Cellulase and Xylanase Production from Rice Straw by a Locally Isolated Fungus *Aspergillus fumigatus* NITDGPKA3 under Solid State Fermentation – Statistical Optimization by Response Surface Methodology

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Abstract: Alkali pretreated rice straw was used as substrate for cellulase production by a locally isolated fungus *Aspergillus fumigatus* NITDGPKA3 under solid state fermentation. Critical process parameters such as incubation period, temperature, basal medium content and pH were statistically optimized for an enhanced cellulase and xylanase yield by response surface methodology. The design predicted an optimum yield of 3.1 IU/g dry substrate, 64.18 IU/g dry substrate and 1040.57 IU/g dry substrate for FPase, CMCase and xylanase respectively under the optimum conditions of incubation period of 90 h, temperature at 33^{o}C, initial basal medium content of 62% and initial pH 4. The experimental values under optimum conditions correlated well with the predicted results. Further, crude enzyme extract from *Aspergillus fumigatus* NITDGPKA3 was used for saccharification of pretreated rice straw and this released 189.50 mg/g of reducing sugar. This work was carried out in the Department of Biotechnology, National Institute of Technology, Durgapur-713209, West Bengal, India, during the period 2010 to 2011.

Keywords: Cellulase, xylanase, solid state fermentation, statistical optimization.

INTRODUCTION

Cellulose is the only renewable carbon source available in large quantities and has no food and feed value. Wide spread attention has been placed on lignocelluloses for the utilization of its fermentable sugars in chemical, food and energy production. Lignocellulosics include agricultural waste, forest waste, municipal solid waste, fruit and vegetable waste, wastes from the pulp and paper industry, as well as herbaceous energy crops [1].

Among the agricultural wastes, rice straw is the most abundant agricultural waste all over the world. Although rice straw contains materials for social benefit, their apparent value is less than the cost of collection, transportation and processing for beneficial use. Usually rice straw is removed from the field at harvest time and is subjected to open field burning. Though straw burning is still practiced in many countries, it is increasingly becoming unacceptable due to environmental and health problems. If the wastes can be utilized for food production, they are no longer wastes but become new resources. With increase in crop yields the management of rice straw is becoming a problem as well as an opportunity for reutilization. It can be used as feedstock for enzyme as well as monosaccharide production.

Acid and enzymatic treatments are two commonly used procedures for production of soluble and low molecular weight hexoses and pentoses. Unlike acid hydrolysis, enzymatic treatment produces no inhibitory compounds and it avoids toxic and corrosive chemicals, high energy input and severe pollution. Cellulase basically hydrolyses the β-1,4-glucosidic linkages of cellulose and produces primary products such as glucose, cellobiose and cello-oligosaccharides. Cellulase is the most extensively studied multiple enzyme complex comprising of endo-glucanases (EG), cellobiohydrolases (CBH) and β -glucosidases (BGL). Endo-glucanases produce nicks in the cellulose polymer exposing reducing and non-reducing ends, cellobiohydrolases acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose units, and β - glucosidases cleave the cellobiose to liberate glucose, the final product of hydrolysis [2].

High market price impedes the large scale application of cellulase enzyme. Hence it is necessary to improve the enzyme yield to make the process economically attractive [3].

Cellulase is generally produced by cellulolytic fungi either in submerged fermentation or in solid state fermentation. Although submerged fermentation is amenable to high levels of process control and monitoring, the method can be complex. Solid state fermentation can be simpler and less energy intensive. In solid state fermentation, the hyphal growth mode of

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the employed filamentous fungi gives them an edge over bacteria which cannot easily access the cellulose inside the substrate in the absence of free water and thus cannot easily contaminate the culture [4, 5]. A crude unprocessed cellulase can be obtained from solid-state fermentation either by using the fermented substrate (koji) directly or by mixing the fermented substrate with water.

Multifactorial system or one-factor at a time is the traditional technique for the optimization of process parameters. However, this type of method is time-consuming and also does not represent the interactive effects between components. In the study presented here, an attempt was made to employ response surface methodology (RSM) to identify the optimum conditions for cellulase production under solid state fermentation by analyzing the relationships among a number of parameters that affect the overall process. Moreover the secreted cellulase was applied for the saccharification of alkali pretreated rice straw.

