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OPTIMIZATION OF PROCESS PARAMETERS FOR CELLULASE AND XYLANASE PRODUCTION USING RICE HUSK

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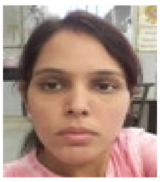
ABSTARCT

The potential of *Trichoderma atroviride* for cellulase and xylanase production using Rice husk was carried out by Response Surface Methodology and Box-Behnken design. The experiment established the optimum conditions of incubation time (5.5 days), temperature (32.5^{0}C), pH (5.5), spore suspension (1.75ml) for solid state fermentation that led to the maximum production of cellulase and xylanase at a level of 90.43 IU/gds, 10.12 IU/gds. Good correlation was observed between the actual and predicted results indicated that the present model was applicable production of cellulase and xylanase.

KEYWORDS: Rice husk, Solid State fermentation, Box-Behnken design, *Trichoderma atroviride*, ANOVA.



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INTRODUCTION

In the planet earth most of the freely available energy rich resources are agricultural residues (wheat bran, rice bran, sugarcane bagasse, corncobs etc.). There has been a vast expansion in agro-industrial field in recent years which makes the sustainable increase in the volume of agricultural residues and if not used or discharged properly, they cause the environmental pollution. Globally 998 tonnes of agricultural waste was produced in a year. In India, about 350 million tonnes of organic waste produced from agricultural sources. Their biotechnological conversion is met only a remedy for environmental problem, but also a source of suitable microbial by-product likes food, fuel and chemicals, enzymes[1]. Rice husk is one example of alternative material that has a great potential as a lignocellulosic material. Rice husk, an abundant agricultural waste produced at a rate of 1.2×10^{8} tonnes/year. Rice husk composed of cellulose, hemicelluloses and lignin. Cellulose is a linear polymer that is composed of glucose subunits linked by β-1,4glycosidic bonds. Hemicellulose is a polysaccharide with a lower molecular weight than cellulose. It contains xylose, mannose, galactose, glucose, arabinose and glucuronic acids, and are linked together by β-1,4 and sometimes by β-1,3-glycosidic bonds[2]. Lignin is physical seal of hemicellulose and cellulose, which is an impenetrable barrier in the plant cell wall[3]. Rice husk contain 35.62% cellulose, 11.96% hemicellulose, 15.39% lignin[4]. Agro-residues are generally considered as suitable substrate for the production of enzymes, especially cellulase, pectinases and xylanase, in solid state fermentation[5]. Solid state fermentation (SSF) offers numerous opportunities in processing of agroindustrial residues. Solid state fermentation (SSF) is identified as a fermentation process occurring in the absence or near absence of free water by employing a natural solid substrate[6]. Many microorganisms are capable of growing on solid substrates such as filamentous fungi, which can grow to a significant extent in the absence of free water[7]. Optimization of process conditions is one of the most critical stages in the development of an efficient and economic bioprocess. The classical method of studying on variable at a time can be effective in some cases, but it is useful consider the combined effects of the entire factor involved[8]. The conventional one-factor-at-a-time approach of optimization is not only tiresome but also ignores to merge interaction of each factor. One of the most common optimization used in the last two decades is the Response Surface Methodology (RSM). RSM is a powerful mathematical model with a collection of statistical techniques by which interaction between multiple processes variables can be identified with fewer experimental trials [9]. It is widely used to examine and optimize the operational variables for experimental design, model developing, and test variable and condition optimization [10]. The aim of this work was to optimize conditions for higher production of cellulase & xylanase under Solid state fermentation (SSF) using statistical methods. The optimum parameters including Incubation time, temperature, pH and spore suspension as cultural variables in the medium were obtained by RSM.

MATERIALS AND METHODS

Preparation of substrate

Rice husk were collected from Rice milling industries and processed to particle size of 1mm and washed with distilled water and dried at 60^{0}C for 8 h in the oven. Processed substrates were collected in the polythene covers and stored at room temperature.

Microorganisms

The microorganism *Trichoderma atroviride* was procured from Institute of microbial Technology (IMTECH) Chandigarh. The strain was well preserved in our laboratory. A spore suspension was obtained for each organism by growing them on potato dextrose agar at 30^{0}C for one week and harvesting the spores with sterile water containing 0.1% Tween-80.

