Response Surface Methodology for the Evaluation and Comparison of Cellulase Production by *Aspergillus nidulans* SU04 and *Aspergillus nidulans* MTCC344 Cultivated on Pretreated Sugarcane Bagasse

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Response surface methodology (RSM) was used to optimize the conditions for the production of endo β-1,4 glucanase – a component of cellulase by *Aspergillus nidulans* SU04 and *Aspergillus nidulans* MTCC344 under solid state fermentation, using pretreated bagasse as chief substrate. A four-factor-five-level central composite design was employed for the experimental design. The endo β-1,4 glucanase produced during the bioconversion of cellulose to glucose by these strains were strongly dependent on the NaOH pretreatment given to bagasse before hydrolysis. Maximum cellulase activity was 32.59 U g^{-1} and 28.96 U g^{-1} (CMCase) for *A. nidulans* SU04 and *A. nidulans* MTCC344 respectively. The optimum conditions for cellulase production are 15 mm bagasse bed height, 60 % moisture content, pH 5 and temperature 40 °C in the solid state fermenter. *A. nidulans* MTCC344 and *A. nidulans* SU04 were able to hydrolyze pretreated sugarcane bagasse completely after 15 days and 6 days of incubation with significant endo β-1,4 glucanase activities. The results of Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD) and Scanning electron microscopy (SEM) of bagasse showed structural changes through pretreatment, in favor of enzymatic hydrolysis. *A. nidulans* SU04 was found to be highly efficient compared to *A. nidulans* MTCC344 in terms of endoglucanase, exoglucanase and β-glucosidase activities.

Key words:
Aspergillus nidulans SU04, *Aspergillus nidulans* MTCC344, CMCase, pretreated bagasse, solid state fermentation, central composite design, response surface methodology

Introduction

Lignocelluloses are chiefly comprised of cellulose, hemicelluloses surrounded by lignin. This complex polyaromatic matrix containing significant amounts of lignin and hemicellulose are generally resistant to enzymatic hydrolysis.[1] Severe pretreatments including steaming and acid hydrolysis removes lignin and hemicellulose, thus making cellulose available for the microorganisms.[2] The pretreatment of lignocelluloses increases the surface area accessibility of cellulose by fungal species capable of synthesizing cellulase for their conversion to glucose.[3] Extracellular cellulase has three components, endoglucanase (endo-1,4-β-D-glucanase, EC 3.2.1.4), exoglucanase (exo-1,4-β-D-glucanase, EC 3.2.1.91) and β-glucosidase (1,4-β-D-glucosidase, EC 3.2.1.21).[4] Cellulases are synthesized by microorganisms during their growth on cellulosic materials.[5–7] They have a wide range of industrial applications in the textile, paper and pulp, food, animal feed, pharmaceutical, fuel and chemical industries.[8,9] These industries demand highly stable cellulase, proficient to outshine at extreme conditions of pH and temperatures. Comprehensive application of cellulase in industry is obstructed by its synthesizing cost. This hike in the could be overcome by exploring new substrates for cellulase production.

Bagasse is the fibrous matter that remains after sugarcane (*Saccharum officinarum)* stalks are crushed to extract their juice. Since the inception of the sugar industry in India in the 1860's, the fibrous and finer dust had been considered a nuisance with little beneficial uses in many Indian states.[10] Until recently, it is used as a renewable resource in the manufacture of pulp and paper products, building materials and cellulosic ethanol.[11] Each ton of cane delivered to processing mills yields 740 kg of juice and 260 kg of moist bagasse.[12] Being an abundant resource, it is easily accessible, cost effective, highly compressible and has moisture retention capability. The present investigation leads to a systemic construction, inculcating a process for natural bagasse degradation combined with cellulase production.

Solid state fermentation (SSF) is as an attractive strategy to produce cellulase using a variety of lignocelluloses as substrates.[13] *Aspergillus nidulans*

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strain appears to be the most promising microorganism; hence it is of interest to examine its role in the breakdown of bagasse for cellulase production.[14–17] In this study, we compare *Aspergillus nidulans* SU04 and *Aspergillus nidulans* MTCC344 on their utilization of bagasse for cellulase production. Optimizing bagasse bed height, moisture content, pH and temperature by using response surface methodology (RSM) employing a four-factor-five-level central composite design (CCD) is the main focus.[18] The crude extract obtained after SSF was partially purified and observations were made on chemical characteristics, physical properties and microstructure of pretreated and hydrolyzed bagasse.

Materials and methods

Microorganism and culture conditions

The strain *Aspergillus nidulans* MTCC344 was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh. Malt Extract Agar (MA) was used as growth medium.[19] The strain *Aspergillus nidulans* SU04 was isolated from decayed, outer shell of *Arachis hypogaea* from Cuddalore district of Tamilnadu, India.[20] Solid media containing 2 % malt extract, 2 % Agar and 1 % CMC was used to screen the cellulase producing strain, followed by 0.01 % congo-red staining and 0.1% NaCl washing.[21] Isolate having clear orange halo zone on CMC agar plate was chosen in subsequent studies. Cultures were stored on Malt Extract Agar slants at 4 °C and as spore suspension in φ = 10 % glycerol at –20 °C. Spore suspension prepared by washing slant cultures with 5 mL sterilized water was transferred into minimal medium (mg L^{-1}), KH_2PO_4, 0.75; NH_4NO_3, 1.5; Thiamine HCl, 0.01; $MgSO_4 \cdot H_2O$, 0.5; $CaCl_2 \cdot 5H_2O$, 0.05 at pH 5. Solid state fermentation was carried out in 250 mL Erlenmeyer flasks containing bagasse as the chief carbon source at 5–25 mm bed height, autoclaved for 30 min at 121 °C, inoculated with three mL of prepared spore suspension. This is then incubated at 50 °C under static conditions for 20 days.

Feedstock pretreatment

The feedstock material, bagasse, was obtained from local sugar mills in Tamilnadu. The substrate was washed with water at room temperature for 4 h, air dried, milled by pulverizing and passed through 60 mesh screen. The initial composition of sugarcane ***bagasse*** was 42.7 % cellulose, 25.4 % hemi-cellulose, 28.0 % lignin and 0.75 % ash.[22] Alkaline pretreatment was used to delignify bagasse.[23] 25 g dry mass bagasse was treated with 200 mL of 0.2 % to 1.0 % NaOH solution for 2, 4, 6, 8, 10 and 12 h. After the treatment, the solution was centrifuged for 20 min at 2500 g, the solid fraction was suspended in hot boiling water for 10 min, followed by centrifugation for 10 min at 5000 g. The fractions were dried and stored in air-tight plastic containers.