MATERIALS AND METHODS

Microorganism and Maintenance Medium

Locally isolated fungus *Aspergillus fumigatus* NITDGPKA3 (Genebank accession number JQ046374) was used for cellulase production. The culture was maintained on Czapek modified agar slants at 4^{o}C and subcultured fortnightly.

Preparation of Rice Straw [6]

Locally procured rice straw was washed, air dried and size fractioned to 0.5mm. It was then pretreated with 0.5 M NaOH at 121^{o}C and 15 psi pressure for 1 hr at the ratio of 1:10 (substrate : NaOH solution). The pretreated rice straw was washed with tap water until the pH of the filtrate reached 7.The solid undigested substrate was dried at 60^{o}C overnight and stored at room temperature for cellulase production and enzymatic saccharification. The pretreated rice straw had the following composition: (cellulose 62.19%, hemicellulose 14.5%, lignin 8.4%, and ash 14.54%).

Inoculum Preparation

The fungal culture was subcultured on Czapek modified agar medium (CMM) and incubated at 30^{o}C. Fully sporulated plates were obtained after 6 days. The sporulated plates were flooded with 20 ml of distilled water containing 0.1% Tween 80. Spores were dislodged by gentle pipetting.

Solid State Fermentation for Enzyme Production

Five grams of rice straw (pretreated) was weighed into 250 ml Erlenmeyer flasks and was moistened with basal medium which had the following composition : 0.2% $NaNO_3$, 0.05% KCl, 0.05% $MgSO_4$, 0.001% $FeSO_4$, 0.1% K_2HPO_4, 0.2% peptone. The flasks were autoclaved at 121^{o}C for 15 minutes at 15 psi pressure. After cooling, the flasks were inoculated with 5 ml suspension per 100 g of dry weight of substrate containing 10^6 spores per ml. The contents were mixed thoroughly and incubated at 30^{o}C.

Analytical Methods

Enzyme Extraction

A known amount of solid fermented matter (0.5 g) was mixed with 5 ml of 0.05 M citrate buffer, pH 4.8 and was shaken at 120 rpm for 2 hours. The mixture was filtered through muslin cloth to obtain filtrate. The filtrate was centrifuged at 8000 rpm for 15 minutes at 4^{o}C. The solid residue was discarded and the supernatant was used for enzyme activity.

Enzyme Assay

Enzyme activities were assayed by estimating total reducing sugar released. Enzyme activity was measured by the methods of International Union of Pure and Applied Chemistry (IUPAC) Commission on Biotechnology [7]. CMCase and xylanase activities were determined using 2 % (w/v) carboxymethyl cellulose (Himedia, India) and 2% (w/v) oat spelt xylan (Himedia, India) solution prepared in 0.05 M, pH 4.8 Na-citrate buffer respectively. The reaction mixture, containing suitably diluted enzyme solution (0.5 ml) and 0.5 ml of substrate solution was incubated at 50^{o}C for 30 minutes and for 10 minutes for CMCase and xylanase respectively. To determine FPase activity 0.5 ml of enzyme solution was incubated with 1 ml of 0.05 M , pH 4.8 sodium citrate buffer containing 1cm X 6cm (=50 mg) Whatman filter paper strip at 50^{o}C for 60 minutes.

In all the cases, after incubation, the released reducing sugar was estimated by the DNS method with some modifications [8]. Dinitrosalycylic acid reagent (1 ml) was added to the reaction mixture and incubated for 5 minutes in a vigorously boiling water bath. Na-K tartarate solution (1 ml) was then added to the mixtures and cooled rapidly. The reducing sugar was estimated from the absorbance measured at 540 nm using glucose (for CMCase and FPase) and xylose (for xylanase) as standards. Enzymatic activities were

defined in International Units (IU). One unit of enzymatic activity is defined as the amount of enzyme that releases 1 µmol reducing sugar/ml/minute.

Optimization of Cellulase Production

The present work involves optimization of different parameters governing cellulase production. The effects of Tween 80 and various nitrogen sources on cellulase production were examined by one factor at a time method.