Medium and culture conditions

Erlenmeyer conical flasks (250ml) containing rice husk 5g and Mendel's media (10ml) of following composition (g/L) $(NH_4)_2SO_4$ (1.4gm), KH_2PO_4 (2gm), $CaCl_2.2H_2O$ (0.4gm), $MgSO_4.7H_2O$ (0.6gm), Urea (0.3gm), Protease peptone (0.75gm) , Yeast extract (10g), $COCl_2.6H_2O$ (3.7mg), $FeSO_4.7H_2O$ (5mg), $MnSO_4.7H_2O$ (1mg), Zinc Sulphate (1.4gm), Tween 80 (2ml) were sterilized at

121^{0}C for 20 min. After cooling, each flask was seeded with the inoculums concentration (0.5-3ml), initial pH (3-8), temperature (25-40^{0}C), incubation period (3-8 days) defined by the experimental design and in a shaker at 200 rpm for throughout the incubation periods. Thereafter, the enzyme was extracted by adding 50ml of 50mM citrate buffer pH 4.8 under shaking at 200 rpm on a rotary shaker and at 30^{0}C for 60min. The resultant slurry was filtered through a muslin cloth and centrifuged at 10000 rpm for 15min at 4^{0}C, and the supernatant was used as a crude enzyme source for Cellulase and Xylanase assay.

Enzyme assay

The fortitude of Cellulase and Xylanase activity was examined according to the method of Ghose[11]. In test tubes, 0.5ml of 1% CMC & 0.5 ml of 1% xylan was added with 0.5ml diluted enzyme was incubated at 50^{0}C for 30min. The reaction was terminated by addition of 2ml DNSA (Dinitrosalicyclic acid) reagent and tubes were kept at boiling water bath for 5min. After cooling the tubes at room temperature, 10ml distilled water was added in each tube. The intensity of the colour was read at 540nm in UV-VIS spectrophotometer. Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1μ mol of glucose per minute under assay condition.

Experimental design

The experimental design and statistical analysis were performed on Box-Behnken design with quadratic model was employed to study the combined effect of four independent variables namely incubation period (A, days), Temperature (B, ^{0}C), pH (C), spore suspension (D, ml) for the dependent variables such as cellulase and xylanase (U/gds) production using Design-Expert software (version 9, Stat-Ease Inc., Minneapolis, USA). A total no. of 29 experimental runs given by this design. The dependent variables were expressed individually as a function of the independent variable known as response function.

This design is interpreted by a second-order polynomial regression model as follows

$$Y = \beta_0 + \sum \beta_i A_i + \sum \beta_{ii} A_i^2 + \sum \beta_{ij} A_i A_j$$

Where, Y is the measured response (cellulase or xylanase yield), β_0 the constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the cross product coefficient Ai and Aj are the levels of the independent variable.

Data analysis

Regression analysis and estimation of the coefficients were performed using Design-Expert software (version 9, Stat-Ease Inc., Minneapolis, USA). The quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by F-test.

RESULTS AND DISCUSSION

Optimization of Cellulase and Xylanase production by *Trichoderma atroviride* using response surface methodology RSM based on the Box-Wilson, which was used to optimize different parameters for cellulase and xylanase production, 29 experimental runs with different combinations of four factors were carried out. The variables such as incubation time, Temperature, pH, spore suspension that influence cellulase & xylanase production in solid state fermentation (SSF) using RSM (Table1). The effect of each factor and their interaction were analyzed using the analysis of variance (ANOVA) (Table2).The calculated regression equation for the optimization of parameters for production of cellulase i.e. CMCase (Y_1, U/gds) and Xylanase (Y_2, U/gds) is a function of the incubation period (A, days), temperature (B, ^{0}C), pH (C), spore suspension (D, ml). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the cellulase and xylanase production as shown below:

Cellulase (U/gds)

$$Y_1 = 85.86 - 0.96A - 0.55B - 1.19C + 1.23D + 2.62AB + 0.16AC - 2.51AD + 9.30\,BC - 0.39BD + 2.12\,CD - 15.27A^2 - 13.76B^2 - 14.41C^2 - 11.75D^2 \quad \text{Equation (1)}$$

