α-arabinofuranosity, acetyl xylan esterase, α-glucuronosity, and feruloyl esterase for hydrolyzing its branches is required for complete hydrolysis of xylan [6]. Researchers focus their attention on microbial enzymes because of its exploitation in commercial and biotechnological applications. Due to their accessibility, structural strength, and simple genetic manipulation, the xylanase production uses microbes other than animal and plant sources [7].

A prospective means of interest in acquiring enzymes is solid state fermentation (SSF). Compared with submerged fermentation, the

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SSF technique using solid substrate has a great advantage, owing to the absence of water to facilitate natural environment for growth of microorganisms, lowering investment expenditure, higher efficiency, and ease of further purification steps [8]. Industrially, commercial xylan as a solid medium that induce enzyme synthesis is not profitable and, therefore, inexpensive and readily available agro-wastes such as sugarcane bagasse, groundnut cake, and rice bran rich in xylan content should be preferred as substrates for xylanase production [9]. Through the rising development of agro-industrialized activities worldwide, wastes of huge capacities are produced per annum. In India, about 625 million tons of agro-industrial wastes are produced annually [10]. The exploitation of agro-industrialized waste materials for high-value enzyme synthesis is satisfactory due to the decreasing cost for the disposal of wastes [1].

Due to sugarcane bagasse's instant accessibility at sugar-processing industries, it is exploited to create added incomes by making the most of it for the production of enzymes [11]. Sugarcane bagasse is employed as a low-priced carbon resource for growth of fungal cultures. In addition, it has also been employed as a good enzyme inducer. At the industrial scale, microorganisms such as fungi, bacteria, and actinomycetes are used for xylanase production [12], but fungi are of immense importance [13]. Filamentous fungi are mainly interesting in view of the fact that they secrete enzymes into the culture media and their enzymatic activity level is significant in comparison with bacteria and yeasts. For the production of xylanase from wastes, filamentous fungi, including *Aspergilli* and *Trichoderma*, are extensively used [14] and they can produce enzymes via SSF. In SSF systems, xylanase production is usually reported using mostly *Aspergillus* species.

Several factors that significantly influence fungal enzymes production during fermentation comprise substrate concentration, incubation time, pH, moisture content, and temperature, which must be optimized for higher productivity [9]. To meet the industrial requirements, more interest is fixed on the stability of the enzyme over various experimental conditions, as well as its ability to hydrolyze xylans [13].

Due to the interlinking nature of the process parameters, the interaction effects between the variables will not be considered while studying each factor alone or grouped with other factors. Hence, usual one factor at a time (OFAT) optimization approach might not subsist consistently [15]. An attractive approach for xylanase production is to cut down the unnecessary steps and treatments to shorten the fermentation time and decrease the production costs by choosing experimental design methods. The statistical optimization method such as central composite design (CCD) of response surface methodology (RSM) is used for the enhancement of process parameters for enzyme synthesis. It results in superior action when compared to the usual methods of the OFAT approach [16]. RSM includes benefits from the usual technique which identifies the relationship among the two self-determining factors that are accountable for superior enzyme production [17]. The mutual effect of all the self-regulating variables and its exchanges would be completely analyzed by RSM during the fermentation process.

Therefore, taking into consideration the significance and applications of xylanases in industries, the current research is carried out to make the most effective use of the process environment for xylanase enzyme synthesis from sugarcane bagasse as the one and only carbon supply for the fungal strain *Aspergillus fumigatus* under SSF.

2. MATERIALS AND METHODS

2.1. Substrate Preparation and Microorganism

Sugarcane bagasse was obtained from a local agricultural field near Chidambaram. It was then sun-dried for a 3-week period, powdered, sieved through 100-mesh screen, treated with 0.2 N NaOH alkali solution, and used as a substrate. The collected substrate was placed in an oven at 50°C for 48 hours to remove the moisture content and stored for further use. The microorganism employed in the present study was *A. fumigatus* [Microbial Type Culture Collection and Gene Bank (MTCC) 343; ITCC2550], obtained from MTCC, Institute of Microbial Technology, Chandigarh, India.