Statistical Design of the Experiment

The significant parameters such as incubation period, temperature, initial pH, basal medium content were statistically optimized using RSM by central composite design (CCD). RSM is an efficient statistical technique for optimization of a complex process. RSM reduces the number of experimental trials as well as evaluates multiple parameters and their interactions. It is less laborious than other approaches. The codes and levels of the selected variables are given in Table **1**. The quadratic model as a response function was constructed according to the following equation:

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 + b_{12}AB + b_{23}BC + b_{24}BD + b_{13}AC + b_{34}CD + b_{14}AD \quad \text{Eq 1.}$$

where Y is the predicted response (dependent variable); b_0 is a constant; b_1, b_2, b_3 and b_4 are the linear coefficients; b_{12}, b_{13}, b_{23}, b_{14}, b_{24}, b_{34} are the cross product coefficients and b_{11}, b_{22}, b_{33}, b_{44} are quadratic coefficients. A, B, C, and D are the coded forms of the selected variables *viz.* temperature, initial pH, incubation period and basal medium content respectively. A total of 30 experiments including 6 centre points were generated by design of experiments using the statistical software Design Expert 7.0.0 (Stat Ease, USA).

Enzymatic Hydrolysis of Alkali Pretreated Rice Straw

Enzymatic hydrolysis of alkali pretreated rice straw was carried out in a 250 ml flask containing 50 ml reaction mixture of 2% (w/v) rice straw, crude enzyme solution and 50 mM citrate buffer (pH 4.8). Streptomycin (40µg/ml) and cycloheximide (30µg/ml) were added to the mixture to prevent microbial contamination. Crude enzyme solution contained 30 IU/g dry substrate of CMCase from *Aspergillus fumigatus* NITDGPKA3. The reaction mixture was incubated at 50°C and 120 rpm for 56 h. Samples (0.5 ml) were withdrawn at regular intervals, centrifuged at 14000 rpm for 15 minutes and the supernatant was analyzed for reducing sugars released.

RESULTS AND DISCUSSION

The effects of various nitrogen sources on cellulase and xylanase production were examined (Table **2**). Addition of tryptone led to an appreciable increase in CMCase (40.39 IU/g dry substrate), FPase (1.97 IU/g dry substrate) and xylanase (393.61 IU/g dry substrate) production compared to other nitrogen sources. Concentration of the effective nitrogen source was varied as enzyme production is also known to be sensitive to the level of nitrogen source in the medium where 0.2 % (w/v) tryptone gave maximum cellulase as well as xylanase production.

For further improvement in enzyme production the concentration of Tween 80 was varied in spore suspension. Tween 80 is a nonionic surfactant that stimulates enzyme production [9] by improving the permeability of the cell membrane [10], so that enzyme synthesized intracellularly can be secreted outside the cell more easily. In the present study 0.4% (w/v) Tween 80 in inoculum resulted in 43.44 IU CMCase/g dry substrate, 2.24 IU FPase/ g dry substrate and 681.22 IU xylanase /g dry substrate (Figure **1**). From the result it is seen that Tween 80 had a greater effect on

Table 1: Experimental Range of the Three Numerical Variables Studied Using Rotatable CCD in Terms of Actual and Coded Factors

Factor	Name	Range and levels (coded)				
		- α	-1	0	+1	+ α
A	Temperature	15	25	35	45	55
B	pH	1.5	3	4.5	6	7.5
C	Incubation period	12	48	84	120	156
D	Basal medium content	40	50	60	70	80

Table 2: Effect of Nitrogen Sources on Enzyme Production

Source	Enzyme yield (IU/g)		
	CMCase	FPase	Xylanase
Nitrogen source			
Peptone	35.14 ± 0.049	1.41 ± 0.006	252.16 ± 2.19
Tryptone	40.39 ± 0.028	1.97± 0.007	393.61 ± 4.2
Yeast extract	21.045± 0.012	0.869 ± 0.01	257.30 ± 5.56
Urea	18.675 ± 0.043	0.583 ± 0.012	146.80 ± 4.52
NH_4NO_3	11.23 ±0.011	0.564 ± 0.014	153.01 ± 3.04
$(NH_4)_2SO_4$	10.771 ± 0.025	0.243 ± 0.091	126.10 ± 2.33
Tryptone (%,w/v)			
0.1	28.86 ± 0.063	1.23 ± 0.012	234.05 ± 3.32
0.2	35.37 ± 0.028	1.95 ± 0.046	393.56 ± 4.03
0.3	33.05 ± 0.024	1.743 ± 0.044	356.19 ± 3.38
0.4	30.95 ± 0.017	1.625 ± 0.023	347.37 ± 3.68

