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A new combinational statistical approach for cellulase optimization in *Aspergillus nidulans*

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Abstract

The enhancement of the cellulase activity of *Aspergillus nidulans* by combinational optimization technique was investigated. The strain isolated from decayed, dry leaf of *Ficus caricus* was compared for the first time for its ability to produce cellulolytic enzyme in submerged fermentation (SmF). The medium ingredients enhancing the cellulase production were optimized by combinational statistical approach by one factor at a time methodology (OFAT), Plackett Burmann methodology (PB) and response surface methodology (RSM). A four-factor-five-level central composite design (CCD) was employed to determine the maximum activity of cellulase at optimum levels of carboxy methyl cellulose (CMC), ammonium nitrate and potassium dihydrogen phosphate at varying pH values. The optimum fermentation parameters were found to be 1.2 g/l CMC, 0.9 mg/l ammonium nitrate and 0.75 mg/l potassium dihydrogen phosphate at pH 6. The optimization of medium by combinational statistical approach led to the fine tuning of the cellulase production thereby enhancing the cellulase activity from 4.91 U/ml to 39.56 U/ml. The predicted results were in agreement with the actual experimental values. The cellulase activity obtained with this strain may be one of the best obtained in *Aspergillus nidulans*.

Keywords: *Aspergillus nidulans*, cellulase activity, submerged fermentation, response surface methodology

Introduction

In recent years, more attention is given to the process of cellulose biodegradation to soluble sugar (Chen *et al*., 2008). Cellulase production in fungi is found to be extracellular and has three components such as endoglucanase, exoglucanase and β-glucosidases (Breznak & Brune, 1994; Yi *et al*., 1999). Until now cellulolytic enzymes have been isolated from bacteria and fungi (Tomme *et al*., 1995), plants (Brummell *et al*., 1994), molds (Blume & Ennis, 1991), microbes from animal intestines (Moriya *et al*., 1998) and herbivorous invertebrates such as arthropods (Watanabe *et al*., 1997; Tokuda *et al*., 1997; Watanabe *et al*., 1998), nematodes (Smant *et al*., 1998) and mollusks (Yokoe & Yasumasu, 1964).

A number of microorganisms particularly fungi possessing cellulose degrading enzymes have been isolated and studied extensively (Kim *et al*., 2003). Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee & Koo, 2001). They are studied extensively due to their application in the hydrolysis of cellulose, the most abundant biopolymer and potential source of utilizable sugars, which serves as a raw material in the production of chemicals and fuel. Cellulases have a wide range of industrial applications. The main applications include textile, paper and pulp, food, animal feed, fuel and chemical industry. Additionally they can be used in waste management, pharmaceutical industry, protoplast production and genetic engineering (Bhat, 2001). However, not many studies are available on the fungi *Aspergillus nidulans* to exploit its potential in the production of cellulase. In the present study, one factor at a time methodology (OFAT) (Skowronek & Fiedurek, 2004), Plackett Burmann methodology (PB) (Berekaa *et al*., 2006) and response surface methodology (RSM) (Amani *et al.,* 2007) have been adopted to optimize the medium requirements for the production of cellulase by *A. nidulans.* The central composite design (Guangrong *et al.,* 2008) was used to optimize the levels of identified controllable factors affecting the medium.

Materials and methods

Microorganism, medium and culture conditions

Aspergillus nidulans isolated from decayed, dry leaf of *Ficus caricus* in the laboratory was maintained on agar slants at 4^0C and sub cultured every 4 weeks. The initial medium (IM) components chosen were CMC, cellulose, sucrose, glucose, yeast extract, ammonium nitrate, peptone, urea, calcium chloride, potassium dihydrogen phosphate, manganese sulphate.H_2O and thiamine hydrochloride. 0.005 mg of streptomycin was added to prevent bacterial growth during incubation. The pH of medium employed for the studies was 3, 4, 5, 6 and 7. *A. nidulans* was grown in shake flask under submerged fermentation (SF) conditions with IM in order to examine the effect of incubation time on cellulase activity and dry cell weight of the fungus. After 8 d of incubation at 30^0C, the culture broth was filtered through glass fiber filter paper (Whatman GF/C) to remove hyphal fragments and residual insoluble cellulose. The culture filtrate was freeze

Indian Journal of Science and Technology Vol. 3 No. 8 (Aug 2010) ISSN: 0974- 6846

dried and resuspended in 100 ml of 0.05 M citrate buffer (pH 4.8). The mixture was stirred overnight and insoluble material was removed by centrifugation (40 min. at 5000 g). Cellulases were then assayed.