2.2. Inoculum Preparation

The stock cultures were preserved as slants of the agar medium at 5°C and sub-cultured at an interval of 3 months and incubated for 120 hours at 35°C. After incubation, 10 ml of sterile water was added to the slants and the spores were removed using a sterile inoculation needle. The spores were shifted from the slants to Czapek Dox broth and incubated for 5 days at 35°C. This spore suspension after filtration through a muslin cloth was used as the inoculum (2–3 ml).

2.3. Xylanase Production Under SSF

SSF experiment was conducted in 250 ml capacity Erlenmeyer flasks by varying the experimental conditions, such as substrate conc., 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 experimental runs were carried out in duplicates and the samples were collected after 100 hours. The flasks were removed and by adding 50.0 ml of 0.05 M Na-citrate buffer (pH 5.3), the contents were mixed at 200 rpm using an orbital shaker for a period of 30 minutes at room temperature. The extraction was conducted by pressing the flask contents using a cotton cloth. It was centrifuged at a speed of 15,000 rpm for 20 minutes and the top clearest content was investigated for xylanase activity.

2.4. Enzyme Assay

Xylanase assay was carried out by assessing the quantity of reducing sugar according to the dinitrosalicylic acid (DNS) method [18] with D-xylose as standard. Xylanase production was stated as IU/g of dry substrate (IU/gds).

Carboxy methyl cellulase (CMcase) was analyzed by evaluating the reducing sugar content according to the DNS method [18] with D-glucose as the standard.

2.5. Optimization of Process Conditions

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 50 runs covering the complete spectrum of combination of variables, was designed for predicting a response surface. These 50 runs were carried out at 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 self-regulating variables are presented in Table 1 and the 50 runs which were carried out are presented in Table.2.

The experiments runs with various substrate concentrations, temperature, pH values, moisture content, and 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 carried out out as a batch process as given in CCD (Table 2). The entire 50 runs were carried out thrice and the response is taken from the average of these values.

The values of the process parameters in the coded form were given by the following equation:

$$p_l = \frac{P_l - P_0}{\Delta P_l} \quad (1)$$

where p_l is the lth variables coded form, p_l is the lth test variable uncoded form, and p_0 is the center point lth test variable uncoded form. The span and extent of the experimental parameters are shown in Table 1.

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

$$Z = \beta_0 + \sum_{l=1}^{n} \beta_m P_m + \sum_{l=1}^{n} \beta_m P_m^2 + \sum_{l=1,l<m}^{n-1} \sum_{m=2}^{kn} \beta_{lm} P_l P_m \quad (2)$$

where Z is the predicted xylanase activity, β_l, β_m, and β_{lm} are constants assessed from regression. They represent the linearized, quadratic, and interaction effects of A, B, C, D, and E on xylanase activity.

The data obtained were subjected to regression analysis using the statistical analysis software Design Expert 8.0.7.1.5 and the constants of Eq. (2) were estimated. The statistical tests called analysis of variance (ANOVA) was employed for validation of the regression equation.

3. RESULTS AND DISCUSSION

From the second-order polynomial Eq. (2), the response xylanase activity was related to the five independent variables by using the multiple regression analysis and statistical importance of the constants of Eq. (2) were evaluated using Design Expert 8.0.7.1.5. The correlation between the five dependent variables and xylanase activity is elucidated as follows:

Xylanase activity, Z (IU/gds) = 415. 017 – 5.318 A + 5.973 B – 1.862 C + 3.208 D + 6.319 E + 10.815 A* B + 9.188 A* C – 3.028 A* D – 0.602 A* E + 3.068 B* C + 0.355 B* D + 8.286 B * E + 4.911 C * D + 1.261 C * E – 4.236 D * E – 15.088 A^2 − 12.411 B^2 – 15.818 C^2 – 9.797 D^2 – 14.427 E^2 (3)

where Z is the xylanase activity and A, B, C, D, and E were symbolic codes of substrate concentration, temperature, initial pH, initial moisture content, and incubation time, correspondingly.

Experimental and predicted xylanase activity values are given in Table 2. The testing of variance (ANOVA) was made to analyze the results and is presented in Table 3. The significance is specified by ANOVA of the quadratic regression model. The value of F (94.426) inferred the corresponding design model to be considerable. p-value < 0.05 states the terms present in the model are important. Coefficient estimates and the resultant values of P imply a linear effect C, D, E; the interaction effect AD, BC, BE, CD, DE and squared effect A^2, B^2, C^2, D^2, and E^2 were found to be highly considerable model conditions for xylanase synthesis.

