

# Statistical Optimization for Cellulase Production with *Aspergillus niger* using lignocellulosic biomass by Response Surface Methodology

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## Abstract

The study focuses on isolation and identification of fungus *Aspergillus niger* (GENBANK accession number: KP164968) and the optimum solid state fermentation conditions required for cellulase production based on Response Surface Methodology (RSM) using Taguchi model. When different lignocellulosic substrates were used, sugarcane baggase supported highest (0.153 U/ml) cellulase production. Statistical optimization using Taguchii model suggested that cellulose production was significantly influenced by culture parameters such as  $MnSO_4$ ,  $FeSO_4$  and Peptone which was the nitrogen source. These culture components were further optimized using RSM, and the optimal concentrations for growth of the organism was found to be 0.085g/l for  $MnSO_4$ , 0.132g/l for  $FeSO_4$  and 1.999g/l for peptone at an optimal temperature of 25°C with pH6. Under optimal media conditions a tenfold increase in cellulase production was observed.

**Keywords:** *Aspergillus niger*, solid state fermentation, cellulase, media optimization.

## I. INTRODUCTION

Lignocellulosic biomass such as agricultural crop residues provides a low cost feedstock for biological production of fuels and chemicals, and offer economic, environmental and strategic advantages [1]. Lignocellulosic biomass contains 75–80% of cellulose and hemicellulose. Lignocellulose is the most abundant, renewable organic material on the biosphere which can be utilized as a substrate for the study of bioethanol production [2]. Many microorganisms including fungi and bacteria have been found to degrade cellulose by producing hydrolytic enzymes collectively known as Cellulases. Cellulases are the enzymes which breaks cellulose into glucose monomers [3]. Cellulase contains endoglucanase and cellobiohydrolases, which act in a synergistic manner in biomass-degrading microbes[4]. *Trichoderma* and *Aspergillus* are known to be potential cellulase producing microorganisms. [5].

Cellulase produced by different microorganisms is affected by various parameters like pH, temperature, aeration, incubation time and growth nutrients [6] Therefore, knowing

the best suited conditions for the enzyme production plays a very important role in success of a process technology [7]. Optimization strategies involving one-factor-at-a-time approach are not adequate as they overlook the multiple factor interactions and may lead to misinterpretation of results. Statistical approach gives better understanding of effects of interacting factors and thus aid in minimizing errors in a significant manner. The main aim of the present study was to optimize the media parameters for maximum production of cellulase by the organism. Optimization was carried out using the statistical tools of Taguchii model and Response Surface Methodology RSM [8, 9].

## II. MATERIALS AND METHODS

The lignocellulosic materials, including rice straw, ragi straw, corn cob, pongamia seed cake, neem seed cake, ash-gourd seeds and sugarcane baggase were obtained from local market of Bangalore, India. Materials were sun dried, chopped into small pieces, milled into smaller particles and then separated by sieve. The flow-through material was used for solid state fermentation.

### A. Isolation and maintenance of microorganism

The wood chips were collected from agricultural fields of GKVK Bangalore. Isolation of the fungal strain was done using serial dilution method and pour plate technique. Samples were poured on Potato dextrose agar (PDA) plates and incubated at room temperature. The fungal isolate demonstrating highest zone of cellulose break down was identified as *Aspergillus niger* on the basis of morphological characteristics. Fungal colonies were preserved for further studies and identification [10].

### B. Screening of cellulose degrading fungi

The purified isolates were inoculated on carboxy methyl cellulose (CMC) agar plates containing 1% CMC agar media. Plates were flooded with aqueous solution of 1% Congo red for 15min at room temperature and the plates were washed properly with 1N NaCl for counter staining the petriplates. Clear zone of hydrolysis observed around the colonies indicates the cellulase activity[11]. Hydrolysis capacity (HC) ratio for the isolate was calculated by dividing the zone

diameter by colony diameter. Isolates which showed the highest ratio were selected for further identification and studies.

**C. Identification of the fungal isolate**

Fungal isolates which gave positive results in screening were selected for further studies and the morphological and molecular identification was carried out. 18s rRNA sequencing was performed and the isolate was found to be *Aspergillus niger* and the sequence was deposited in GEN Bank USA Collection Centre and the accession number (KP164968) was received.