Materials

CMC and Cellulose were procured from Sigma chemicals (Mumbai, India). Agar and other medium components were purchased from Hi-media (Mumbai, India). All other analytical grade reagents were supplied by Merck (Mumbai, India) and SD Fine chemicals (Mumbai, India).

Enzyme assay

Gel diffusion assay (Saczi & Radford, 1986) was performed to assess the magnitude of the cellulase activity. Assay for endo beta-1, 4 glucanase, exo beta-1, 4 glucanase and β-glucosidase was carried out by carboxy methyl cellulose (CMC) assay, filter paper (FP) assay and cellobiase assay according to IUPAC recommendation (Ghose, 1987). One unit of CMC activity was defined as the amount of enzyme needed to liberate one µmol of glucose/min. from 1 ml of culture broth under assay conditions during the hydrolysis of CMC. One unit of FP degrading activity was defined as the amount of enzyme needed to liberate 1 µmol of glucose/min. during hydrolysis of 50 mg of Whatman filter paper. One unit of cellobiase activity was defined as the amount of enzyme needed to liberate 2 µmol of glucose/min. during hydrolysis of cellobiose. Glucose in the culture supernatant was analyzed by using UV Visible spectrophotometer (Hitachi Model-100-40) at 540 nm.

One factor at a time methodology (OFAT)

In the medium selected, glucose, sucrose, CMC and cellulose were substituted as four different carbon sources. All carbon sources were used at 2% (w/v) concentration. The effect of different nitrogen sources on cellulase production was studied by employing yeast extract, peptone, urea and ammonium nitrate at 0.5% (w/v) concentration. To study the effect of mineral salts on cellulase production, calcium chloride, potassium dihydrogen phosphate, manganese sulphate.H_2O and thiamine hydrochloride were employed at 0.5 mg/l concentration. The fermentation experiments were carried out at initial pH values varying between 3 and 7.

Table 1. Medium components for screening using PB design

Variables	Medium component	Levels	
		L(−1)	H(+1)
A	KH_2PO_4 (mg/ l)	0.25	0.75
B	Urea (mg/l)	0.3	0.5
C	NH_4NO_3 (mg/l)	0.3	1.5
D	$MnSO_4.H_2O$ (mg/l)	0.1	0.1
E	Thiamine HCl (mg/l)	0.01	0.01
F	Peptone (mg/l)	0.1	5.0
G	CMC (g/l)	0.2	2.0

Plackett Burmann screening design (PBSD)

Plackett Burmann screening design (PBSD) was used to screen (n) variables in just (n+1) number of experiments (Plackett & Burman, 1946; Ghanem *et al.,* 2000) resulting in a tremendous decrease in the number of total experiments. PBSD was employed to evaluate the relative importance of various components in promoting cellulase activity and to screen the important variables affecting the cellulase production. The preliminary information for the trails was taken from the results of OFAT trails. The PBSD was set up for 7 variables in 2 levels, high and low. The high level of each variable was set far enough from low level to identify the ingredients of the media having significant influence on cellulase production. The PBSD matrix was developed using SAS package version 8.01. The medium components used in the PBSD trials are shown in Table 1.

Central composite design (CCD)

After optimizing the values of medium components by OFAT and PBSD 4 most important variables namely, CMC at 2 mg/l, ammonium nitrate at 0.3 mg/l and 1.5 mg/l, potassium dihydrogen phosphate at 0.75 mg/l and pH at 5 were observed to mainly control the cellulase production by *A. nidulans* under SmF conditions. Based on the results from PBSD, CMC, ammonium nitrate, potassium dihydrogen phosphate and pH were selected for further evaluation of their effects on cellulase activity by CCD, a very useful tool for determining optimal level and interaction of medium constituents. RSM consists of a group of empirical techniques devoted to the evaluation of existing relation between a cluster of controlled experimental factors and the measured responses according to 1 or more selected criteria. A prior knowledge and understanding of the process as well the process variables under investigation are necessary for achieving a more realistic model. For RSM analysis based on CCD, 30 experiments were performed in triplicate. The coded levels of the independent variables are given in Table 2. A 2^4 factorial CCD was developed by design expert package version 7.1.6 with 8 axial points and 6 replicates at the center points leading to 30 runs. The variables were coded according to the following equation.

$$x_i = \frac{X_i - X_0}{\Delta X}, \text{ i=1,2,3,...,k} \quad (1)$$

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 polynomial model was used to fit the quadratic resulting in the equation,

Indian Journal of Science and Technology Vol. 3 No. 8 (Aug 2010) ISSN: 0974- 6846