Xylanase (U/gds)

$$Y_2 = 9.09 - 0.57A - 0.54B - 0.73C - 0.30\,D - 0.33AB + 0.72\,AC + 1.00\,AD + 0.50BC - 0.16BD + 1.08CD - 3.06A^2 - 1.53B^2 - 2.35C^2 - 1.66D^2 \quad \text{Equation (2)}$$

The predicted levels of cellulase and xylanase production in rice husk substrate using the above equations are given in Table 1 along with the experimental data. Several indicators were used to evaluate the adequacy of the fitted model i.e., Determination coefficient (R^2), Coefficient of variation (CV), Model significance (F-value). The quadratic regression model illustrate that Equation 1 and 2 are highly significant statistical models for Cellulase and Xylanase production responses in rice husk, as it was evident from the Fisher's F-test with a very low probability value [(P model >F)(Table 2).

Table 1

Result of BBD showing observed and predicted response for Cellulase and Xylanase

Run	Factor A Incubation Time	Factor B Temperature	Factor C pH	Factor D Spore suspension	Response 1 Cellulase	Predicted value Cellulase	Response 2 Xylanase	Predicted value Xylanase
	(Days)	(0C)		(ml)	(IU/gds)	(IU/gds)	(IU/gds)	(IU/gds)
1	3	25	5.5	1.75	62.26	60.94	7.12	5.29
2	8	32.5	3	1.75	57.34	56.25	3.34	3.12
3	5.5	32.5	5.5	1.75	90.43	85.86	10.12	9.09
4	5.5	25	5.5	3	70.23	62.51	8	6.29
5	5.5	32.5	5.5	1.75	87.23	85.86	9.12	9.09
6	5.5	40	8	1.75	62.32	65.24	4.34	4.44
7	5.5	32.5	8	3	64.43	61.85	5.55	5.13
8	5.5	32.5	3	3	53.23	60	3.99	4.42
9	8	32.5	8	1.75	50.21	54.19	4.23	3.1
10	3	32.5	8	1.75	56.67	55.77	2.34	2.81
11	3	32.5	5.5	3	60.65	63.53	2.56	3.65
12	5.5	25	3	1.75	63.37	68.72	6.33	6.97
13	8	25	5.5	1.75	58.34	53.79	3.99	4.79
14	5.5	40	5.5	3	61.23	60.63	4.34	4.9
15	5.5	25	8	1.75	38.11	47.74	2.11	4.52
16	5.5	32.5	8	0.5	68.21	55.16	4.99	3.57
17	5.5	32.5	5.5	1.75	86.12	85.86	8.23	9.09
18	5.5	32.5	5.5	1.75	89.95	86.86	8	9.09
19	5.5	32.5	3	0.5	65.48	61.78	7.77	7.2
20	5.5	40	5.5	0.5	53.23	58.96	3.88	5.83
21	3	40	5.5	1.75	56.34	54.61	6.66	4.87
22	5.5	40	3	1.75	50.4	49.03	6.56	4.9
23	3	32.5	5.5	0.5	49.03	56.05	5.55	6.25
24	3	32.5	3	1.75	64.46	58.49	4.32	5.7
25	8	32.5	5.5	3	55.35	56.59	4.44	4.49
26	8	40	5.5	1.75	62.89	57.93	2.23	3.07
27	5.5	32.5	5.5	1.75	75.56	85.86	9.99	9.09
28	5.5	25	5.5	0.5	60.67	59.28	6.89	6.58
29	8	32.5	5.5	0.5	53.78	59.16	3.45	3.11

Table 2

ANOVA for the experiment

Term	Response Y1, Cellulase	Response Y2, Xylanase
F-value	4.59	3.23
P>F	0.0037	0.0179
R^2	0.822	0.76
Mean	63.02	5.53
Adjusted R^2	0.642	0.527
Adequate precision	7.01	5.468
Coefficient of variance(%)	12	28.89
Lack of fit	30.05	12.68