The model fitness is verified by R^2. R^2-value of 0.9848 illustrates the correctness of the model for xylanase production using sugarcane bagasse. The predicted R^2-value of 0.9423 for xylanase activity is in practical agreement with the adjusted R^2-values of 0.9744.

Adequate precision value, 32, for xylanase designates a satisfactory signal. A low CV value, 1.45, indicates a superior consistency of the tests executed.

Three-dimensional response surfaces were drawn to explore the combined effects of the experimental parameters and its most favorable intensity on xylanase synthesis. Figures 1–10 correspond to the most important joint effects of the five experimental parameters and their best possible ranges are explored using 3D response surface curves.

Figure 1 shows the simultaneous outcome of substrate concentration and temperature on xylanase activity. The nature of the response curves confirms excellent relationships between the above-mentioned variables. The xylanase activity increases, as the

Table 1: Span and extent of experimental parameters.

Variables		Levels				
	Symbolic code	−2.38	−1	0	1	2.38
Substrate concentration (g)	A	5.2	8.0	10.0	12.0	14.8
Temperature (°C)	B	29.1	32.5	35.0	37.5	40.9
Initial pH	C	2.7	4.0	5.0	6.0	7.3
Initial moisture content (%)	D	58.2	65.0	70.0	75.0	81.8
Incubation time (hours)	E	108.1	115	120	125	131.9

Table 2: CCD along with xylanase and CMCase activity as response.

Run No	Symbolic coded values					Xylanase activity (IU/gds)		CMCase activity (IU/gds)
	A	B	C	D	E	Exp	Pred.	Exp
1	−1	−1	−1	1	−1	379.34	379.59	107.46
2	−1	1	1	−1	1	345.65	349.17	97.92
3	−1	−1	−1	−1	−1	368.90	369.17	104.50
4	−1	1	−1	−1	1	375.45	372.43	109.36
5	−1	1	1	1	1	365.09	363.71	103.42
6	1	1	−1	−1	−1	331.32	335.95	96.86
7	0	−2.38	0	0	0	326.43	330.49	92.47
8	1	−1	−1	1	1	305.57	307.96	82.56
9	0	0	0	0	−2.38	317.04	318.25	89.81
10	−1	1	1	−1	−1	317.19	307.76	89.86
11	1	1	1	1	−1	368.40	363.76	104.36
12	−1	1	−1	−1	−1	331.03	336.07	97.78
13	1	−1	1	1	1	336.09	328.82	95.21
14	0	0	0	0	0	415.34	415.06	117.66
15	1	1	1	−1	−1	347.90	344.39	94.56
16	0	0	2.38	0	0	310.87	320.98	88.07
17	0	0	0	0	0	410.23	415.06	116.21
18	−1	−1	1	−1	1	342.89	336.85	97.14
19	−1	1	1	1	−1	336.69	339.24	95.38
20	0	2.38	0	0	0	356.80	358.93	101.08
21	0	0	0	0	0	415.12	415.06	117.60
22	1	−1	1	1	−1	338.31	339.90	95.84
23	−2.38	0	0	0	0	340.56	342.20	96.48
24	−1	1	−1	1	1	366.14	367.32	103.72
25	1	1	−1	−1	1	373.90	369.90	108.92
26	0	0	0	0	0	415.34	415.06	117.66
27	1	−1	−1	−1	1	324.65	326.60	97.97
28	−1	−1	1	−1	−1	327.45	328.59	92.76
29	0	0	0	0	0	414.24	415.06	117.35
30	0	0	0	0	0	415.09	415.06	117.59
31	0	0	0	0	2.38	343.35	348.33	97.27
32	0	0	0	0	0	415.45	415.06	117.69
33	1	1	−1	1	1	355.78	352.68	100.79
34	1	1	1	1	1	383.60	385.81	108.67
35	0	0	0	−2.38	0	345.06	351.88	97.75
36	−1	−1	1	1	−1	358.90	358.65	101.67
37	−1	−1	−1	−1	1	375.34	372.39	106.33
38	1	−1	−1	−1	−1	332.56	325.79	94.21
39	0	0	0	0	0	415.21	415.06	117.62
40	−1	−1	−1	1	1	360.98	365.86	102.26
41	0	0	0	2.38	0	367.78	367.15	104.19
42	1	1	−1	1	−1	331.32	335.67	93.86
43	1	−1	−1	1	−1	325.67	324.09	92.26
44	1	−1	1	−1	1	327.45	327.82	92.76
45	−1	1	−1	1	−1	352.25	347.90	99.79
46	0	0	−2.38	0	0	333.76	329.84	94.55
47	2.38	0	0	0	0	312.34	316.89	88.48
48	−1	−1	1	1	1	355.45	349.97	100.69
49	1	−1	1	−1	−1	324.27	321.96	91.86
50	1	1	1	−1	1	388.67	383.39	110.10