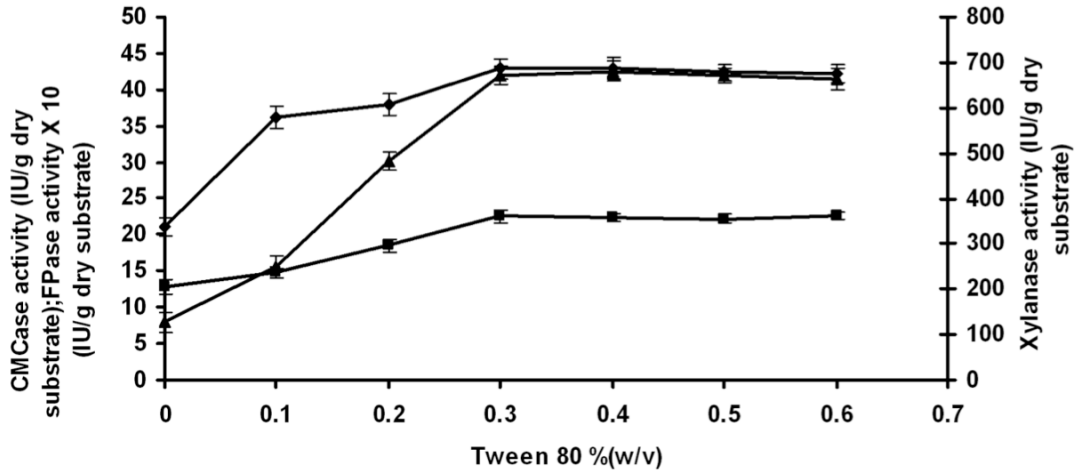


Figure 1: Effect of Tween 80 on enzyme production. FPase (■), CMCase (♦), Xylanase (▲). Results are presented as the mean of three replicates with standard deviation.

xylanase production than on the production of CMCase and of FPase.

Response Surface Methodology

Central Composite Design (CCD) was used to investigate the effects of four independent variables on cellulase and xylanase production where 30 experimental runs with different combinations of four factors were carried out. The experimental design and the actual response along with the predicted response are given in Table **3**. The quadratic regression equation was analyzed for predicted response. With the 30 design conditions the experimental values of CMCase, FPase and xylanase activities markedly varied in the range of 9.6 – 66 IU/g dry substrate, 0.483 – 3.3 IU/g dry substrate and 142.32 – 1054.70 IU/g dry substrate respectively. The multiple correlation coefficient (R^2) values for CMCase, FPase and xylanase varied between 98.29% and 98.74% which represents appreciable fitness of the model as well as significant effects of temperature, initial pH, incubation period and basal medium content on cellulase and xylanase production. Analysis of variance (ANOVA) was employed to signify the variables and their interaction effects on enzyme production. The ANOVA of the quadratic regression model is summarized in Table **4**.

CMCase

The Model F value (83.95) with low probability value ($p < 0.0001$) and high R^2 value (0.9874) statistically

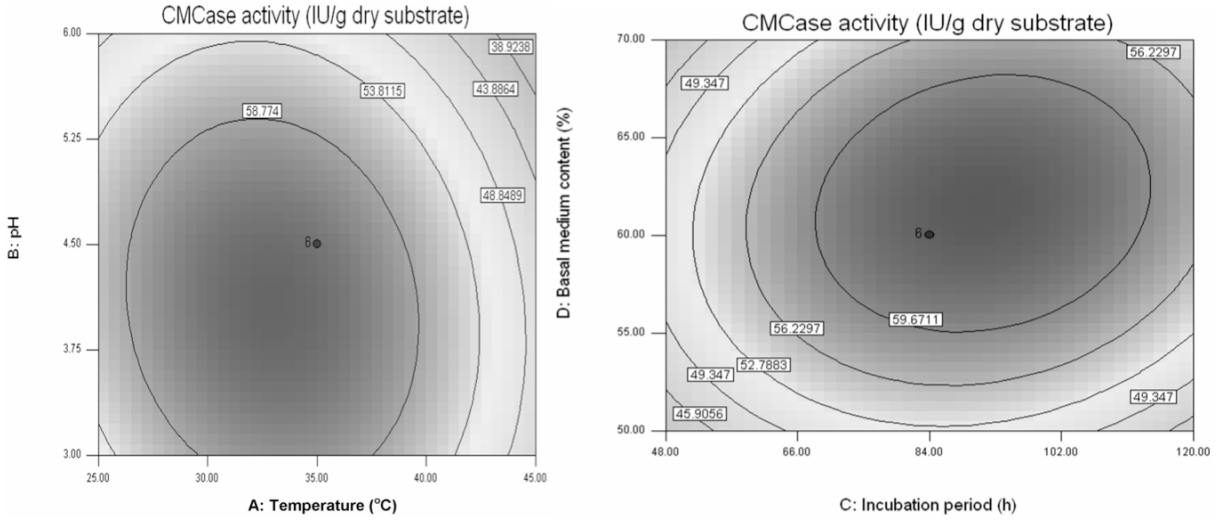
Table 3: Observed and Predicted Responses in the Experiments Obtained by Central Composite Design (CCD)