The p-value is a tool for evaluating the significance and contribution of each parameter. Model term having values of Prob > F less than 0.05 are considered significant, whereas those greater than 0.10 are insignificant[12]. ANOVA indicated the R^2-value of 0.821, 0.763 respectively, for responses Y_1, Y_2. For good statistical model, the R^2 value should be in the range of 0-1.0, and the values are obtained in the data analysis indicates the model is good. This again ensured a satisfactory adjustment of the quadratic model to the experimental data, and indicated that the model could explain 85-90% of the variability in the response. The coefficient of variation (CV) indicates the degree of precision with which the experiments are compared. Generally, the higher the value of the CV is, the lower the reliability of the experiment. Here the value of coefficient of variation is 12, 28.89 for responses Y_1, Y_2 indicate good reliability of the experiment performed. The adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The adequate precision was 7.01, 5.46 for Y_1, Y_2 responses that indicates an adequate signal [13]. The lack of fit, F-value of 30.05%, 12.68% for responses Y_1, Y_2 which is the ratio of mean square due to regression to the mean square due to error and indicates the influence of each controlled factor on tested model, was significant at high confidence level. Non significant lack of fit is good. (Fig.1, 2) shows that the actual response values agree well with the predicted response values of Cellulase & xylanase. The 3D response surface plot described the regression model was drawn to illustrate the combined effects of the independent variables and combined effects of each independent variable upon the response variable. The optimum conditions for maximum production of enzyme production was determined by response surface analysis and also estimated by the regression equation. The optimum conditions are namely: Incubation time (5.5 days), Temperature (32.5°), pH (5.5) and spore suspension (1.75 ml). The optimal values for variables as predicted are found to be within design region. This shows that the model correctly explains the influence of the chosen variables on enzyme production. The response surface curves were plotted to understand the interaction of the variables and to determine the optimum levels of each variables for maximum response (fig 3-4). Validation of the model: The suitability of the model equation for predicting the optimum response values was tested using the optimum conditions mentioned above. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to experimentally validate and predict the value of the responses using model equations. The experimental values were found to be in accord with the predicted ones (Table 1). The cellulase and xylanase activity reached 87.23 IU/gds for cellulase and 8.89 IU/gds for xylanase under the optimal conditions.

Figure 1
Predicted VS Actual values for Cellulase

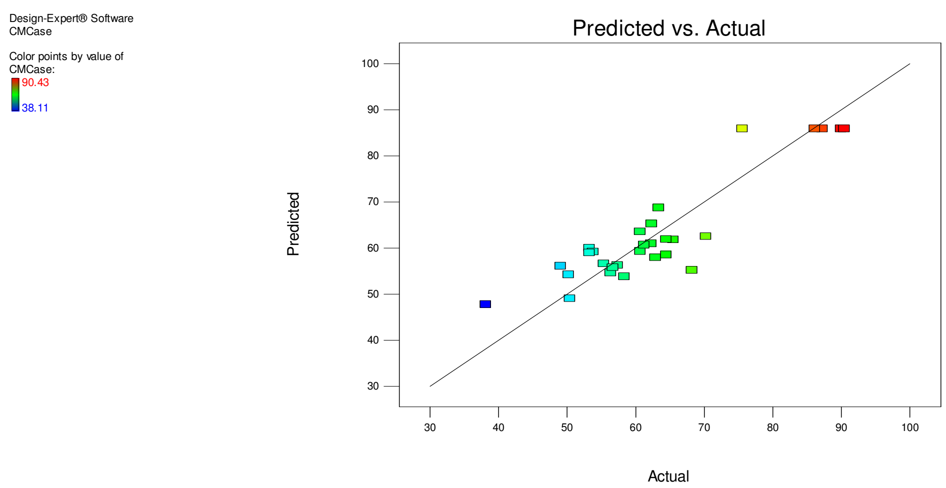
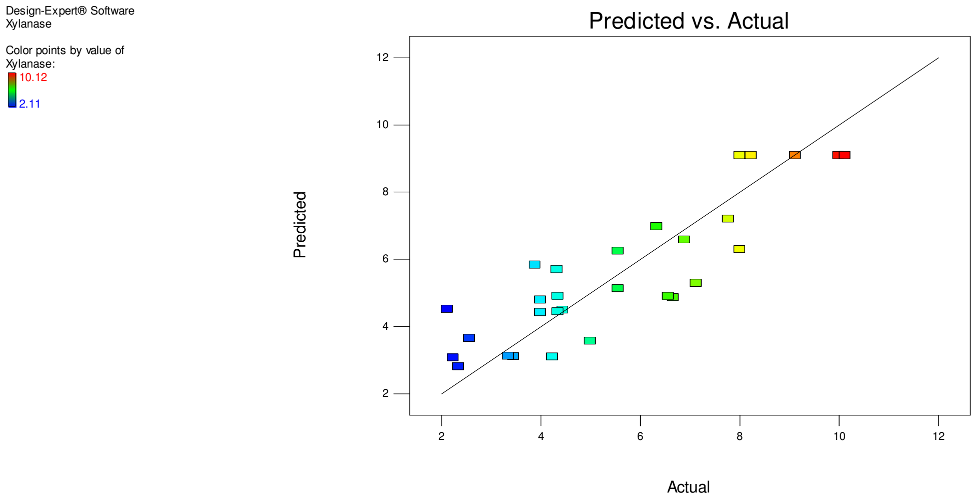