Std. Dev = 5.1715; R^2 = 0.9848; Mean = 356.48; Adj R^2 = 0.9744; C.V = 1.45; Pred R^2 = 0.9423; Adeq Precision = 32.00.

Table 3: ANOVA for xylanase production using sugarcane bagasse.

Source	Coeffestimate	Sum of squares	df	Mean squares	*Fvalue*	*p*-value Prob>*F*
Model	415.017	50,509.087	20	2,525.4544	94.4261	< 0.0001
A	−5.3185	1,225.6110	1	1,225.6110	45.8253	< 0.0001
B	5.9739	1,546.2800	1	1,546.2800	57.8150	< 0.0001
C	−1.8620	150.2228	1	150.2228	5.6168	0.0247
D	3.2088	446.1315	1	446.1315	16.6807	0.0003
E	6.3195	1,730.4007	1	1,730.4007	64.6992	< 0.0001
AB	10.8150	3,742.8552	1	3,742.8552	139.9444	< 0.0001
AC	9.1888	2,701.8601	1	2,701.8601	101.0219	< 0.0001
AD	−3.0288	293.5465	1	293.5465	10.9756	0.0025
AE	−0.6025	11.6162	1	11.6162	0.4343	0.5151
BC	3.0688	301.3513	1	301.3513	11.2674	0.0022
BD	0.3550	4.0328	1	4.0328	0.1508	0.7006
BE	8.2863	2,197.1821	1	2,197.1821	82.1521	< 0.0001
CD	4.9113	771.8520	1	771.8520	28.8594	< 0.0001
CE	1.2613	50.9040	1	50.9040	1.9033	0.1783
DE	−4.2363	574.2661	1	574.2661	21.4717	< 0.0001
A^2	−15.0889	12,679.284	1	12,679.284	474.0753	< 0.0001
B^2	−12.4116	8,579.0319	1	8,579.0319	320.7678	< 0.0001
C^2	−15.8189	13,935.808	1	13,935.808	521.0563	< 0.0001
D^2	−9.7979	5,346.2539	1	5,346.2539	199.8951	< 0.0001
E^2	−14.4277	11,592.494	1	11,592.494	433.4404	< 0.0001
Residual		775.6137	29	26.7453		
Lack of fit		753.7629	22	34.2620	10.9760	0.0016
Pure error		21.8508	7	3.1215		
Corr total		51,284.701	49			

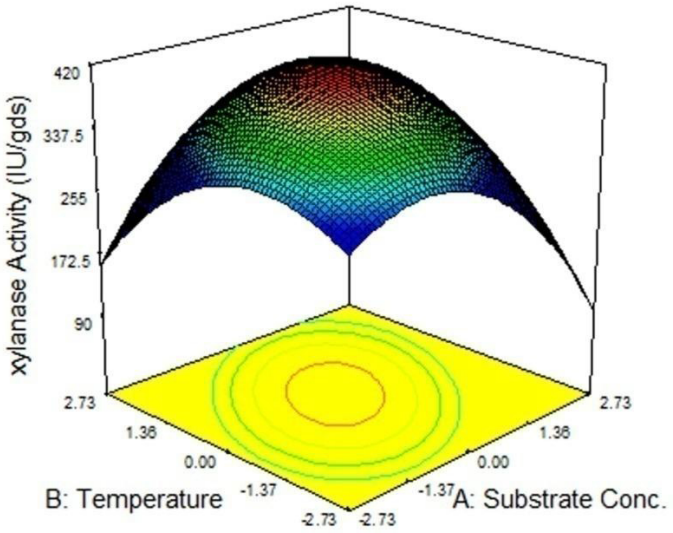

Figure 1: Three-dimensional simultaneous effect plot of substrate concentration and temperature.