Cellulose, hemicellulose, lignin estimation

The NaOH extract-free oven dry bagasse dust was examined for the cellulose, hemicellulose and lignin content after the alkali treatment.[24–27] 5 g of NaOH extract-free oven-dried bagasse dust was refluxed with 1.5 g sodium chlorite and 0.5 mL of φ = 0.3 % acetic acid at 100 °C. The process was repeated until bagasse became white. The mixture was filtered through G3 crucible and washed with hot distilled water. The crucible was dried to constant mass at 105±3 °C. The cellulose % was determined using

$$\text{Cellulose (\%)} = \frac{ODMC}{ODMS} \cdot 100$$

Hemicellulose percentage was determined by treating 25 g NaOH extract-free oven-dried bagasse dust samples with 1 L of 4 % NaOH at room temperature for 24 h. The filtered alkaline extract was neutralized with acetic acid and the hemicellulose precipitated by adding an equal volume of 95 % ethanol. It was washed with gradient strengths of alcohol and ether and finally dried overnight at 100 °C.[25,28] The hemicellulose % was determined using

$$\text{Hemicellulose (\%)} = \frac{ODMH}{ODMS} \cdot 100$$

To determine the percentage of lignin, 1 g of NaOH extract free oven-dried bagasse dust was treated with 15 mL of 72 % H_2SO_4 for 2 h at 20 °C with occasional stirring. The mixture was then transferred to 1 L flask and the acid concentration is brought down to 3 % by refluxing with distilled water for 4 h. The mixture was filtered through G3 crucible and washed with hot distilled water until acid free. The crucible was dried to constant mass at 105±3 °C.[25] The lignin % is determined using

$$\text{Lignin (\%)} = \frac{ODML}{ODMS} \cdot 100$$

Bagasse fermentation

Bagasse fermentation was performed using untreated bagasse and pretreated bagasse.[29] Bagasse was layered in 250 mL Erlenmeyer flasks and sterilized. After bed layering and sterilizing, the bed height was maintained as 5, 10, 15, 20 and 25 mm. The corresponding mass of the bagasse was ob-

served to be 1.05 g, 1.29 g, 2.15 g, 2.75 g and 3.24 g respectively. 3 mL minimal media containing 10^5 spores mL^{-1} was suspended in 2 mL sterile water and inoculated into the untreated and pretreated bagasse separately. The inoculum addition contributed to 1.5 % to 2 % variation in the moisture levels (Moisture Analyzer-Ohaus MB35). Moisture content was varied between 40 % and 80 % by the addition of sterile water, added with the inoculum. The cultures were incubated at 20, 30, 40, 50 and 60 °C. Fermentation was initiated at various initial pH values from 3 to 7. Extracts were drawn every 24 h by the addition of 10 mL distilled water to the solid bed, pressing, followed by filtering the extract through nylon cloth.

Experimental design

Adoption of central composite design employed here requires prior knowledge on the upper and lower limits of the parameters and awareness of the enzymatic hydrolysis and its factors.[30,31] Four independent variables, namely, bed height (5–25 mm), moisture content (40–80 %), initial pH (3–7), and growth temperature (20–60 °C) were chosen and their lowest and highest levels were fixed. The design was employed to investigate the performance of the response variable, cellulase activity (U g^{-1}). Each independent variable had five levels –2, –1, 0, +1 and + 2. CCD with a total of 30 different combinations was constructed by Design Expert software (Version 8.0.1, Stat-Ease Inc., Minneapolis, USA) statistical package to evaluate the production of cellulase by these strains. All the experimental sequences were performed in triplicate. The coded values of independent variables were found from equation

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad i = 1,2,3,\ldots,k \tag{1}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the value of X_i at the center point and ΔX is the step change. A second-order quadratic model was used to fit the quadratic equation,

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \\ & + \beta_5 x_1^2 + \beta_6 x_2^2 + \beta_7 x_3^2 + \beta_8 x_4^2 + \\ & + \beta_9 x_1 x_2 + \beta_{10} x_1 x_3 + \beta_{11} x_1 x_4 + \\ & + \beta_{12} x_2 x_3 + \beta_{13} x_2 x_4 + \beta_{14} x_3 x_4 \end{aligned} \tag{2}$$

where Y is the measured response (cellulase activity, U g^{-1}), x_1, x_2, x_3, x_4 are the coded independent input variables, β_0 is the intercept term, $\beta_1, \beta_2, \beta_3, \beta_4$ are the linear coefficients showing the linear effects, $\beta_5, \beta_6, \beta_7, \beta_8$ are the quadratic coefficients showing the squared effects and $\beta_9, \beta_{10}, \beta_{11}, \beta_{12}, \beta_{13}, \beta_{14}$ are the cross product coefficients showing the interaction effects. The optimum values of the factors were obtained by solving the regression equation, analyzing the surface of the three-dimensional response surface plot and also by the setting up of constraints for the levels of the variables. The response surface graphs and contour plots are the graphical representations of the regression equation. The optimum values of factors were obtained by solving the regression equation by Monte Carlo optimization.[32] The goodness of fit of the model was evaluated by coefficient of determination R^2. The statistical significance of the model was investigated by Analysis of variance (ANOVA), tested using Fisher's statistical analysis. The significance of each coefficient was determined by applying the *t*-test.

Analytical methods

Assay for Endo β-1,4 glucanase (CMCase) was carried out according to IUPAC recommendation by measuring the release of reducing sugars in the reaction mixture of 1 mL crude enzyme and 1 mL of y = 0.01 g mL^{-1} CMC solution in 0.05 mol L^{-1} sodium citrate buffer (pH 4.8) incubated at 40 °C for 1 h.[33] One unit of CMC activity is defined as the amount of enzyme needed to liberate one μmol of glucose min^{-1} from 1 mL of culture broth under assay conditions. Filter paper cellulase (Fpase) activity was assayed by measuring the release of reducing sugars in a reaction mixture containing Whatman No.1 filter paper 50 mg of dimension (1.0 × 6.0 cm) as substrate in 0.05 mol L^{-1} sodium citrate buffer (pH 4.8) at 50 °C for 1 h. One unit of Fpase activity is defined as the amount of enzyme needed to liberate 1 μmol of glucose min^{-1} during hydrolysis reaction. β-Glucosidase (cellobiase) activity was measured by the release of reducing sugars in a reaction mixture containing 1 mL crude enzyme and 1 mL of 15mmol L^{-1} cellobiose solution incubated at 50 °C for 1 h. One unit of cellobiase activity is defined as the amount of enzyme needed to liberate 2 μmol of glucose min^{-1} during hydrolysis of cellobiose. Glucose in the culture supernatant was analyzed using UV-Visible spectrophotometer (Hitachi Model: 100–40) at 540 nm.[34] X-ray diffraction analysis was carried out from 0–70°; 2θ to examine the structural properties of untreated, pretreated and hydrolyzed bagasse samples (Miniflex Model, Rigaku, Japan). The FTIR spectrum of samples was recorded in the region of 400–4000 cm^{-1} (Nicolet 20DXB).