Figure 2
Predicted VS Actual values for Xylanase



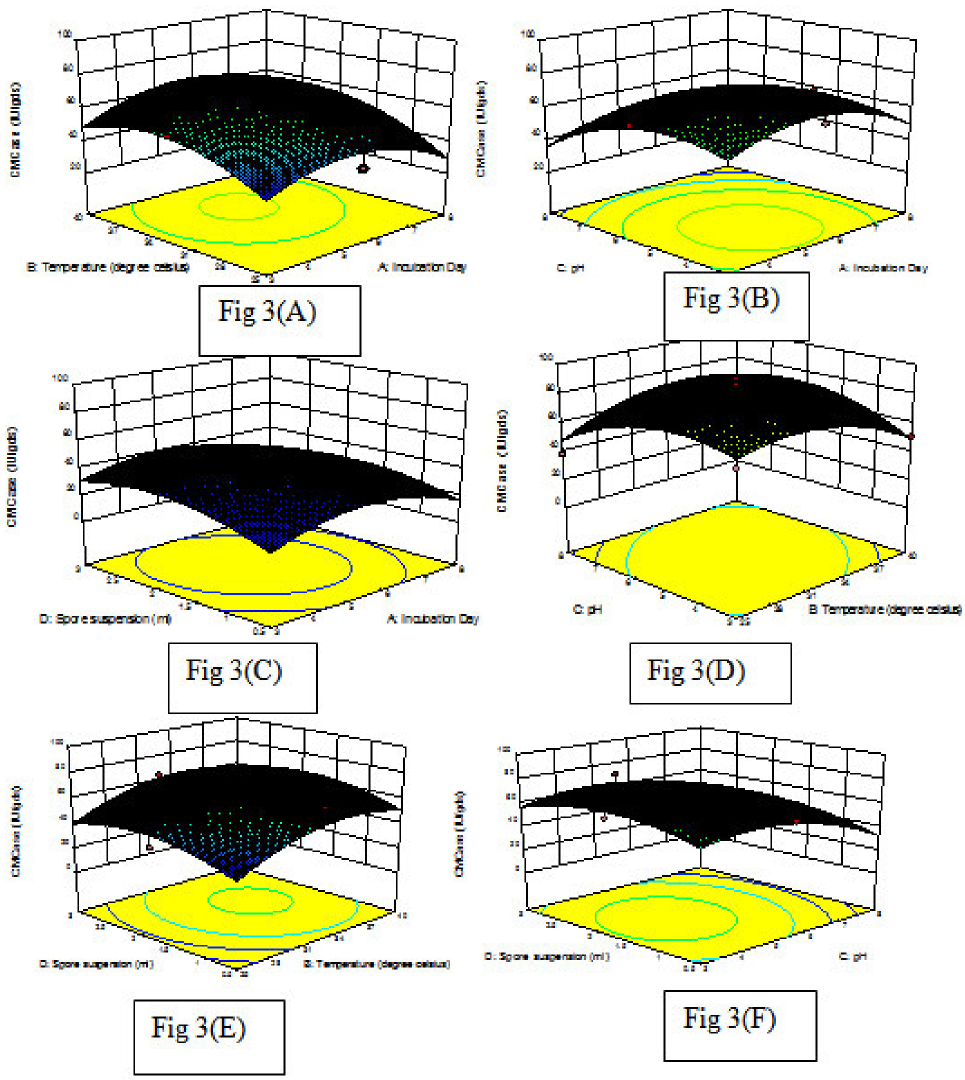


Figure 3(A-F)
Three dimensional response plots for Cellulase activity showing interactive effects of variables