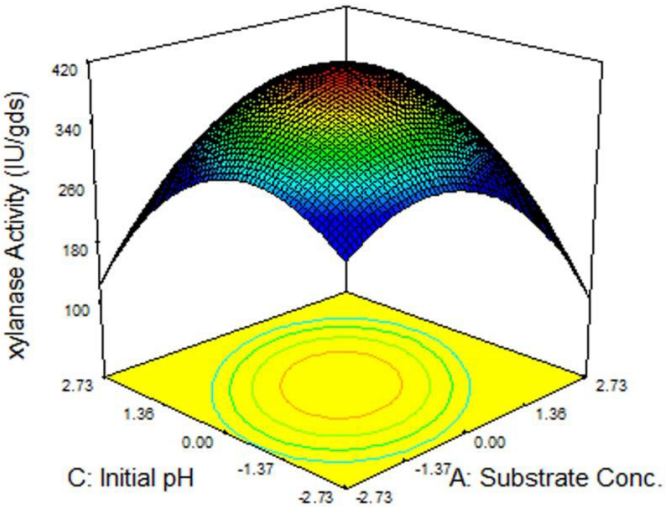

Figure 2: Three-dimensional simultaneous effect plot of substrate concentration and initial pH.

substrate concentration increases, and reaches maximum activity at 9.88 g of substrate. Thereafter, the xylanase activity decreases. This may be owing to the reality that elevated concentrations of substrate leads to an increase in stickiness of the medium, which affects the constituents present in the medium and the transportation of oxygen to the cells through substrate gets decreased due to poor aeration rate [19]. This is evident from Figures 2–4.

The consequence of temperature on xylanase activity is investigated by carrying out experiments at different temperatures

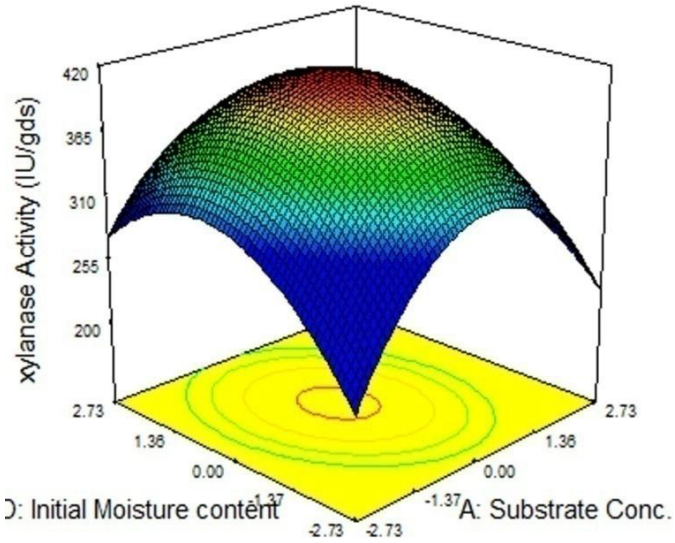

Figure 3: Three-dimensional simultaneous effect plot of substrate concentration and Initial moisture content.

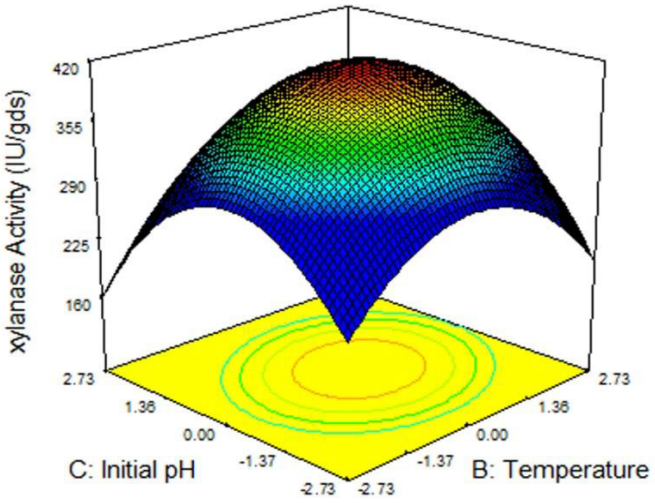

Figure 5: Three-dimensional simultaneous effect plot of temperature and initial pH.