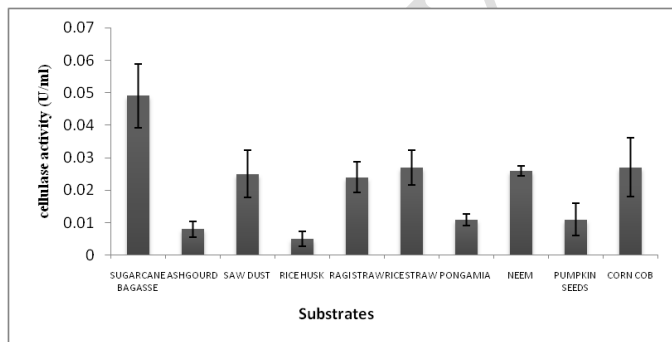
**D. Inoculum preparation**

The fungi selected for the study were grown on PDA slants and spores were harvested aseptically from 5-days-old slants. [12] was added to fungal slant and vortexed for some time. Spore count was measured with haemocytometer and adjusted to  $2 \times 10^6$  spores/ml by adjustment of optical density.

**E. Screening substrate and substrate preparation**

Various agricultural wastes were collected from the local market of Bangalore, India. The collected lignocellulosic wastes were dried in sunlight for a week, and sieved to mesh size ranging from 0.45mm to 1mm, crushed and used for further studies. Different substrates selected for screening included ragi straw, rice straw, pongamia cakes, neem cakes, pumpkin seeds, rice husk, saw dust, ash gourd seeds, sugarcane baggase and corn cob. Sugarcane baggase gave best results amongst the substrates screened as shown in Fig. 1 and was therefore selected for cellulase production.

Figure 1: Cellulase production by inoculation of *Aspergillus niger* on different substrate



**F. Enzyme production under solid state fermentation**

Solid state fermentation was carried out in Erlenmeyer flasks (250 mL) with 5g of substrate in it. The substrate moistened with minimal media, which included  $MnSO_4$ ,  $FeSO_4 \cdot 7H_2O$ ,  $CaCl_2 \cdot 2H_2O$  pH – 6, temperature 25°C, carbon source

(sucrose), and nitrogen source (peptone), was autoclaved at 120°C for 30 min. After cooling the substrate flasks were inoculated with *Aspergillus niger* and kept at room temperature for growth. The samples were withdrawn at regular intervals to determine the enzyme activity. Preliminary experiments were carried out for 7days and it was founded out that maximum activity was obtained on day four.

**G. Enzyme Assays**

Total cellulase activity was assayed using filter paper assay [13, 14]. The reaction mixture contained Sodium citrate buffer (0.5 ml, pH 4.8), filter paper (0.05g) and culture filtrate of 0.5 ml. After the incubation at 50°C the reaction was stopped using 3 ml of DNS [15] and the activity was estimated spectrophotometrically by reading the absorbance at 540nm.

**H. Optimization of cellulase production**

Optimization was first performed with single factor optimization. Further screening of the variables for maximum enzyme activity was then performed by using the Taguchii model. This experimental design assumes that there is no interactions between the different variables in the range under consideration. It calculates the difference between average value of the measurements made at highest level (+1) and the lowest (-1) of the variable factor under consideration and is therefore suitable to identify the variable which affect the cellulase activity significantly. In the present study seven variables such as carbon source, nitrogen source,  $MnSO_4$ ,  $FeSO_4$ ,  $CaCl_2$ , temperature and pH were studied along with a control. Variables which were found insignificant were eliminated and only the significant variables were retained for further optimization.

The statistical software package “Design-Expert software (version 9.0.3.1, stat-Ease. Inc, Minneapolis, USA), was used for analyzing the experimental data. Once the critical factors were identified through preliminary screening the central composite design (CCD) was used to obtain a quadratic model [9].