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \beta_5 x_1^2 + \beta_6 x_2^2 + \beta_7 x_3^2 + \beta_8 x_4^2 + \beta_9 x_1 x_2 + \beta_{10} x_1 x_3 + \beta_{11} x_1 x_4 + \beta_{12} x_2 x_3 + \beta_{13} x_2 x_4 + \beta_{14} x_3 x_4 \quad (2)$$

Where Y is the measured response (cellulase activity (U/ml)), x_1, x_2, x_3 *and* x_4 are the coded independent input variables, β_0 is the intercept term, β_1, β_2, β_3 *and* β_4 are the coefficients showing the linear effects, β_5, β_6, β_7 and β_8 are the quadratic coefficients showing the squared effects and β_9, β_{10}, β_{11}, β_{12}, β_{13}, *and* β_{14} are the cross product coefficients showing the interaction effects.

The optimum values of CMC, NH_4NO_3, KH_2PO_4 and pH were obtained by solving the regression equation by Monte Carlo optimization (Conley, 1984). The goodness of fit of the model was evaluated by coefficient of determination R^2 and analysis of variance (ANOVA).

Table 2. Levels of the variables tested in CCD

Variables & symbol code		Coded levels				
		-2	-1	0	+1	+2
CMC (g l^{-1})	X_1	0.40	0.8	1.20	1.60	2.00
NH_4NO_3 (mg l^{-1})	X_2	0.30	0.6	0.9	1.20	1.50
KH_2PO_4 (mg l^{-1})	X_3	0.25	0.5	0.75	1.00	1.25
pH	X_4	4.0	4.5	5.0	5.5	6.0

Results and discussion

Visualization of zones of CMC hydrolysis by gel diffusion assay

CMC agar plates examined after gel diffusion assay produced zones of hydrolysis within 5 days. *A. nidulans* showed activity against CMC (zone of clearing diameter 1.3 cm). CMC agar plated with *A. nidulans*, stained with 1% congo red and fixed with NaOH. This enhanced the contrast between the halo and the background. Halo formation on CMC agar plates resulted from cleavage of CMC into fragments smaller than cellohexaose to which congo red did not bind.

Moreover, halos could result from cleavage of CMC into fragments small enough to be washed out of the plates during the staining procedure (Saczi & Radford, 1986). In either case only endoglucanase activity would be expected to produce a zone of hydrolysis. The fungi used in this study exhibited strong cellulolytic activity which got confirmed by the production of zone of hydrolysis. Fig. 1 depicts the growth profile and activity of cellulase produced by *A. nidulans.* There was a significant increase in the biomass and cellulase activity (4.91 U/ml), which were at their peak on the 6th day of incubation. The initial pH variation from 3 to 7 had significant effect on cellulase activity (Fig. 2a).

Cellulase activity was found to be predominant at pH value of 5 (Robson & Chambliss, 1984; Zverlov *et al.*, 1998). Earlier reports also suggested maximum production of cellulase at this value of pH (Geeraerts & Vandamme, 2008).

Optimization by OFAT

The experimental combinations for OFAT have been shown in Table 3. During SmF, carbon source was seen to act as major constituent for the synthesis of cellulase. The effect of different carbon sources on cellulase production has been shown in Fig. 2b. It was seen that carbon source that provided free glucose, did not aid cellulase production, whereas the source that provided complex carbon (CMC) aided in the production of cellulase. Glucose, being the easily assimilated carbon source supported growth without the need or with less need for producing cellulose (Niranjane *et al.*, 2007). When used as carbon source, it was seen to provide maximum dry cell weight of 4.523 g/l and cellulase activity of 4.32 U/ml, whereas CMC supported maximum cellulase activity of 20.5 U/ml with dry cell weight of 3.412 g/l after 5 d of incubator shaking.

Fig. 2c shows the effect of different nitrogen sources on cellulase activity. The organic nitrogen sources, yeast extract and peptone gave more biomass with least cellulase activity, whereas the inorganic nitrogen sources, NH_4NO_3 and Urea supported least biomass and higher cellulase activity. This may be attributed to the fact that complex substances like amino acids and vitamins in organic nitrogen sources could trigger the biomass production, thus making it unnecessary for the fungus to produce cellulase (Wen *et al.*, 2005). Absence of these in the inorganic nitrogen sources instigated *A. nidulans* to make the most use of carbon source for cellulase production but with less increase in biomass. Maximum cellulase activity of 14.31 U/ml was observed when NH_4NO_3 was employed in the medium as the chief nitrogen source. The maximum activity of cellulase was obtained when the fungal strain was supplied with an optimum level of carbon and nitrogen source.