Serial No	Temperature	pH	Incubation period	Basal medium content	FPase Actual/Predicted	CMCase Actual/Predicted	Xylanase Actual/Predicted
1	25	3	48	50	1.532(1.659)	31.76(33.576)	504.393(541.228)
2	45	3	48	50	1.382(1.341)	26.78(26.083)	444.078(431.514)
3	25	6	48	50	1.454(1.374)	29.39(28.204)	477.623(448.598)
4	45	6	48	50	0.564(0.674)	12.28(13.324)	180.292(214.802)
5	25	3	120	50	1.491(1.594)	29.81(30.460)	475.540(509.704)
6	45	3	120	50	1.251(1.335)	25.42(26.895)	399.903(423.676)
7	25	6	120	50	1.707(1.606)	32.14(30.471)	545.671(514.742)
8	45	6	120	50	0.864(0.965)	17.28(19.518)	276.192(304.633)
9	25	3	48	70	1.893(1.793)	37.86(35.967)	605.129(573.547)
10	45	3	48	70	1.266(1.378)	25.32(26.431)	402.698(436.109)
11	25	6	48	70	1.401(1.327)	29.02(26.988)	447.853(426.562)
12	45	6	48	70	0.633(0.530)	10.37(10.065)	202.349(165.043)
13	25	3	120	70	2.247(2.147)	43.94(42.338)	718.219(686.264)
14	45	3	120	70	1.710(1.790)	35.20(36.730)	546.630(572.513)
15	25	6	120	70	1.935(1.977)	37.70(38.742)	627.525(636.948)
16	45	6	120	70	1.356(1.239)	28.12(25.746)	433.468(399.115)
17	15	4.5	84	60	1.335(1.430)	25.70(28.029)	426.755(458.639)
18	55	4.5	84	60	0.483(0.374)	9.66 (7.541)	142.320(111.092)
19	35	1.5	84	60	2.448(2.319)	46.53(45.227)	783.544(744.266)
20	35	7.5	84	60	1.368(1.483)	27.36(28.872)	438.304(478.238)
21	35	4.5	12	60	1.029(1.057)	20.58(21.542)	328.937(342.112)
22	35	4.5	156	60	1.743(1.701)	34.86(34.107)	557.179(544.660)
23	35	4.5	84	40	1.443(1.295)	27.86(25.916)	461.279(418.346)
24	35	4.5	84	80	1.569(1.703)	32.38(34.534)	501.557(545.147)
25	35	4.5	84	60	2.976(3.106)	59.52(62.620)	951.328(1014.888)
26	35	4.5	84	60	3.060(3.106)	65.00(62.620)	994.40(1014.888)
27	35	4.5	84	60	3.300(3.106)	66.00(62.620)	1054.70(1014.888)
28	35	4.5	84	60	3.240(3.106)	63.80(62.620)	1035.30(1014.888)
29	35	4.5	84	60	3.060(3.106)	61.40(62.620)	1022.60(1014.888)
30	35	4.5	84	60	3.000(3.106)	60.00(62.620)	1031.00(1014.888)

signify the model. An insignificant lack of fit implies that the model was robust enough to explain the effects of the four parameters on CMCase production. The linear terms of temperature, initial pH and incubation period were significant at $p<0.0001$ level but basal medium content was not ($p<0.005$). All the quadratic terms were significant ($p< 0.0001$). Enzyme production was positively affected by the interaction coefficients between temperature and initial pH as well as basal medium content and incubation period at ($p< 0.05$) and ($p<0.005$) respectively. Moisture content which is affected by basal medium content influences biosynthesis and enzyme production by interfering with the physical properties of the solid substrate [11]. High moisture content reduces the porosity of solid particles, thus limiting oxygen transfer [12, 13] whereas low moisture content reduces solubility of the nutrients of the solid substrate which results in low degree of swelling [14]. CV value obtained from the model was 7.27. "Pred R-Squared"(0.9477) was also in reasonable agreement with the "Adj R-Squared"(0.9756). Contour plots showing interaction