Results and discussion

Chemical composition of pretreated bagasse

The effect of NaOH concentration (0.2–1.0 %) on cellulose, hemicelluloses and lignin content of bagasse at different time intervals is shown in

Table 1 – *Effect of alkali treatment on the cellulose, hemicellulose and lignin content of bagasse*

Treatment time (h)	NaOH (%)														
	0.2			0.4			0.6			0.8			1.0		
	C^a	H^a	L^a	C^a	H^a	L^a	C^a	H^a	L^a	C^a	H^a	L^a	C^a	H^a	L^a
2	41.29	24.5	27.2	41.1	20.12	14.51	40.36	19.02	7.52	39.26	18.02	7.00	39.10	7.05	7.14
4	41.10	19.6	21.32	40.63	19.2	18.51	40.32	17.42	6.52	39.20	11.02	6.00	39.17	6.02	6.25
6	41.05	18.52	11.12	40.51	17.17	8.12	40.30	9.21	7.12	39.17	8.14	5.00	39.15	6.00	5.11
8	40.96	6.69	9.58	40.45	6.02	7.42	40.28	5.11	6.12	39.02	5.02	4.20	39.00	5.12	4.25
10	40.90	7.02	7.25	40.39	5.23	6.25	40.29	4.21	5.00	38.89	4.08	4.00	38.90	4.02	4.00
12	40.80	6.42	6.02	40.38	5.20	6.02	40.28	4.14	5.00	39.10	4.01	4.00	39.12	4.02	3.98

C: % cellulose; *H*: % hemicellulose; *L*: % lignin

[a] values are mean ± SD of three determinations.

Table 1. Pretreatment using NaOH was seen to decrease the cellulose, hemicelluloses and lignin content of bagasse. Cellulose content showed a placid decrease from 41.29 % to 39.10 % as the alkali used in the treatment was increased from 0.2 % to 1.0 %. The time of treatment was seen to have a negligible effect on the cellulose content of bagasse. The lignin content and hemicellulose content was seen to decrease drastically from 24.5 % to 7.05 % and 27.2 % to 7.14 % respectively when treated with 0.2 % NaOH, as the treatment time was increased from 2 to 12 h. The content of cellulose, hemicelluloses and lignin was seen to be consistent after 0.8 % NaOH treatment irrespective of the treatment time. But from the observations made, optimum time of 10 h was found to be favorable for the alkaline pretreatment of bagasse.

Response surface approach by Central Composite Design

The range and levels of the variables used in experimental design matrix is presented in Table 2. The results of CCD experiments with different combinations for bed height (x_1), moisture content (x_2), initial pH (x_3) and growth temperature(x_4) are presented along with mean predicted and experimental responses for cellulase activity of *A. nidulans* MTCC344 and *A. nidulans* SU04. There was considerable variation in the responses at different values of selected variables. Maximum cellulase activities (28.96 U g^{-1} and 32.59 U g^{-1}) were observed in run no. 25 and run no. 27 for *A. nidulans* MTCC344 and *A. nidulans* SU04. Data obtained were studied by applying the multiple regression analysis based on eqs. (1) and (2). The predicted response Y_{R1} and Y_{R2} for the cellulase activity of *A. nidulans* MTCC344 and *A. nidulans* SU04 is expressed by eq. (3) and eq. (4) as a function of bed height, moisture content, initial pH and growth temperature. The predicted response variable was obtained as below,

$$Y_{R1} = 28.84 + 1.72x_1 + 0.82x_2 - 2.83x_3 + 0.18x_4 - 4.46x_1^2 - 4.53x_2^2 - 2.37x_3^2 - 1.80x_4^2 - 0.28x_1x_2 - 1.98x_1x_3 - 0.11x_1x_4 + 0.13x_2x_3 + 0.12x_2x_4 + 2.29x_3x_4 \quad (3)$$

$$Y_{R2} = 32.65 + 1.25x_1 + 0.62x_2 - 1.29x_3 + 0.20x_4 - 4.69x_1^2 - 4.45x_2^2 - 2.48x_3^2 - 1.95x_4^2 - 0.48x_1x_2 - 2.13x_1x_3 - 0.14x_1x_4 + 0.23x_2x_3 + 0.17x_2x_4 + 2.38x_3x_4 \quad (4)$$

ANOVA for the model shows the *F*-value of 9992.21 and 9989.63 for *A. nidulans* MTCC344 and *A. nidulans* SU04 implying its significance. Values of Prob>*F* less than 0.05 indicate significance of the model terms.[30,35] The regression equation obtained after the analysis of variance (ANOVA) indicates the coefficient of determination (R^2) as 0.9999 and 0.9989 for cellulase activity of *A. nidulans* MTCC344 and *A. nidulans* SU04 respectively. Correlation coefficient (R) values of 0.99 and 0.98 indicate direct proportionality between the experimental and predicted values of responses. The predicted determination of coefficient (Pred R^2) of 0.998 and 0.9997 is in reasonable agreement with the Adj R^2 of 0.9967 and 0.9998. Adequate precision (Adeq Pre) ratio of 301.24 and 501.29 for the response indicates an adequate signal. Hence, this model could be used to navigate the design space. It indicates the degree of precision with which the experiments are compared. A lower coefficient of variation (CV) of 0.54 % and 0.32 % for *A. nidulans* MTCC344 and *A. nidulans* SU04 indicates the precision with which the experiments

Table 2 – *Levels of the variables tested in CCD matrix in coded terms showing the levels of tested variables, cellulase activity (CMCase) in terms of experimental and predicted values for A. nidulans MTCC344 and A. nidulans SU04*