Table 3. OFAT design matrix with experimental values

Run	Component				Medium component				U/ml
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	
1	A1	B1	C1	D1	G	Y	CC	3	4.32
2	A2	B1	C1	D1	S	Y	CC	3	6.32
3	A3	B1	C1	D1	C	Y	CC	3	20.5
4	A4	B1	C1	D1	C	Y	CC	3	5.41
5	A1	B1	C1	D1	G	Y	CC	3	3.21
6	A1	B2	C1	D1	G	A	CC	3	14.31
7	A1	B3	C1	D1	G	P	CC	3	2.14
8	A1	B4	C1	D1	G	U	CC	3	12.41
9	A1	B1	C1	D1	G	Y	CC	3	1.03
10	A1	B1	C2	D1	G	Y	K	3	5.92
11	A1	B1	C3	D1	G	Y	M	3	4.91
12	A1	B1	C4	D1	G	Y	T h	3	4.51
13	A1	B1	C1	D1	G	Y	CC	3	2.13
14	A1	B1	C1	D2	G	Y	CC	4	3.14
15	A1	B1	C1	D3	G	Y	CC	5	5.92
16	A1	B1	C1	D4	G	Y	CC	6	4.01

Indian Journal of Science and Technology Vol. 3 No. 8 (Aug 2010) ISSN: 0974- 6846

Fig.1 Time course of dry cell weight (Δ) & cellulase activity (▲).

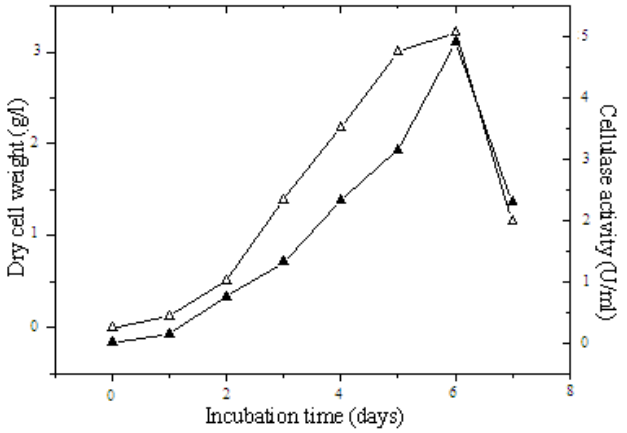


Fig.2.a.Effect of pH on dry cell weight ( ) and cellulase activity ( ) of A. nidulans grown on Initial medium

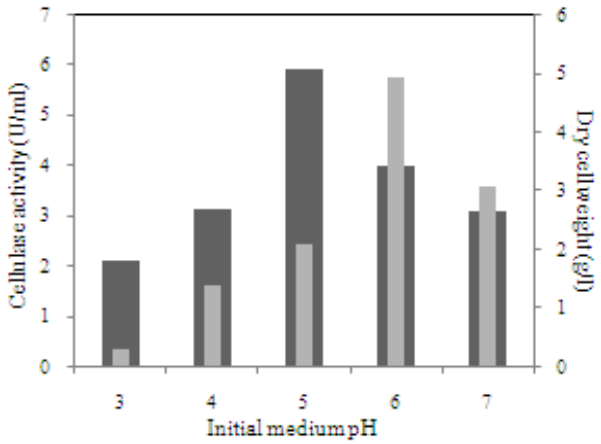


Fig.2.b.Effect of carbon source on dry cell weight ( ) and Cellulase activity ( )

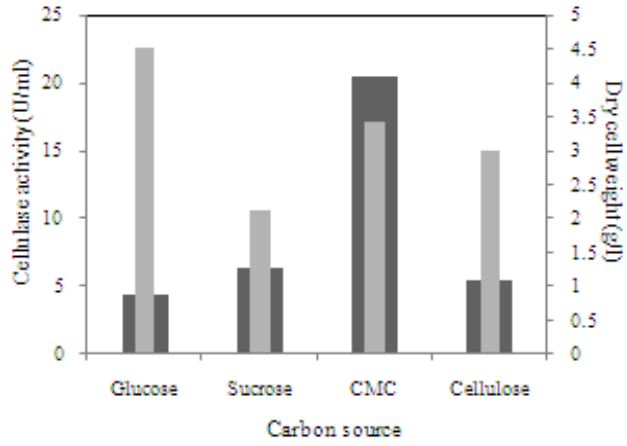


Fig.2.c. Effect of Nitrogen sources on dry cell weight ( ) and Cellulase activity ( )