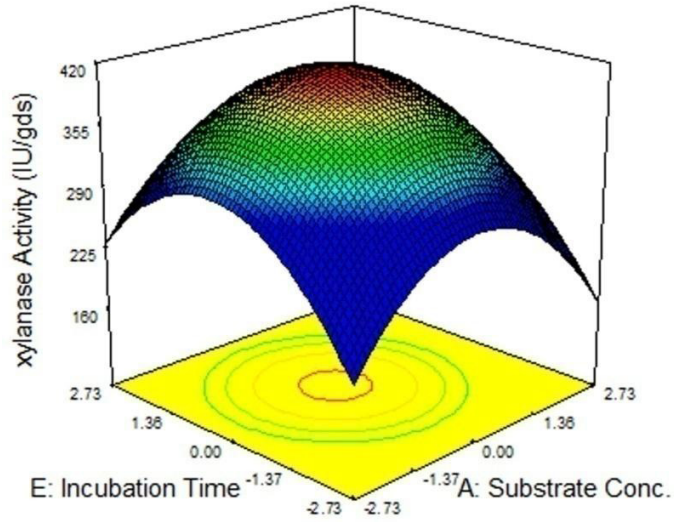

Figure 4: Three-dimensional simultaneous effect plot of substrate concentration and incubation time.

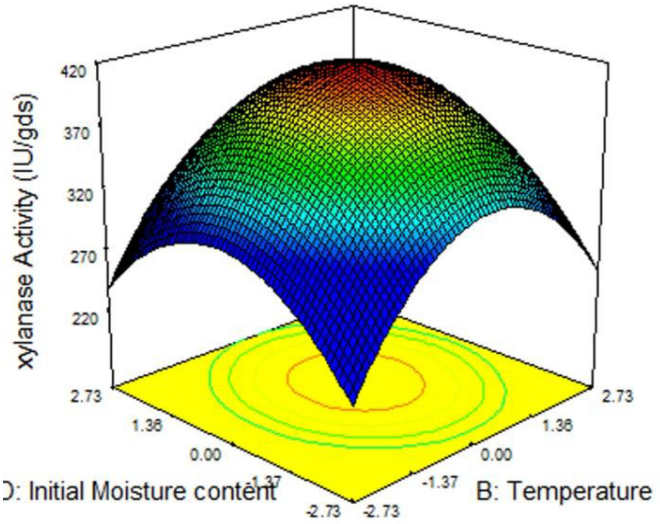

Figure 6: Three-dimensional simultaneous effect plot of temperature and initial moisture content.

starting from 29.1°C to 40.9°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 29.1°C to 40.9°C. The temperature perhaps increases the secretion of the enzyme by altering the properties of the cell wall of the fungi [20]. The xylanase activity decreased considerably even for slight increase in the temperature from 35.73°C.

The consequence of initial pH on xylanase activity is studied by conducting experiments from pH 2.7 to 7.3, and the results are shown in Figures 2, 5, 8, and 9. The maximum xylanase activity was obtained at pH 4.98. This indicates that *A. fumigatus* was resistant to acidic operating conditions. At this pH value, high xylanase activities were obtained when compared to that for other pH values which also confirms a usual profile of an acidophilic enzyme [21]. However, the xylanase activity decreases when the pH is raised above 4.98. Cultivation of the microorganism at adverse pH restricts the growth of fungi, xylanase production, and accessibility of the substrate.

The consequence of the initial moisture content on xylanase activity is studied by carrying out experiments ranging from 58.2% to 81.8%. The results are revealed in Figures 3, 6, 8, and 10. The enzyme activity was found to increase as initial moisture content is elevated from 71.2% to 76.1%, and it was found to decrease with further increase in moisture percentage of the medium. The maximum activity of 415.45 IU/gds xylanase activity was attained with the initial moisture content of 71.30%. The decrease in the enzyme activity for initial moisture content greater than 71.30% might be due to the fact that higher wetness intensity time and again