Trials	Variables and experimental values				Cellulase activity (U g^{-1})			
	X_1	X_2	X_3	X_4	*A. nidulans* MTCC344		*A. nidulans* SU04	
	Levels (values)	Levels (values)	Levels (values)	Levels (values)	Expt*	Pred	Expt*	Pred
1	–1(10)	–1(50)	–1(4)	–1(30)	15.50	15.53	16.52	16.62
2	+1(20)	–1(50)	–1(4)	–1(30)	23.69	23.70	25.63	25.48
3	–1(10)	+1(70)	–1(4)	–1(30)	17.30	17.23	18.21	18.53
4	+1(20)	+1(70)	–1(4)	–1(30)	24.30	24.28	25.35	25.79
5	–1(10)	–1(50)	+1(6)	–1(30)	9.85	9.88	10.25	10.65
6	+1(20)	–1(50)	+1(6)	–1(30)	10.18	10.13	11.35	11.63
7	–1(10)	+1(70)	+1(6)	–1(30)	12.10	12.10	13.52	13.96
8	+1(20)	+1(70)	+1(6)	–1(30)	11.18	11.22	12.58	12.68
9	–1(10)	–1(50)	–1(4)	+1(50)	11.28	11.29	13.52	13.65
10	+1(20)	–1(50)	–1(4)	+1(50)	19.05	19.03	20.53	20.58
11	–1(10)	+1(70)	–1(4)	+1(50)	13.42	13.45	14.53	14.65
12	+1(20)	+1(70)	–1(4)	+1(50)	20.04	20.06	21.56	21.69
13	–1(10)	–1(50)	+1(6)	+1(50)	14.80	14.80	15.63	15.78
14	+1(20)	–1(50)	+1(6)	+1(50)	14.50	14.62	15.69	15.75
15	–1(10)	+1(70)	+1(6)	+1(50)	17.45	17.49	18.56	18.96
16	+1(20)	+1(70)	+1(6)	+1(50)	16.23	16.18	17.86	17.96
17	–2(5)	0(60)	0(5)	0(40)	7.60	7.58	8.62	9.02
18	+2(25)	0(60)	0(5)	0(40)	14.45	14.44	15.62	15.96
19	0(15)	–2(40)	0(5)	0(40)	9.15	9.11	10.52	10.63
20	0(15)	+2(80)	0(5)	0(40)	12.35	12.37	13.65	13.75
21	0(15)	0(60)	–2(3)	0(40)	24.11	24.13	25.62	25.96
22	0(15)	0(60)	+2(7)	0(40)	14.65	14.60	15.62	15.96
23	0(15)	0(60)	0(5)	–2(20)	21.24	21.27	22.59	22.67
24	0(15)	0(60)	0(5)	+2(60)	22.04	21.98	23.56	23.68
25	0(15)	0(60)	0(5)	0(40)	28.96	28.84	32.56	32.54
26	0(15)	0(60)	0(5)	0(40)	28.95	28.84	32.58	32.55
27	0(15)	0(60)	0(5)	0(40)	28.85	28.84	32.59	32.68
28	0(15)	0(60)	0(5)	0(40)	28.75	28.84	32.54	32.58
29	0(15)	0(60)	0(5)	0(40)	28.94	28.84	32.55	32.53
30	0(15)	0(60)	0(5)	0(40)	28.59	28.84	32.56	32.54

Factors: X_1, bagasse bed height (mm); X_2, moisture content (%); X_3, initial pH; X_4, temperature (°C).

**The observed values of cellulase activity were the means of triplicates*

were conducted. The smaller predicted residual sum of squares (PRESS) value of 0.15 and 0.13 for *A. nidulans* MTCC344 and *A. nidulans* SU04 proves validity of the model. The model has high R^2, significant *F*-values and an insignificant lack-of-fit indicating its excellency in predicting the solution to our problem. This also shows the best degree of fitness for the model equations. The effects of four variables on the enzymatic hydrolysis were studied and the relevant conditions for the process were optimized. As far as the CCD is concerned, all the variables were aptly predicted, confirming its adequacy.

Response surface graphs and contour plots

The most significant three-dimensional response surfaces and their corresponding contours for cellulase activity against any two independent variables by keeping the other independent variables at zero levels are presented in Fig. 1a-c. A total of six response surfaces were obtained by considering all the possible combinations for each strain. These plots show the type of interaction between the tested variables, thereby permitting optimum conditions.[36] The maximum predicted value of the response under the optimum experimental condition is represented by the surface confined in the smallest ellipse in the contour diagram.[37,38] Figs. 1a-c represents the significant plots for the strain, *A. nidulans* MTCC344. Interaction between moisture content and bagasse bed height is shown in Fig. 1a for CMCase. The result demonstrated the maximum point on the response surface. The CMCase was 27.59 U g^{-1} at bed height 15 mm and moisture content 60 % for all the pH ranges and temperatures studied. The highest CMC-ase activity 27.89 U g^{-1}, was obtained at initial pH 5 and 60 % moisture content (Fig. 1b). The optimum levels for the temperature and initial pH were found to be 37.5 °C and 4.25 as evident from Fig. 1c. Contour diagrams of Fig. 1c are elliptical but not tilted, indicating a a good deal of negligible interaction between the factors (X_3 and X_4). The significant plots for the isolated *A. nidulans* SU04 are shown in Figs. 2a-c. Fig. 2a depicts the three-dimensional response surface graphical representation and contour plot showing the effect of initial pH and bagasse bed height on CMCase by *Aspergillus nidulans* SU04. Maximum CMCase of 28 U g^{-1} was achieved at initial pH value of 5 and 15 mm bed height. Maximum CMCase of 26.45 U g^{-1} was seen at 43.8 °C and 15 mm bed height (Fig. 2b). When moisture content was varied from 50 % to 70 %, maximum CMCase was seen to be 26.5 U g^{-1} at 63 % moisture content, 15 mm bed height and initial pH 5 (Fig. 2c). In the response plots and contour diagrams of Figs. 1a-c and Figs. 2a-c, contour