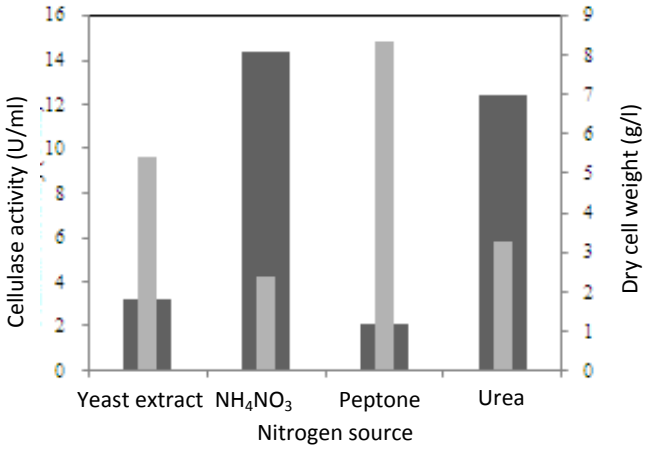


Fig.2.d. Effect of Mineral salts on dry cell weight ( ) and cellulase activity ( )

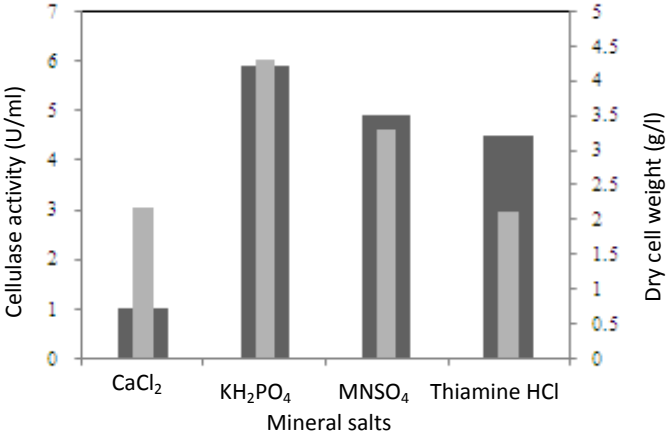
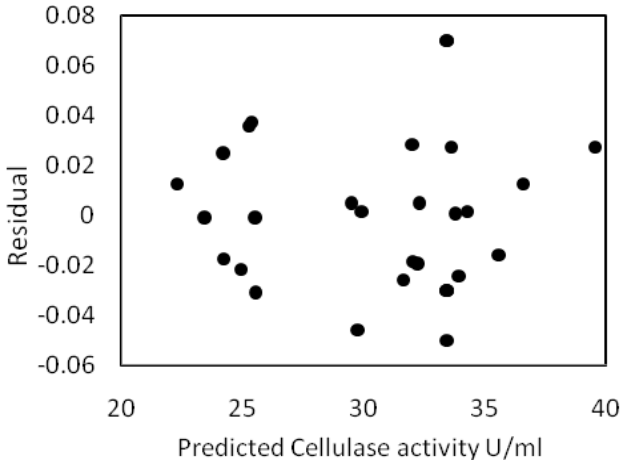


Fig.3.Residuals versus predicted cellulase activity



Indian Journal of Science and Technology Vol. 3 No. 8 (Aug 2010) ISSN: 0974- 6846

Table 4. PB design matrix with experimental values for cellulase activity

Run	Variables							(U/ml)
	A	*B*	*C*	*D*	*E*	*F*	*G*	
1	+1	+1	+1	−1	+1	−1	+1	40.32
2	−1	+1	+1	+1	−1	+1	−1	10.31
3	−1	−1	+1	+1	+1	−1	+1	29.18
4	+1	−1	−1	+1	+1	+1	−1	17.21
5	−1	+1	−1	−1	+1	+1	+1	17.10
6	+1	−1	+1	−1	−1	+1	+1	35.61
7	+1	+1	−1	+1	−1	−1	+1	20.19
8	−1	−1	−1	−1	−1	−1	−1	11.12

Fig. 2d shows the effect of different mineral salts on the activity of cellulase. KH_2PO_4, $MnSO_4.H_2O$ and thiamine hydrochloride were seen to support the maximum activity of cellulase compared to $CaCl_2$. $2H_2O$.

Optimization by PBSD

The second optimization was done by using 8 run PBSD to identify the significant factors affecting cellulase activity of *A. nidulans*. The experimental design for screening the medium components has been shown in Table 4. The experimental data for cellulase activity in Plackett Burmann trials were also listed. The results showed a wide variation from 11.12 U/ml to 40.32 U/ml, which reflected the importance of medium optimization for higher cellulase activities.