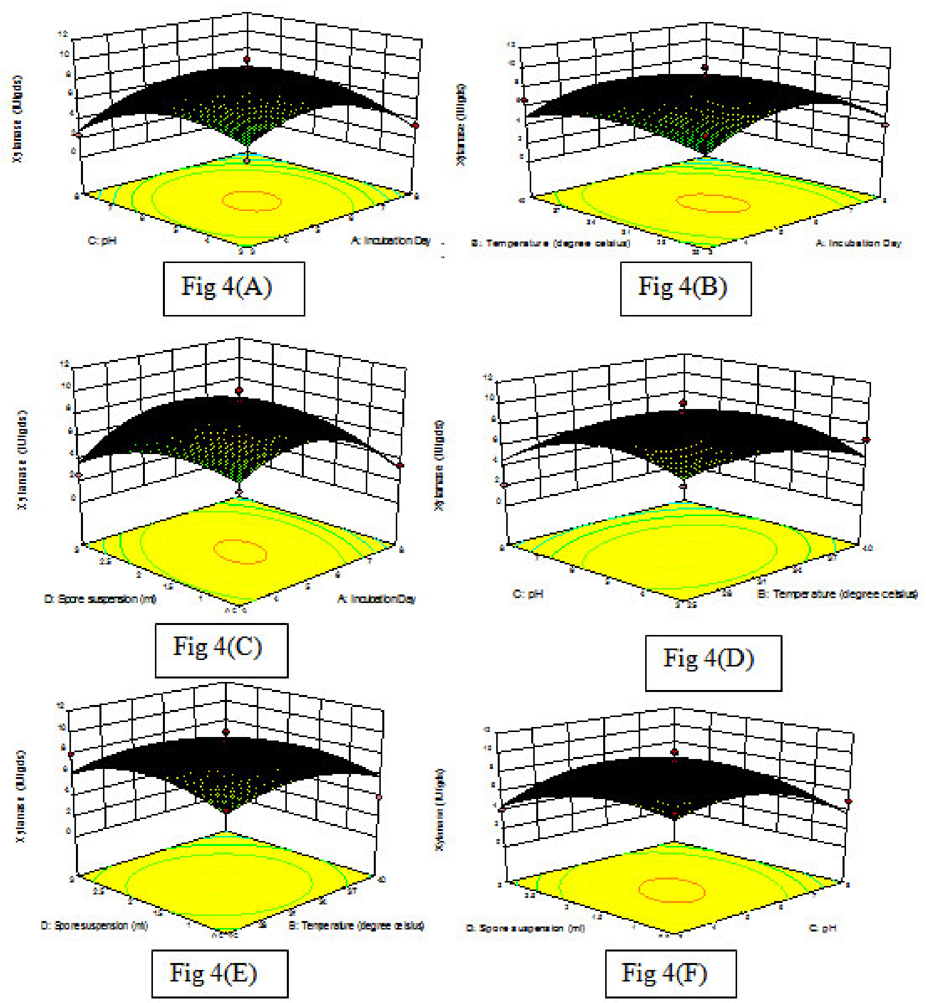


Figure 4 (A-F)
Three dimensional response plots for Xylanase activity showing interactive effects of variables

CONCLUSION

In the present work, the applied response surface methodology (RSM) proved to be efficient in optimizing process parameters for cellulase and xylanase production using a very cheap substrate rice husk. From the optimization studies, the optimum experimental conditions are incubation period (5.5 days), temperature (32.5^{0}C), pH (5.5) and spore suspension (1.75ml). Using the optimized conditions the maximum cellulase and xylanase production of 90.43 IU/gds, 10.12 IU/gds was obtained. Comparison of predicted and experimental values revealed good correspondence between them, implying that Box-Behnken models derived from RSM can be used to adequately describe the relationship between the dependent and independent variables in cellulase and xylanase production.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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