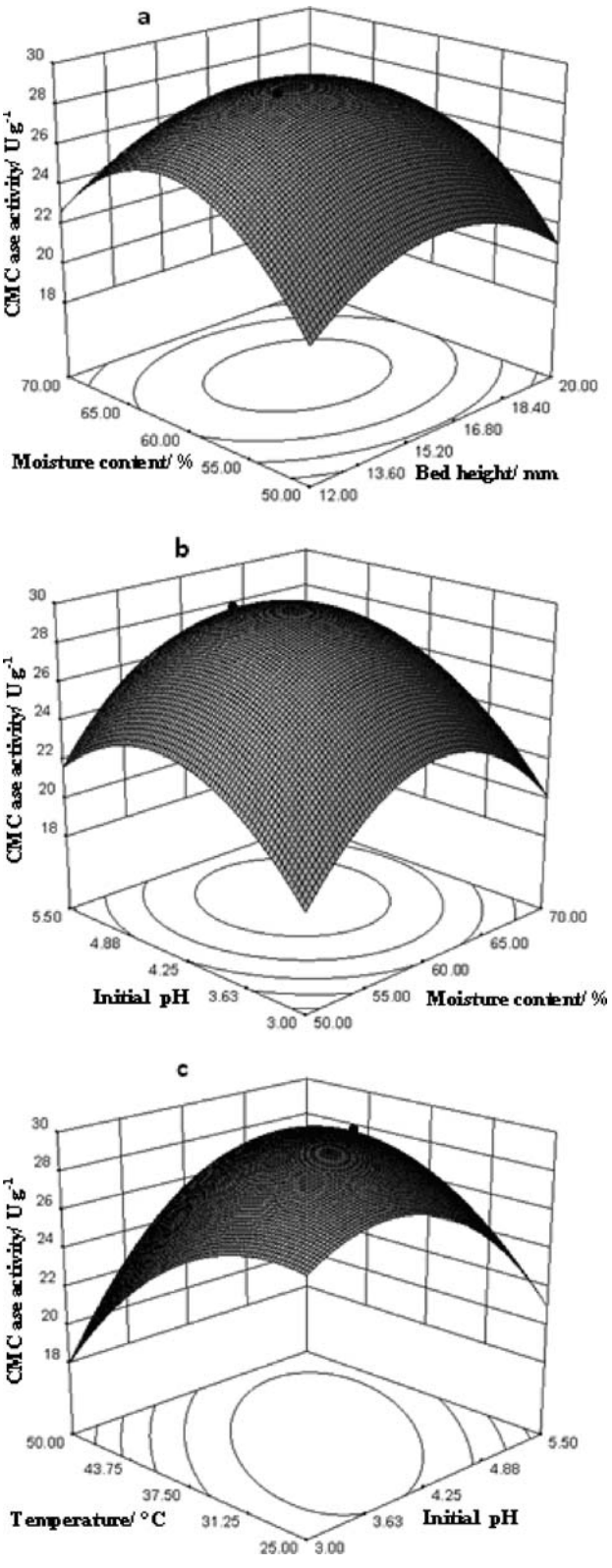
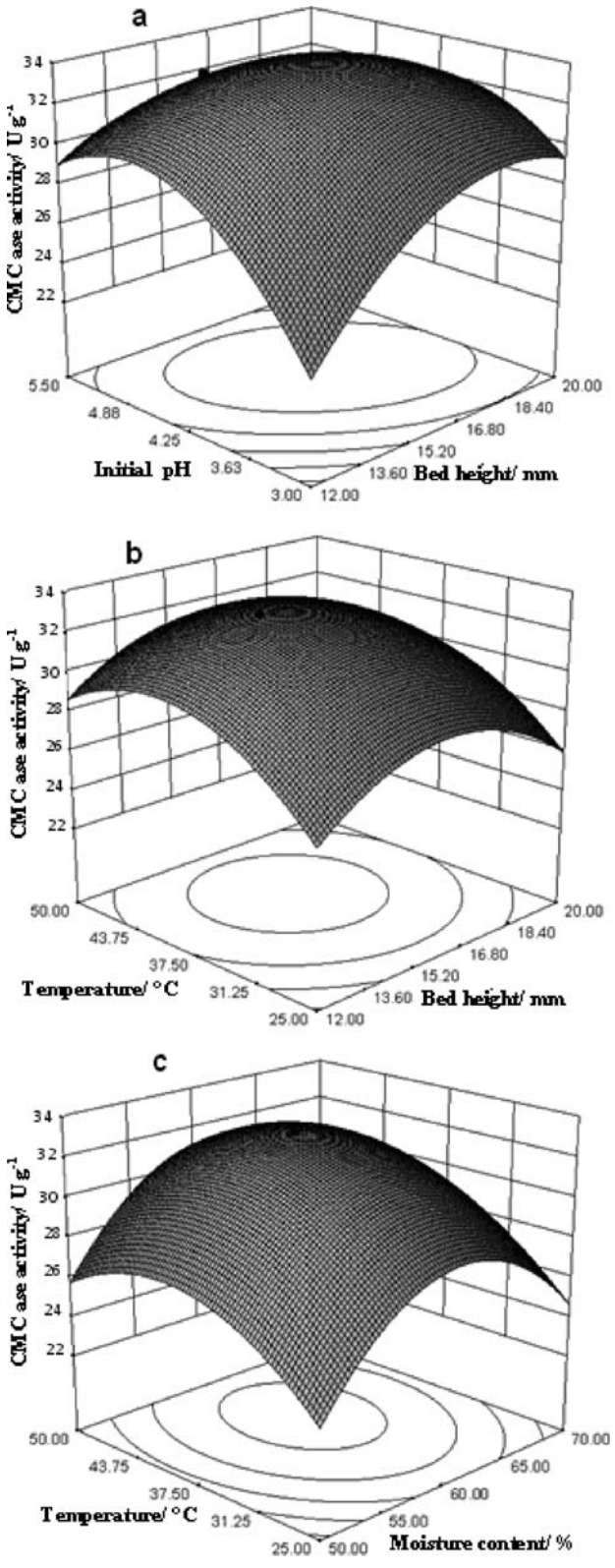


Fig. 1 – *a) Response surface plots, moisture content/% vs bagasse bed height/mm at pH 4 and temperature 40 °C for CMCase from A. nidulans MTCC344; b) Response surface plots, initial pH vs moisture content/% at temperature 40 °C, bagasse bed height 15 mm for CMCase from A. nidulans MTCC344; c) Response surface plots, temperature/°C vs initial pH at moisture content 60 % and bagasse bed height 15 mm for CMCase from A. nidulans MTCC344/U g^{-1}*