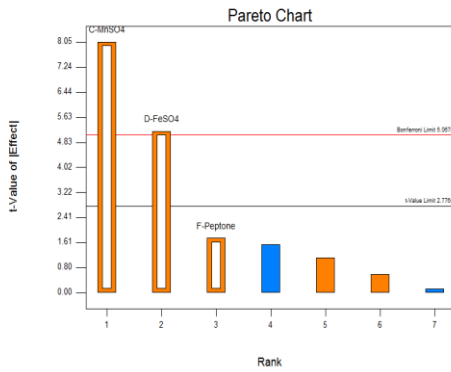
**III. RESULTS AND DISCUSSION**

Taguchii experiments showed a wide range of variations in cellulase production with different combinations of culture components. From the pareto chart (Fig. 2) the variables namely,  $MnSO_4$ ,  $FeSO_4$  and Peptone as nitrogen source were found to be positive factors in the final response, i.e. cellulase activity, while the other factors such a pH, temperature, C-source and  $CaCl_2$  were eliminated from further studies as being unimportant.

Figure 2: Pareto chart showing the effect of different media components on cellulase activity

Design-Expert® Software  
Script (Cellulase activity)

- A: maltose
- B: Temperature
- C: MnSO<sub>4</sub>
- D: FeSO<sub>4</sub>
- E: CaCl<sub>2</sub>
- F: Peptone
- G: pH
- Positive Effects
- Negative Effects



11	0	0	0	0.515
12	0	0	-1.68179	0.365
13	1	1	1	0.361
14	0	0	0	0.483
15	-1	-1	-1	0.407
16	-1.68179	0	0	0.353
17	-1	1	-1	0.423
18	0	0	0	0.515
19	-1	1	1	0.392
20	0	-1.68179	0	0.402

Table 1: Ranges of variables used in RSM

Variables	Code	-1.6817	-1	0	+1	+1.6817
MnSO <sub>4</sub>	A	0.029	0.05	0.1	0.15	0.25
FeSO <sub>4</sub>	B	0.029	0.05	0.1	0.15	0.25
Peptone	C	0.594	1	2	3	5.04

The ANOVA table for the model for cellulase production is given in (Table 3). The exactness of the model was examined by the F value recorded for the model. An F value obtained in 932.48 obtained in the present study suggests that the model is significant. The model terms for which the p value is less than 0.05 are considered to be significant while p values greater than 0.1 suggests that the model terms are not significant. The model level can also be assessed by a comparison between the coefficients of determination R<sup>2</sup> for the experimental and the predicted responses. The "Adj R-Squared" value 0.997 obtained in the present study. Also the "Adeq Precision" value, which is a measure of the signal to noise ratio, was 101.92 for the present study. The model was therefore suitable for navigation through the design space.

Table 3: Analyses of variance (ANOVA) for response surface quadratic model for the production of cellulase

ANOVA for Response Surface Quadratic Model					
Analysis of variance table [Partial sum of squares - Type III]					
Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	0.14	9	0.015	932.48	< 0.0001
A- MnSO <sub>4</sub>	0.032	1	0.032	1930.10	< 0.0001
B- FeSO <sub>4</sub>	6.714E-003	1	0.007	404.41	< 0.0001
C- Peptone	1.973E-004	1	0.000	11.88	0.0063
AB	6.661E-004	1	0.001	40.12	< 0.0001
AC	4.371E-003	1	0.004	263.27	< 0.0001
BC	1.361E-004	1	0.000	8.20	0.0169
A^2	0.081	1	0.081	4885.77	< 0.0001
B^2	3.058E-003	1	0.003	184.20	< 0.0001
C^2	0.021	1	0.021	1270.31	< 0.0001
Residual	1.660E-004	10	0.000		
Lack of Fit	1.585E-004	5	0.000	21.14	0.9950
Pure Error	7.500E-006	5	0.000		
Cor Total	0.14	19	0.015	932.48	

The concentrations of MnSO<sub>4</sub>, FeSO<sub>4</sub> and Peptone were further optimized using CCD of RSM (Table1). The results of CCD study are summarized in (Table2). Response from 20 different combinational design depicted wide variation in cellulase activity from 0.049U/ml to 0.515U/ml. Deduction of optimal point at which maximum response was obtained was evaluated by fitting the experimental data to a second order polynomial equation using regression analysis and ANOVA. Regression analysis is yielded the following polynomial as the best fit.

$$Y = 0.49 - 0.048A + 0.022B + 0.0038C + 0.009AB + 0.023AC + 0.004BC - 0.075A^2 - 0.015B^2 - 0.038C^2$$

Where, Y is the cellulase activity (IU/ml) and A, B, C are the coded variables MnSO<sub>4</sub>, FeSO<sub>4</sub> and Peptone respectively.