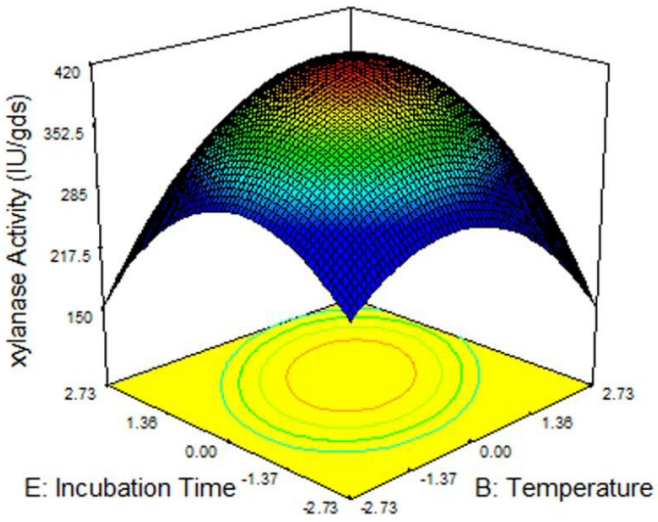

Figure 7: Three-dimensional simultaneous effect plot of temperature and incubation time.

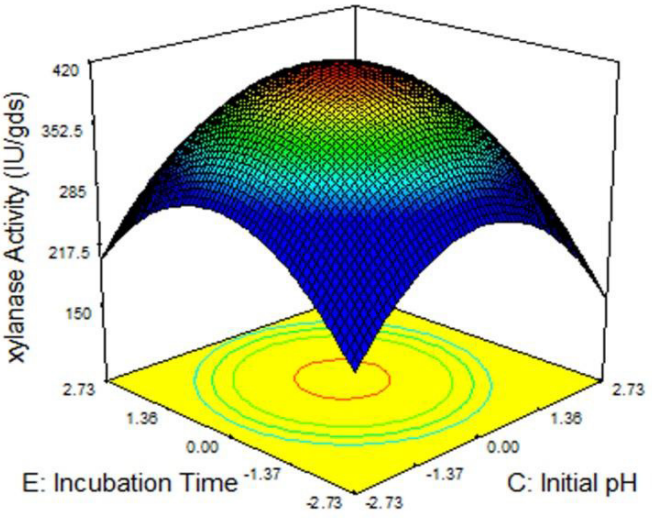

Figure 9: Three-dimensional simultaneous effect plot of pH and incubation time.

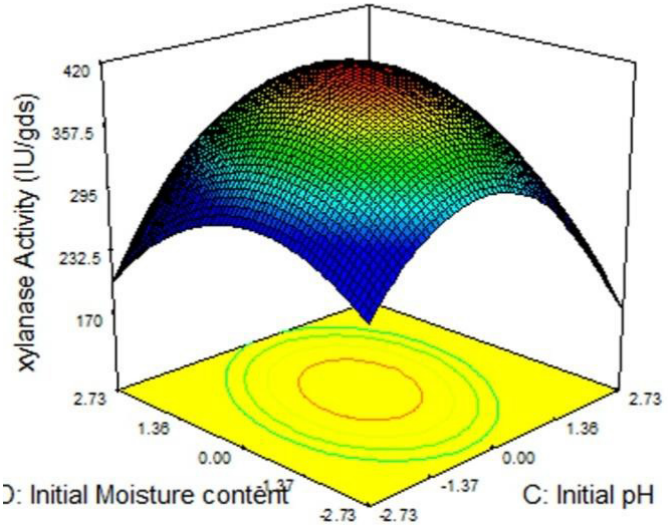

Figure 8: Three-dimensional simultaneous effect plot of initial pH and initial moisture content.

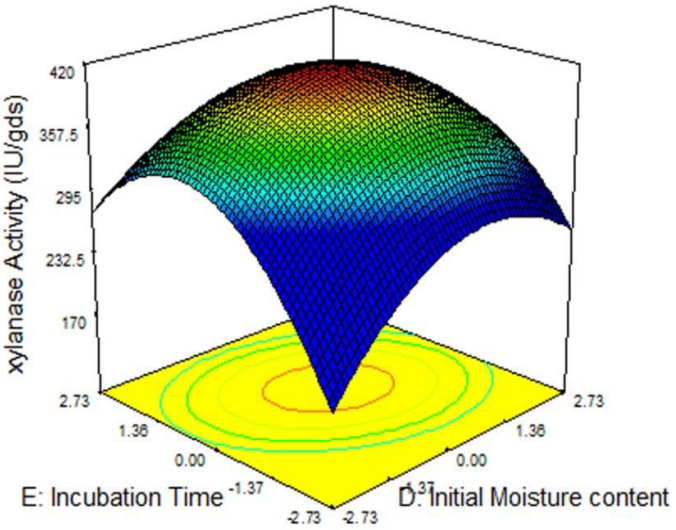

Figure 10: Three-dimensional simultaneous effect plot of initial moisture content and incubation time.

guides to constituents of sugarcane bagasse attaching together to the flask which reduced porosity of substrate and preventing the transfer of oxygen into the substrate as a barrier. Moreover, high moisture occupies the spaces between the particles not allowing oxygen and formulates the medium to be further susceptible to bacterial infection [22].