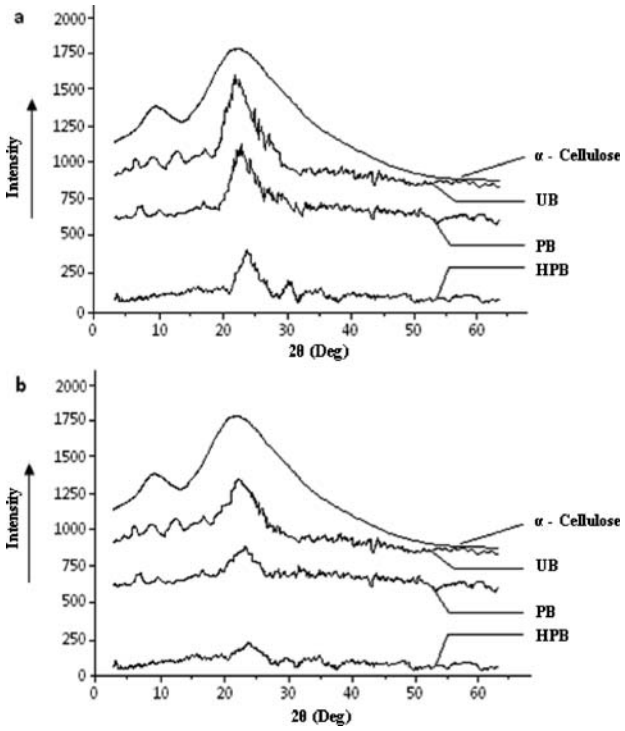


F i g . 2 – *a) Response surface plots, initial pH vs bagasse bed height/mm at moisture content 60 % and temperature 40 °C for CMCase from A. nidulans SU04; b) Response surface plots, temperature/°C vs bagasse bed height/mm for initial pH 4 and moisture content 60 % for CMCase from A. nidulans SU04; c) Response surface plots, temperature vs moisture content for initial pH 4, and bagasse bed height 15 mm for CMCase from A. nidulans SU04*

diagrams of Figs. 1a, 2a, 2b and 2c were elliptical and tilted, indicating significant cross-product interaction between the factors, X_1 and X_2 for *A. nidulans* MTCC344 and the factors, X_1 and X_3, X_1 and X_4, X_2 and X_4 for *A. nidulans* SU04 respectively. Cellulase activity of *Aspergillus* strains in solid state fermentation using bagasse is sensitive to minor changes in the test variables.

XRD pattern of bagasse samples

Pretreatment of bagasse and bagasse fermentation by the *Aspergillus* strains had a tremendous effect on the physical properties and cellulose microstructure of bagasse. In order to understand the structural properties of untreated, pretreated and hydrolyzed bagasse samples, X-ray diffraction study was carried out in the 0–70° range using Cuk$_\alpha$ radiation. Fig. 3 shows an XRD pattern of bagasse samples with 2θ verses intensity having several peaks of cellulose indicating random orientation for the crystalline nature. The measured interplanar distances agreed with the values reported for cellulose in the literature.[39] The higher peak intensities of an XRD pattern are due to the better crystallinity. The XRD of α-cellulose, untreated bagasse (UB), pretreated bagasse (PB), *A. nidulans* MTCC344 cellulase hydrolyzed pretreated bagasse and *A. nidulans* SU04 cellulase hydrolyzed pretreated bagasse (HPB) confirm a decrease in the crystallinity of samples in the order of α-cellulose standard, UB,



F i g . 3 – *X-ray diffraction pattern of bagasse hydrolyzed by (a) A. nidulans MTCC344 and (b) A. nidulans SU04*

PB and HPB respectively. Cellulose content was slightly affected by the alkali pretreatment but fermentation seems to tremendously decrease the cellulose content of the bagasse possibly due to the breakdown of cellulose to glucose by cellulase synthesized by *Aspergillus* strains. Cellulase synthesized by *A. nidulans* SU04 seems to have a better influence on the crystallinity of bagasse samples when compared with *A. nidulans* MTCC344 as observed from the decrease in the intensity of the peaks in the XRD pattern.

FTIR spectra of bagasse samples

The Fourier transform infrared (FTIR) spectra of bagasse showed peaks corresponding to cellulose and lignin.[40–42] The lignocellulosic composition of the bagasse could be identified from the peak presence between 1600 cm^{-1} and 1000 cm^{-1}. The FTIR spectra of the bagasse samples are shown in Fig. 4. Band intensities of lignin peaks were predominant in the untreated bagasse 1238 cm^{-1}. These peaks from 1600 cm^{-1} to 1000 cm^{-1} were not apparent in HPB. This region is associated with the carbonyl groups including ketones, esters and aldehydes which are cellulose related. The FTIR band at 2900 cm^{-1}, assigned to C-H stretching vibration is a measure of the general organic material content of the bagasse. The peak at 1735 cm^{-1} attributed to C=O stretching of the carbonyl and acetyl groups in the 4-O-methyl – glucano acetyl xylan component of hemicellulose in the bagasse, disappears on pretreatment and more readily on hydrolysis. Complete disappearance of 1650 cm^{-1} and 1540 cm^{-1} peaks, attributed to the primary and secondary amides in the solid structure of bagasse could also be inferred from the FTIR spectra. The band 1508 cm^{-1} indicating the presence of aromatic rings of lignin decreases more in intensity on hydrolysis than on pretreatment. 1433 cm^{-1} band assigned to CH_2 symmetrical deformation was absent in untreated bagasse but appeared in the bagasse after fermentation indicating the change in environment of C_6 carbon atom due to the formation of hydrogen involving O_6 atom. Peak at 1370 cm^{-1} bandwidth attributed to the aryl aldehyde or organic sulphates disappears after pretreatment and hydrolysis. Peak at 1267 cm^{-1} bandwidth attributed to C-O-C bond in the cellulose chain becomes weak on pretreatment and shifts to 1298 cm^{-1} possibly due to the depolymerization by alkali treatment.[41] Total disappearance of peak at 1230 cm^{-1} responsible for CH deformation of CH_3 or CH_2 bending was observed in the pretreated bagasse. 1170 cm^{-1} bandwidth assigned to the C-O-C stretching vibrations decrease in intensity due to fermentation. The lignin peak band intensity 1238 cm^{-1} was seen to shift after the alkali pretreatment proving delignification effect of sodium hydroxide and it totally disappeared in samples after the hydrolysis during the fermentation. There was a drastic change in the band intensity of cellulose in the untreated, pretreated and fungal hydrolyzed samples. The peak intensity was high in the untreated and alkali treated samples whereas in the fungal treated samples the peak intensity showed an incredible decline. The band intensities of all the cellulose peaks of fungal treated samples were lower than those of alkali samples, providing evidence for the hydrolysis effect of cellulase produced by *A. nidulans* strains on bagasse. It is quite obvious that crystallinity of bagasse decreases during fermentation involving depolymerization as evidenced by the decrease in the intensity of absorption band at 1267 cm^{-1}.