Table 2: Central composite design in coded levels with cellulase yield as response

Run	Factor 1 A:MnSO <sub>4</sub> g/l	Factor 2 B:FeSO <sub>4</sub> g/l	Factor 3 C:Peptone g/l	Response 1 Cellulase activity U/ml
1	0	1.681793	0	0.478
2	1	-1	1	0.292
3	0	0	0	0.478
4	1	1	-1	0.301
5	-1	-1	1	0.357
6	1	-1	-1	0.246
7	0	0	1.681793	0.381
8	0	0	0	0.475
9	0	0	0	0.473
10	1.681793	0	0	0.185

The ANOVA table suggests that all the linear, quadratic and the interaction terms in the present study are significant (Table 3). The role of interaction between the variables in cellulase production was studied by contour plots of the response function against any two independent variables as shown in Figures 3a, 3b and 3c.

The optimal values of the 3 significant variables MnSO<sub>4</sub>, FeSO<sub>4</sub> and Peptone were determined by using the principles of maxima and minima. Differentiating Y in equation (1) with respect to the 3 variables A, B and C yields the following equations:

$$-0.048 + 0.009B + 0.0023C - 0.158A = 0 \quad (2)$$

$$0.022 + 0.009A + 0.0041C - 0.038B = 0 \quad (3)$$

$$0.0038 + 0.023A + 0.004B - 0.084C = 0 \quad (4)$$

Also, since the second derivatives of Y with respect to A, B and C are all negative, equations (1), (2) and (3) yield the values of these variables which give a maxima for Y. The values of A, B and C obtained from the above equations can be converted to the actual concentrations of the corresponding variables from the simple equations:

$$A = \frac{Z_1 - 0.1}{0.05} \quad (5)$$

$$B = \frac{Z_2 - 0.1}{0.05} \quad (6)$$

$$C = \frac{Z_3 - 2}{1} \quad (7)$$

Where Z<sub>1</sub>, Z<sub>2</sub> and Z<sub>3</sub> are the concentrations of MnSO<sub>4</sub>, FeSO<sub>4</sub> and Peptone respectively. In the present study these concentrations were found to be 0.085 g/l, 0.132 g/l and 1.99 g/l respectively.

Figure 3(a): Contour plot of FeSO<sub>4</sub> and MnSO<sub>4</sub> interaction on cellulase activity

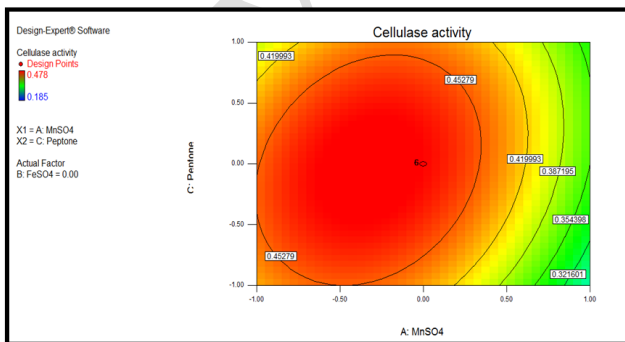


Figure 3(b): Contour plot of Peptone and MnSO<sub>4</sub> interaction on cellulase activity

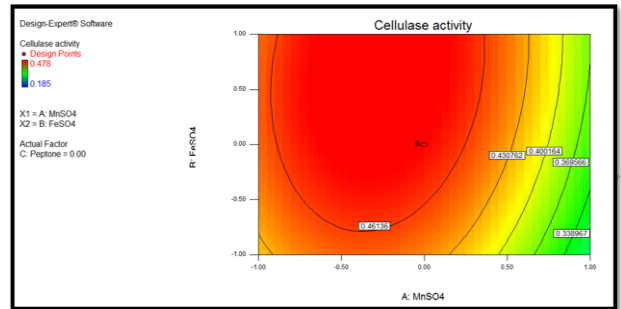
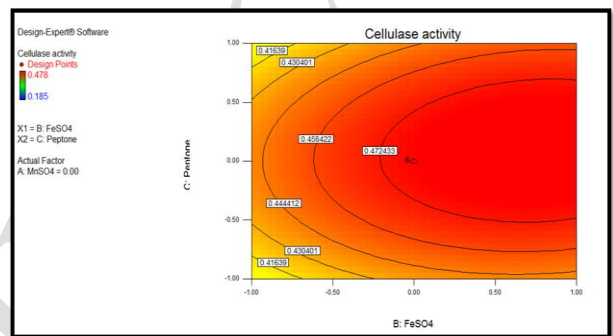