According to the resulting effects of the 7 variables on cellulase activity, associated significant levels have been shown in Table 5. CMC *(G)* showed large effect followed by NH_4NO_3 *(C)* and KH_2PO_4 *(A).* Thiamine HCl *(E)* showed least effect followed by Urea *(B)*.It could be seen with the high concentration of CMC and with least concentration of urea, the cellulase activity of the enzyme produced by the fungus was predominating, provided, NH_4NO_3 and KH_2PO_4 were held at high concentration and pH was maintained at 5. $MnSO_4.H_2O$ *(D)* and Peptone *(F)* are dummy variables.

Table 5. PBSD results and significant levels

Trials	*A*	*B*	*C*	*D*	*E*	*F*	*G*
∑H	113.3	87.9	115.4	76.89	103.8	80.23	142.4
∑L	67.71	93.1	65.62	104.2	77.23	100.8	38.64
∑H-∑L	45.62	-5.2	49.8	-27.3	26.58	-20.6	103.8
Effect	11.40	1.3	12.45	-6.82	6.645	-5.15	25.94
MS	260.2	3.38	310.0	92.88	88.31	52.94	1345.8
F	2.872	0.04	3.422	1.025	0.975	0.584	14.86

MS-mean square; EMS-error mean square=90.60

Optimisation by RSM

The effect of 4 variables on cellulase activity was studied by the third optimization technique, the response surface methodology.

Fig.4(a).Response surface plot showing the effect of CMC and NH_4NO_3 on cellulase activity of A. nidulans (KH_2PO_4 0.75 mg/l and pH 5.0)

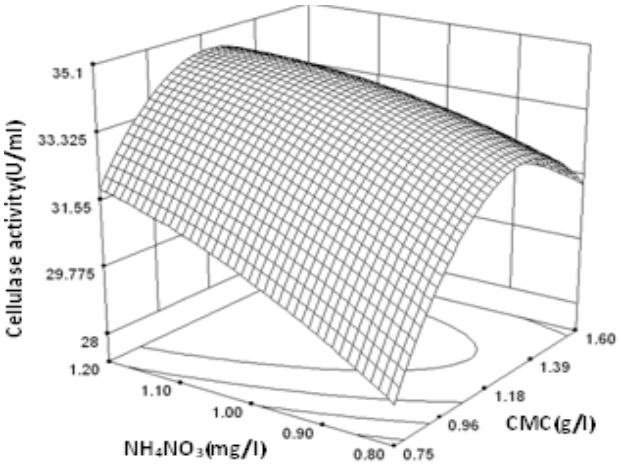


Fig.4 (b). Response surface plot showing the effect of CMC and KH_2PO_4 on cellulase activity by A. nidulans (NH_4N0_3 0.90 mg/l and pH 5.0)

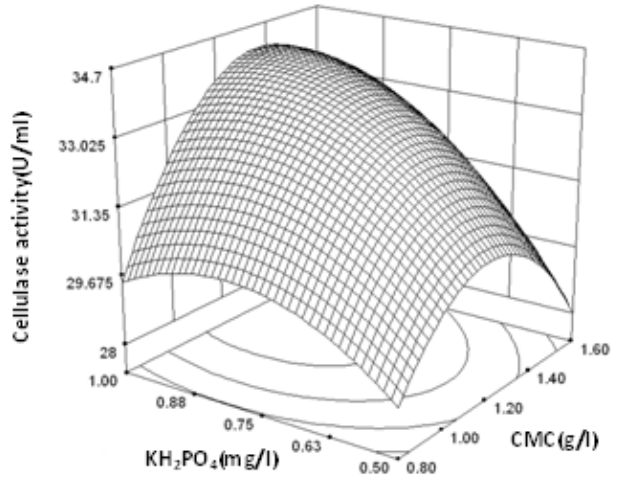
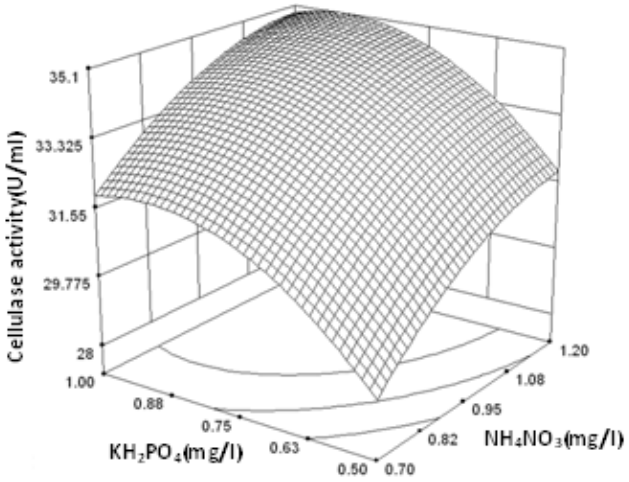