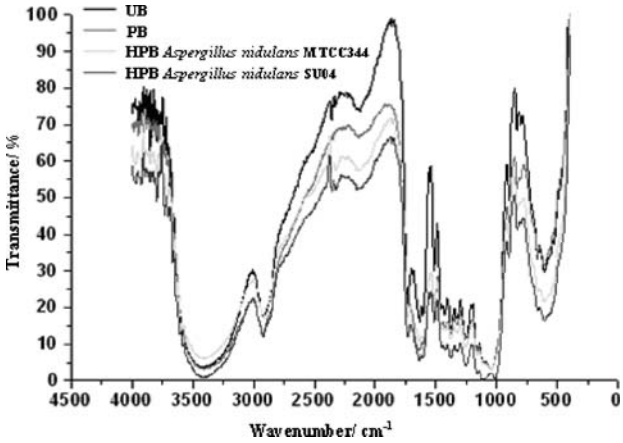
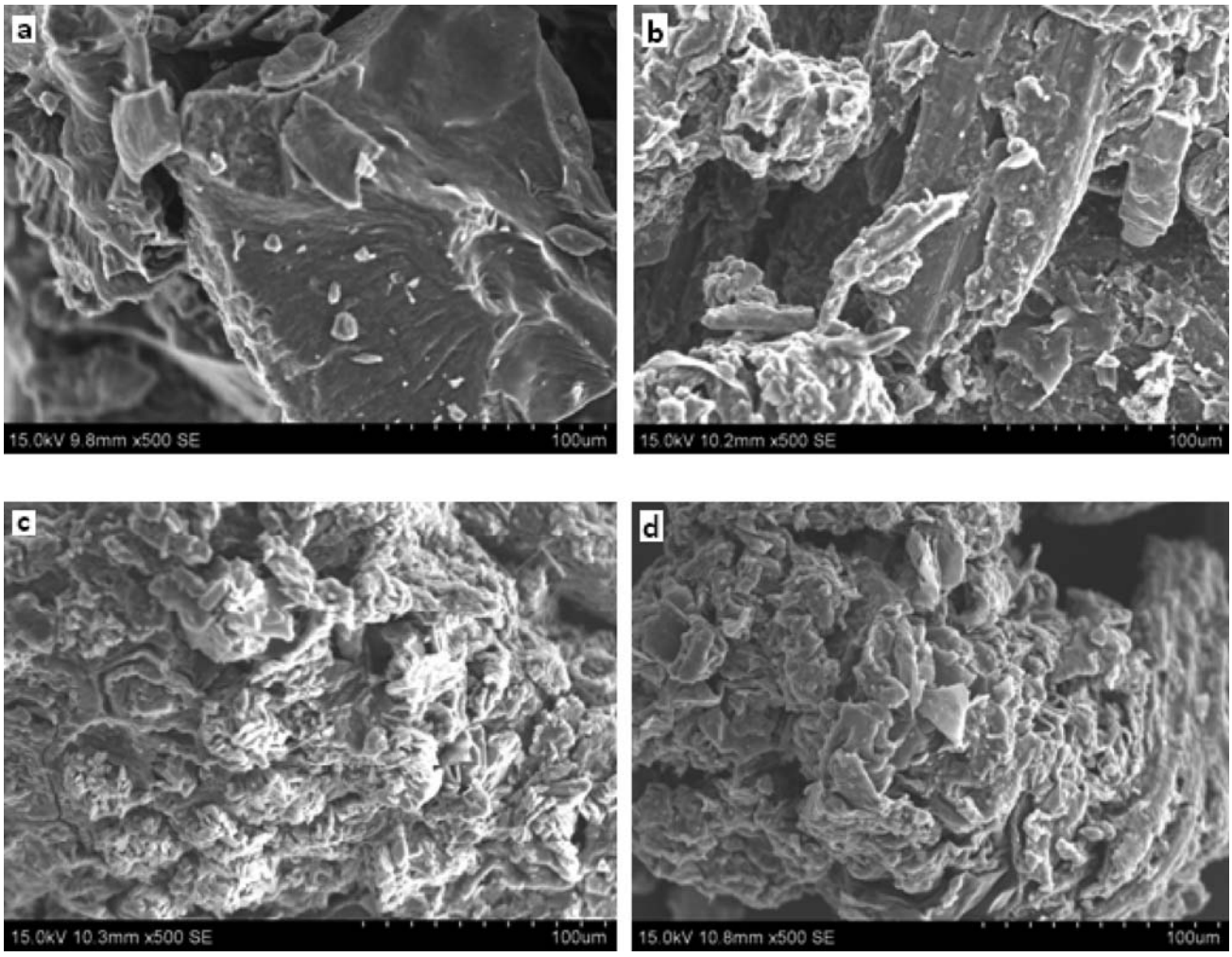


Fig. 4 – *FTIR spectra of bagasse*

Scanning electron microscopy of bagasse

A change in the surface morphology was observed by subjecting the treated samples to scanning electron microscopy (SEM) using Philips XL30 Scanning electron microscope with electron acceleration voltage of 20 kV and probe current of $5 \cdot 10^{-11}$A after subjecting the fibers to gold-sputtering in a Denton vacuum desk I for 1.5 min under a 26.66 Pascal Argon atmosphere and a current of 30 mA. A large fraction of lignin and hemicellulose was removed by pretreatment. Further, a large fraction of cellulose was removed by hydrolysis. This resulted in physical changes in the coir pith. SEM micrographs of UB, PB, *A. nidulans* MTCC344 hydrolyzed bagasse and *A. nidulans* SU04 hydrolyzed bagasse, shown in Fig. 5a, Fig. 5b, Fig. 5c and Fig. 5d, provide a qualitative confirmation change in the morphology of bagasse due to the treatment. From these SEM micrographs one could thus expect the effect of enzyme hydrolysis on bagasse to be governed largely by mechanical interlocking and by Van der Waals interactions. Bagasse was seen to develop a smoothened surface initially and later a