Figure 3(c): Contour plot of Peptone and FeSO<sub>4</sub> interaction on cellulase activity



#### IV. VALIDATION OF EXPERIMENTATION

Predicted results suggested by numerical modeling was validated by performing the experiment in triplicate with the optimized concentrations of the 3 variables. These three sets of experiments yielded an average cellulase activity of 0.486 U/ml, which is in good agreement with the predicted value of 0.502 U/ml. giving a 10 fold increase in enzyme production when the optimal medium is used.

#### REFERENCES

- [1] C.E Wyman, "Handbook on Bioethanol: Production and Utilization," Washington, DC, Taylor and Francis.
- [2] C.K Ramesh, G. Rishi, P.K Yogender, S. Ajay, P. Zhang, "Bioethanol production from pentose sugars: Current status and future prospects," Renew Sust Energy Rev, vol. 15, pp. 4950– 4962, 2011.
- [3] J.C. Yi, J.C. Sandra, A.B. John and T.C. Shu, "Production and distribution of endoglucanase, cellobiohydrolase, and β-glucosidase components of the cellulolytic system of Volvariella volvaceae, the edible straw mushroom," Appl Environ Microbiol, vol.65, pp. 553-559, 1999.
- [4] M. Dashtban, M. Maki, K. T. Leung, C. Mao and W. Qin, "Cellulase activities in biomass conversion: measurement methods and comparison," Crit Rev in Biotechnol, pp. 1–8, 2010.



[5] R. Kumar, S. Singh and O.V. Singh, "Bioconversion of lignocellulosic biomass: biochemical and molecular perspectives," J Ind Microbiol Biotechnol, vol. 35, pp.377–391, 2008.

[6] Sharma, R. Tewari and S. K. Soni, "Application of Statistical Approach for Optimizing CMCase Production by *Bacillus tequilensis* S28Strain via Submerged Fermentation Using Wheat Bran as Carbon Source," Int J Biol, Food Vet Agri Eng, vol. 9(1), 2015.

[7] B.A. Cantwell, P.M. Sharp, E. Gormley, D.J. McConnell, "Molecular cloning of *Bacillus* b-glucosidase. In: Aubert JP, Beguin P, Millet J (eds) Biochemistry and genetics of cellulose degradation. Academic press, San Diego, pp 181–201,1988.

[8] T. Shankar and L Isaiarasu, "Statistical optimization for cellulase production by *Baillus pumilus* EWBCM1 using rsonse surface methodology," Global J. Biotech & Biochem, vol 7(1), 1-6, 2012.

[9] P. Pachauri, S.B Sullia and S. Deshmukh "Statistical optimization for enhanced production of cellulase from sugarcane bagasse using response surface methodology," J Sci Ind Res, vol. 75, pp.181-187.

[10] M.A. Rifai, "A revision of the genus *Trichoderma*," Mycol. Pap, vol. 116, pp. 1-116, 1969

[11] Sazci A , A. Radford and K. Erenle, "Detection of cellulolytic fungi by using Congo red as an indicator: a comparative study with the dinitrosalicylic acid reagent method," J Appl Bacteriol, vol. 61, pp. 559-562, 1986.

[12] J.P. Smits, A. Rinzema, J. Tramper, H. M. Van, W. Knol, "Solid-state fermentation of wheat bran by *Trichoderma reesei* QM9414: substrate composition changes, C balance, enzyme production, growth and kinetics," Appl Microbiol Biotechnol, vol. 46(5–6), pp. 489–496, 1996.

[13] T.K. Ghose, "Measurement of cellulase activities," Pure Appl Chem, vol. 59, pp. 257–268, 1987.

[14] Hankin and S.L. Anagnostakis, "Solid media containing carboxymethylcellulose to detect Cmcellulase activity of micro organisms," J Gen Microbiol, vol. 98(1):109–115, 1977

[15] G.L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugar," Anal Chem, Vol. 9(31), pp. 426-428, 1959.