Fig.4(c). Response surface plot showing the effect of NH_4N0_3 and KH_2PO_4 on cellulase activity by A. nidulans(CMC 1.2g/l and pH 5.0)



Indian Journal of Science and Technology Vol. 3 No. 8 (Aug 2010) ISSN: 0974- 6846

$$Y_i = 33.43 + 0.80x_1 + 2.17x_2 + 1.57x_3 + 0.99x_4 - 2.38x_1^2 - 0.96x_2^2 - 1.23x_3^2 + 1.04x_4^2 - 1.08x_1x_2 + 1.02x_1x_3 + 1.54x_1x_4 - 0.065x_2x_3 + 1.13x_2x_4 - 1.37x_3x_4 \quad (3)$$

Table 6 gives the CCD matrix with experimental and predicted values for cellulase activity. The regression equation showed the cellulase activity as an empirical function in terms of coded factors, where Y_i is the predicted cellulase activity in U/ml. ANOVA for response surface quadratic model gave F value = 23338.52, with p-values of all the coefficients (p< 0.0001), implying the significance of the model. The coefficient of variation of the model was (C.V=0.14%). The goodness of fit of the model was examined by determination coefficient (R^2=0.999) which implied that sample variation of more than 99.9% was attributed to the variables and only 0.1% of total variance could not be explained by the model (Haaland,1989). The adjusted determination coefficient (Adj R^2 =0.999) was also satisfactory to confirm the significance of the model. The "Pred R-Squared" of 0.9998 was in reasonable agreement with the "Adj R-Squared" of 0.9999. Adeq precision measured the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 575.478 obtained in this model indicated an adequate signal which could be used to navigate the design space. The results of the response surface quadratic model in the form of analysis of variance (ANOVA) with significance of each coefficient, determined by student's t- test and p- value are listed in Table 7. The larger the magnitude of the t in t-test and smaller the p-value, the more significant is the corresponding coefficient. KH_2PO_4 has a strong positive linear effect on the response (p<< 1.4E-25). NH_4NO_3 also showed significant effect. Significant interaction was seen between CMC with KH_2PO_4 and pH of the medium and between NH_4NO_3 and pH of the medium (Elisashvili & Penninckx, 2008). The model predicted maximum cellulase activity of 39.56 U/ml with 1.2 g/l CMC, 0.9 mg/l NH_4NO_3 and 0.9 mg/l KH_2PO_4 at pH 5 (Adsul *et al.*, 2007; Martinsa *et al.*, 2008). Fig. 3 shows the variation of residuals against the predicted cellulase activity. Response surface contours plots and three dimensional graphs are employed to understand the relationship between the response and experimental levels of each variable.

Table 6. Central composite design matrix in coded terms showing the experimental and predicted values for cellulase activity

Trials	Experimental values				Cellulase activity (U/ml)	
	X_1	X_2	X_3	X_4	Experimental *	Predicted
1	-1	-1	-1	-1	25.50	25.53
2	1	-1	-1	-1	24.20	24.17
3	-1	1	-1	-1	29.90	29.89
4	1	1	-1	-1	24.20	24.21
5	-1	-1	1	-1	29.51	29.50
6	1	-1	1	-1	32.20	32.21
7	-1	1	1	-1	33.64	33.61
8	1	1	1	-1	32.03	32.00
9	-1	-1	-1	1	24.90	24.92
10	1	-1	-1	1	29.70	29.74
11	-1	1	-1	1	33.80	33.79
12	1	1	-1	1	34.30	34.29
13	-1	-1	1	1	23.40	23.40
14	1	-1	1	1	32.30	32.29
15	-1	1	1	1	32.00	32.01
16	1	1	1	1	36.60	36.58
17	-2	0	0	0	22.30	22.28
18	2	0	0	0	25.50	25.50
19	0	-2	0	0	25.30	25.26
20	0	2	0	0	33.90	33.92
21	0	0	-2	0	25.40	25.36
22	0	0	2	0	31.60	31.62
23	0	0	0	-2	35.57	35.58
24	0	0	0	2	39.59	39.56
25	0	0	0	0	33.38	33.43
26	0	0	0	0	33.40	33.43
27	0	0	0	0	33.50	33.43
28	0	0	0	0	33.40	33.43
29	0	0	0	0	33.50	33.43
30	0	0	0	0	33.40	33.43

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

These plots also showed the type of interaction between test variables and helped to obtain the optimum conditions (Haaland, 1989). Fig. 4a shows the cellulase activity as a result of interaction between CMC and ammonium nitrate with potassium dihydrogen phosphate and pH maintained at 0.75 mg/l and 5 respectively. A high carbon source and low nitrogen source was seen to enhance the activity of cellulase produced by the organism. Interaction between CMC and potassium dihydrogen phosphate at constant values of Ammonium nitrate 0.9 mg/l and pH 5.0 has been shown in Fig. 4b.