Table 4: Analysis of Variance for the Response Surface Quadratic Model

Source	F value			Prob > F		
	FPase	CMCase	Xylanase	FPase	CMCase	Xylanase
Model	61.40255	83.95052	72.77379	< 0.0001	< 0.0001	< 0.0001
A-temp	76.97462	96.77444	90.73566	< 0.0001	< 0.0001	< 0.0001
B-pH	48.25079	61.66865	53.16244	< 0.0001	< 0.0001	< 0.0001
C-incubation period	28.60997	36.39913	30.81813	< 0.0001	< 0.0001	< 0.0001
D-basal medium	11.48328	17.1245	12.07803	0.0041	0.0009	0.0034
AB	6.693348	8.387428	7.710352	0.0206	0.0111	0.0141
AC	0.160088	2.370502	0.280976	0.6947	0.1445	0.6038
AD	0.432711	0.641348	0.384885	0.5206	0.4357	0.5443
BC	4.029376	4.453194	4.77711	0.0631	0.0520	0.0451
BD	1.506647	1.999934	1.479536	0.2386	0.1777	0.2426
CD	8.054624	13.83388	10.41927	0.0125	0.0021	0.0056
A^2	382.7059	529.6311	457.5236	< 0.0001	< 0.0001	< 0.0001
B^2	114.3328	172.2642	139.8686	< 0.0001	< 0.0001	< 0.0001
C^2	234.9335	318.9854	280.3989	< 0.0001	< 0.0001	< 0.0001
D^2	203.406	276.4982	244.02	< 0.0001	< 0.0001	< 0.0001
Lack of fit	1.35	0.84	1.71	0.3883	0.621	0.2883



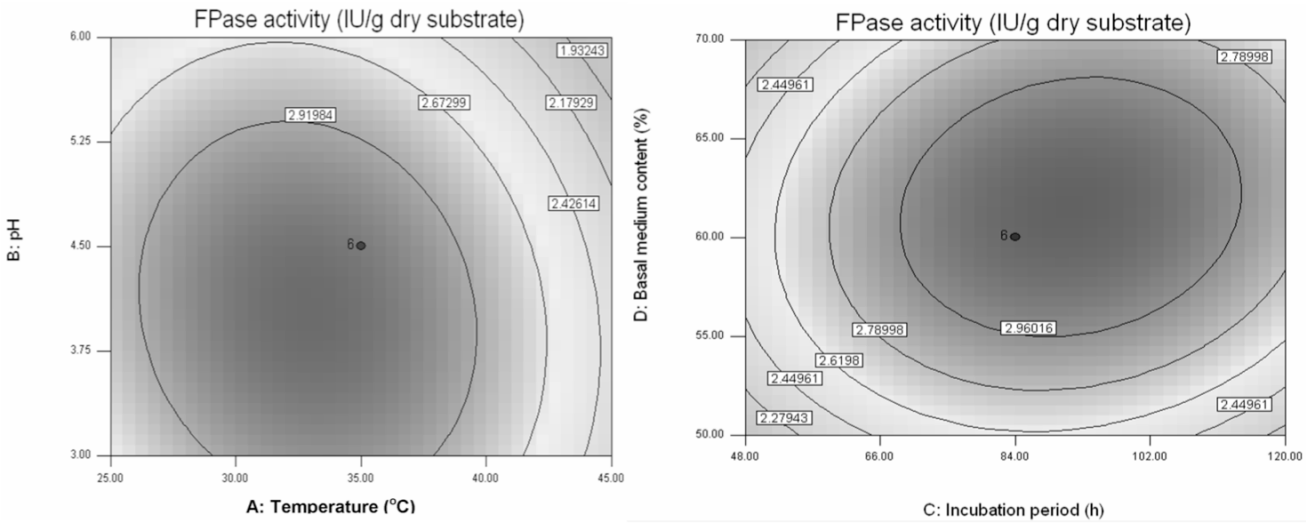
a **b**

Figure 2: a. Three dimensional plot of CMCase (response), effect of pH and temperature.
b. Three dimensional plot of CMCase (response), effect of basal medium content and incubation period.

between temperature and initial pH as well as basal medium content and incubation period on CMCase production have been depicted in Figure **2a** and **2b** respectively where optimum response is observed near the central value of temperature, initial pH, incubation period and basal medium content.