The consequence of incubation time on xylanase activity is investigated by conducting experiments with incubation period ranging from 108.1 to 131.9 hours, which are shown in the Figures 4, 7, 9, and 10. The maximum xylanase of 415.45 IU/gds is obtained with the incubation period of 120.05 hours. Additional increase in incubation time results in the decline of xylanase activity. This might be due to the decrease in the exhaustion of the constituents present in the medium that leads to decreased growth of the cells as well as enzyme production. With prolonged incubation, the decrease in enzyme activity could be appropriate to the fact that xylanases are frequently articulated at the closing stages of the exponential phase and yielding period is related to the medium under consideration [23].

Optimum conditions are the lone points from where the highest xylanase activity is achieved. By solving Eq. (3) using RSM, the most favorable situation for production of xylanase enzyme is acquired. The most favorable experimental values of the tested variables for highest xylanase activity are substrate concentration = 9.88 g, temperature = 35.73°C, pH = 4.98, initial moisture content = 71.30%, and incubation time = 120.05 hours. The most favorable values for the parameters as predicted are within the design region. Figure 11 confirms the experimental values be in

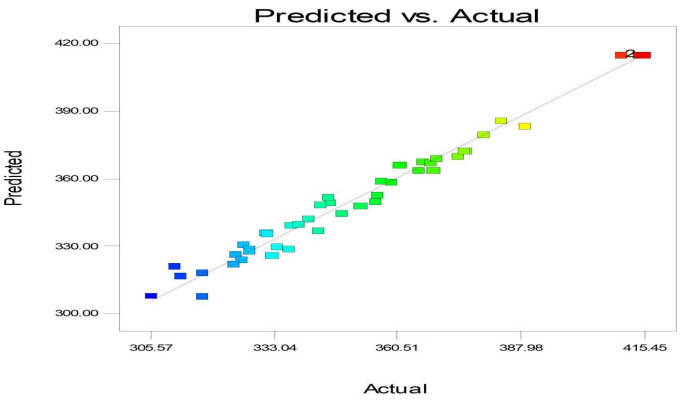

Figure 11: Predicted versus experimental xylanase activity.

agreement with the predicted values. Very poor CMCase activity is also found along with xylanase production.

Using *Aspergillus* sp. under SSF, the optimum values for substrate concentration, temperature, initial pH, initial moisture content, and incubation time for maximum xylanase activity as reported by previous studies [24–28] were 10 g, 35°C°, 3.5–5.5, 70%–72%, and 100–120 hours, respectively, which are in excellent agreement with the present research data also.

3.1. Model Validity

Validity of the model is checked by conducting the group experimentation under more favorable operating circumstances. After conducting three repetitive experiments, the xylanase activities are compared. The experimental xylanase activity is found to be 417.501 IU/gds, which is very close to that of 417.121 IU/gds, predicted by the regression model. Also it confirms the model validity.

4. CONCLUSION

The optimization of xylanase production using inexpensive agricultural waste under cheaper SSF technology by RSM was investigated in the current research. The usage of sugarcane bagasse as low-cost agro-wastes brought down the xylanase enzyme production cost and also reduces environmental pollution. To formulate xylanase production, further cost-effective, experimental parameters optimization were carried out through RSM. Enhanced xylanase activity of 417.501 IU/gds was obtained with the following optimum process parameters: substrate concentration = 9.88 g, temperature = 35.73°C, initial pH = 4.98, initial moisture content = 71.31%, and incubation time = 120.05 hours. It is concluded that SSF technique using inexpensive agro-waste (sugarcane bagasse) as substrate was more effective, as well as economical, for production of xylanase by *A. fumigatus*.

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CONFLICTS OF INTEREST

The authors state that they do not have any conflicts of interest.

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