The cellulase activity was seen to increase with an increase in the concentration of CMC upto 1.2 g/l after which it started to decrease significantly. Similar trend was observed with KH_2PO_4 whose concentration of 0.75 mg/l was seen to provide a maximum cellulase activity. Fig. 4c shows the interaction between NH_4N0_3 and KH_2PO_4. The response surface clearly indicated the poor performance of the fungi in the production of cellulase with significant activity at low values of this nitrogen source and mineral salt.

Final optimized conditions are obtained by solving inverse matrix from eqn. (3) and through statistical analysis of the constraints. By both means the optimum values of the tested variables in uncoded (natural) units obtained were CMC 1.2 g/l, NH_4NO_3 0.9 mg/l, KH_2PO_4 0.75 mg/l at pH 5. At these optimized conditions the model predicted 39.56 U/ml of cellulase activity. The

Indian Journal of Science and Technology Vol. 3 No. 8 (Aug 2010) ISSN: 0974- 6846

subsequent experiments with optimized medium were observed to yield consistent results with prediction. Cellulase activity obtained by optimizing the medium contents was found to be significantly affected by the interaction of *A. nidulans* with the designed medium (Jorgensen *et al*., 2005). The present study using one factor at a time methodology, Plackett Burmann methodology and response surface methodology enabled us to find the optimum levels of the ingredients of the medium designed to produce cellulase of increasing activity.

Table 7. Analysis of variance (ANOVA), regression coefficient estimate and test of significance for cellulase activity (response surface quadratic model)

Factor	Sum of squares	Mean squares	Coefficient estimate	d.f	Computed t-value	F-value	P-value
Intercept	588.86	42.06	33.43	14	107.421	23338.52	4.539E-23
X_1	15.46	15.49	0.80	1	-1.209	8593.98	0.2453
X_2	112.49	112.49	2.17	1	0.657	62419.27	0.5207
X_3	58.84	58.84	1.57	1	158.256	32650.81	1.365E-25
X_4	23.72	23.72	0.99	1	-137.974	13161.98	1.089E-24
X_1*X_2	18.71	18.71	-1.08	1	-294.100	10379.20	1.257E-29
X_1*X_3	16.56	16.56	1.02	1	-118.303	9191.37	1.069E-23
X_1*X_4	38.19	38.19	1.54	1	-152.229	21191.84	2.442E-25
X_2*X_3	0.068	0.068	-0.065	1	127.812	37.51	3.357E-24
X_2*X_4	20.34	20.34	1.13	1	-101.878	11286.12	1.004E-22
X_3*X_4	30.20	30.20	-1.37	1	95.871	16754.33	2.495E-22
X_1^2	155.88	155.88	-2.38	1	145.574	86495.23	4.774E-25
X_2^2	25.22	25.22	-0.96	1	-6.124	13995.69	1.945E-05
X_3^2	41.76	41.76	-1.23	1	106.236	23173.72	5.360E-23
X_4^2	29.44	29.44	1.04	1	-129.438	16336.13	2.777E-24
Residual	0.027	1.802E-003		15			

Conclusion

The present study demonstrates the usage of combination optimization methodology to produce cellulase with significant activity. In this investigation one factor at a time methodology, Plackett Burmann methodology and response surface methodology based on four-factor-five-level central composite design (CCD) was used to solve the problem. The results presented here confirm the feasibility of medium optimization and cultivation conditions to improve cellulase activity. Optimization of process parameters resulted in increase in cellulase activity from 4.91 U/ml to 39.56 U/ml by *A. nidulans*. To the best of our knowledge, this magnitude of enzyme activity produced by *A. nidulans* has not been reported so far. A continued multidisciplinary research on the basic and applied aspects was essential to meet the growing demand for cellulase. This work on cellulase production would be continued with experiments on purification and batch fermentations aiming at higher yields. The data obtained in the present and future studies would be used to model the cellulase production by *A. nidulans.*

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