FPase

FPase is regarded as the total cellulolytic activity. Maximum FPase activity (3.3 IU/g dry substrate) was obtained at the combination of incubation temperature 35°C, initial pH 4.5, incubation period 84 hours and basal medium content 60%. A high Model F-value (61.40) along with high multiple correlation coefficient (R^2) value of 0.9829 and an insignificant lack of fit signify a good effect of the process parameters on FPase production. FPase production was significantly affected by linear terms of the temperature, initial pH, incubation period at ($p < 0.0001$) whereas basal medium content had little effect ($p<0.005$). The



a **b**

Figure 3: a. Three dimensional plot of FPase (response), effect of pH and temperature.
b. Three dimensional plot of FPase (response), effect of basal medium content and incubation period.

interaction between temperature and pH as well as basal medium content and incubation period affected enzyme production at ($p<0.05$). A reasonable agreement between the "Pred R-Squared"(0.9212) and "Adj R-Squared"(0.9668) indicates a good corelation between the observed and predicted values. The contour plots (Figure **3a** and **3b**) represent the interaction between temperature and initial pH and between basal medium content and incubation period on enzyme production respectively where optimum level of FPase activity was obtained under the similar culture conditions observed for CMCase production.

Xylanase

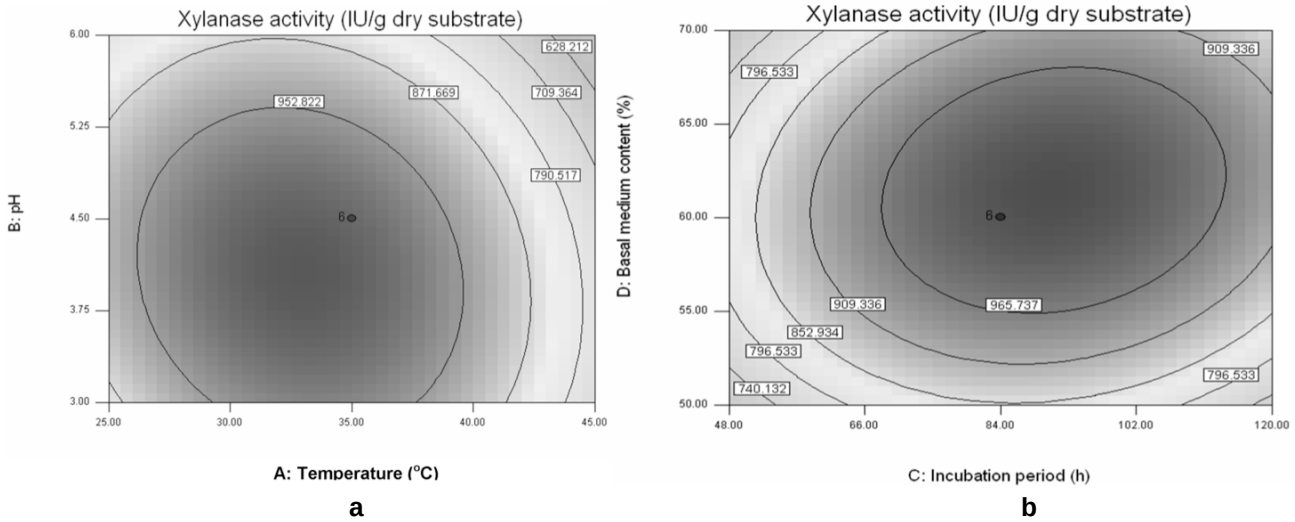
ANOVA of the experimental values resulted in a high model F value (72.77) and a low probability value $p < 0.0001$ (Table **4**). In this case, obtained R^2 value (0.9855) and an insignificant lack of fit revealed high significance of the model where only 0.014% of total variation was not explained by the model. The predicted multiple correlation coefficient (Pred R^2 = 0.9306) value was in reasonable agreement with the adjusted R^2 value of 0.9719. The proximity of adjusted R^2 to predicted R^2 signifies a good adjustment of the theoretical values to the experimental data by the model. Xylanase production was affected by linear, squared and interaction coefficients of variables in a way similar to CMCase and FPase production. The contour plots of the interaction effects of temerature and initial pH as well as basal medium content and incubation period on xylanase activity (Figure **4a** and **4b** respectively) showed that optimum enzyme was produced under culture conditions similar to those for CMCase as well as FPase production.