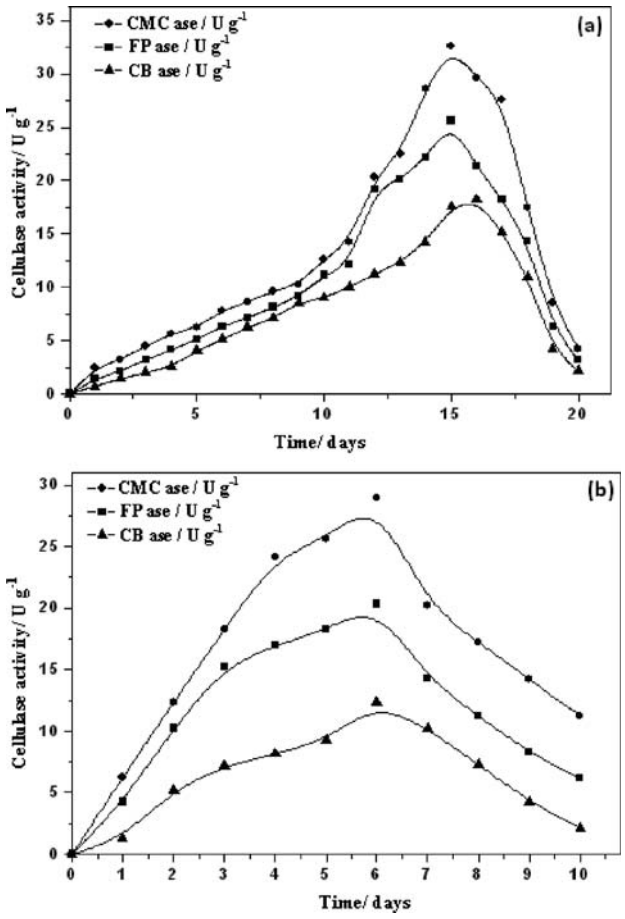


F i g . 5 – *SEM images of (a) untreated bagasse, (b) pretreated bagasse (c) A. nidulans MTCC344 hydrolyzed bagasse (d) A. nidulans SU04 hydrolyzed bagasse*

rough surface possibly due to the accumulation of the enzyme in the matrix of the pith and removal of the portion of micro fibrils. The increase in surface roughness, however, is relatively small and is found to be localized and very superficial, as expected from the long treatment time. A linear reduction in weight change was seen possibly due to the roughening of the bagasse surface by the formation of cavities and stripping off of the wall. Bagasse is made up of highly ordered crystalline region and less structured amorphous region. The degree of departure from crystallinity is variable to form the less structured purely amorphous region with all degrees of order in-between. A single type of cellulase preferentially endoglucanase may probably be sufficient for degradation of cellulose.[43,44] Application of bagasse as substrate in enzyme hydrolysis using *A. nidulans* strains may probably have an added advantage mainly in the reuse of bagasse which might otherwise be dumped as waste. *A. nidulans* SU04 seems to be more promising compared to *A. nidulans* MTCC344.

Model validation

Validation of this statistical model was carried out by taking x_1(15 mm), x_2(60 %), x_3(pH 5) and x_4(40 °C) in the validation experiments (Fig. 6a and Fig. 6b). Under these conditions, *A. nidulans* MTCC344 and *A. nidulans* SU04 produced cellulase of 28.94 U g^{-1} and 32.57 U g^{-1} activity respectively, in agreement with the predicted value of 28.84 U g^{-1} and 32.68 U g^{-1} activity suggested by the model. The exoglucanase activity was observed to be 20.3 U g^{-1} and 25.4 U g^{-1} respectively for *A. nidulans* MTCC344 and *A. nidulans* SU04 respectively. The β-glucosidases activities were observed to be 10.98 U g^{-1} and 18.52 U g^{-1} respectively for *A. nidulans* MTCC344 and *A. nidulans* SU04 respectively. These activities were revealed on the 15th day and 6th day for *A. nidulans* MTCC344 and *A. nidulans* SU04 respectively, proving the efficiency of *A. nidulans* SU04 over *A. nidulans* MTCC344 in terms of activity and synthesizing time.[45–47] The entire solid state fermentation of



F i g . 6 – *Time course of cellulase activity of (a) A. nidulans SU04 and (b) A. nidulans MTCC344 on bagasse fermentation*

pretreated bagasse by *A. nidulans* SU04 could be scaled up in future studies for improvement in the yield of cellulase.

Conclusions

Alkali pretreatment using NaOH on bagasse was seen to increase the feasibility of cellulose exposure to *Aspergillus* strains and hence the production of cellulase of significant activity and yield. The techniques adopted including response surface methodology, solid state fermentation and alkali pretreatment facilitated the fabrication cellulase of desired activity in a shorter period and therefore helped in reducing the cost of cellulase production. As revealed by XRD studies, FTIR spectra and SEM analysis, *A. nidulans* SU04 was highly efficient compared to *A. nidulans* MTCC344 in terms of degrading the substrate, bagasse. The adopted approach represents a novel solution to the necessity of reusing one of the few dumped waste 'bagasse'.

ACKNOWLEDGMENTS

The gift of bagasse from Kothari sugar mills, Ariyalur, Tamilnadu, India is gratefully acknowledged.

List of abbreviations and symbols

RSM – Response surface methodology
MTCC – Microbial Type Culture Collection
CMCase – Carboxy methyl cellulase or Endo β-1, 4 glucanase activity, U g^{-1}
FTIR – Fourier transform infrared spectroscopy
XRD – X-ray diffraction
SEM – Scanning electron microscopy
SSF – Solid state fermentation
CCD – Central composite design
MA – Malt Extract Agar
ODMC – oven dry mass of cellulose after pretreatment, mg
ODMH – oven dry mass of hemicelluloses after pretreatment, mg
ODML – oven dry mass of lignin after pretreatment, mg
ODMS – oven dry mass of bagasse sample before pretreatment, mg
Y – measured response, U g^{-1}
x_1, x_2, x_3, x_4 – coded independent input variables
β_0 – intercept term
β_1, β_2, β_3, β_4– linear coefficients
β_5, β_6, β_7, β_8– quadratic coefficients
β_9, β_{10}, β_{11}, β_{12}, β_{13}, β_{14} – cross product coefficients
ANOVA – Analysis of variance
y – mass concentration, g mL^{-1}
φ – volume fraction, %
UB – untreated bagasse
PB – pretreated bagasse
HPB – enzyme hydrolyzed pretreated bagasse
R^2 – coefficient of determination
R – correlation coefficient
Pred R^2 – predicted determination of coefficient
Adeq Pre – adequate precision
CV – coefficient of variation
PRESS – predicted residual sum of squares

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