Optimization of the Process Parameters

Numerical optimization step in CCD was used to predict the optimum values of CMCase, FPase and xylanase activities by the adjusted model. The goals for variables of temperature, initial pH, incubation period and basal medium content were set as ‘‘in range”. The goal for the responses (FPase, CMCase and xylanase) was set as ‘‘maximize” because the maximum value of yield is the aim. A number of solutions was produced by the software Design Expert.7.0.0 and ranked according to their desirability. The solutions with the highest desirability were chosen and the optimum values were 3.1 IU/g dry substrate, 64.18 IU/g dry substrate and 1040.57 IU/g dry substrate for FPase, CMCase and xylanase respectively. The optimized conditions were temperature 33^{o}C, initial pH 4, incubation period 90 hours and basal medium content 62%. Under the optimized culture conditions, experimental values of CMCase, FPase and xylanase activities obtained were 62.33 IU/g dry substrate, 3.04 IU/g dry substrate and 1056.28 IU/g dry substrate respectively. Experimentally optimized values were in good agreement with the predicted values ensuring valid significance of the developed model and its application in predicting the experimental results.

Enzymatic Hydrolyisis

The efficacy of secreted cellulases from *Aspergillus fumigatus* NITDGPKA3 in saccharifying alkali



a **b**

Figure 4: a. Three dimensional plot of xylanase (response), effect of pH and temperature.
b. Three dimensional plot of xylanase (response), effect of basal medium content and incubation period.

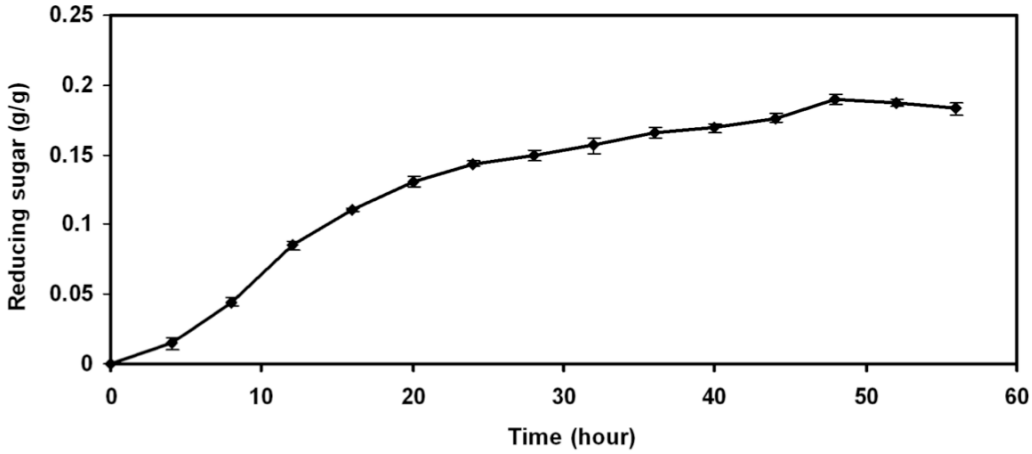


Figure 5: Enzymatic hydrolysis of alkali pretreated rice straw.

pretreated rice straw was evaluated. From Figure **5** it is observed that the release of sugars increased with the increase of saccharification time studied. The maximum reducing sugar (189.50 mg/g) was obtained at 48 hours.

CONCLUSION

Statistical optimization of cultivation conditions using central composite design appears to be an efficient tool for the cellulase production under solid state fermentation by *Aspergillus fumigatus* NITDGPKA3. The optimal FPase, CMCase and xylanase activities under optimal conditions were 3.1 IU/g dry substrate, 64.18 IU/g dry substrate and 1040.57 IU/g dry substrate respectively. The present study indicates that the strain produced cellulase enzyme which hydrolyzed rice straw to fermentable sugars.

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