RESEARCH PAPER:

Statistical optimization of endoglucanase enzyme production by a local isolate, *Aspergillus heteromorphus* using response surface methodology

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SUMMARY

The production of endoglucanase enzymes by the local isolate of the fungus, *Aspergillus heteromorphus* was investigated. The fungus was cultivated under solid state fermentation condition at 30 0C for 120 hours and endoglucanase production was studied. The effect of initial pH, sugarcane bagasse as substrate and peptone concentration as nitrogen source were optimized using Box- Behnken design as independent variables. The optimal level of each parameter for maximal endoglucanase production by the fungus was determined. Endoglucanase activity was positively influenced by linear increase of peptone concentration and decrease at axial concentration of peptone, bagasse and pH. Results showed that endoglucanase activity was highest 61.7 U/ml when the peptone conc. was 1.75 g/l, bagasse conc. 2.5 g/l and pH was 5 respectively. Validation of predicted results was done, and the experimental values correlated well with that of the predicted.

Key words : *Aspergillus heteromorphus*, Endoglucanase, Sugarcane bagasse, Box-Behnken, Solid-state fermentation

Due to dwindling of fossil fuels, national security, long- term economic and environmental problems are motivating the continuous search for new and sustainable source of energy. Liquid fuels for transportation can be derived from lignocellulosic biomass by enzymatic hydrolysis and subsequent fermentation to produce bioethanol (Lynd *et al.*, 1999). Enzymatic hydrolysis of cellulosic biomass is considered as the most efficient and least polluting methods for generating glucose from lignocellulosics, but the production economics of bioethanol is largely dependent on the cost of cellulases (Reith *et al.*, 2002). Cellulase is a deceptively complex concept, a convenient shorthand term for four enzyme activities and molecular entities, required for the complete hydrolytic breakdown of macromolecular cellulose to glucose : Endoglucanases, Cellodextrinases, Cellobiohydrolaese and finally β- glocosidases (Singh *et al.*, 2006). The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling. In the present study, sugarcane bagasse was used as a substrate for enzyme production. Sugarcane converts approximately 2 % of solar energy into chemical bonds of carbohydrates where in two third of these carbohydtates are in the form of lignocelluloses (Singh *et al.*, 2008). Sugarcane contains 12-17% total sugars of which 90% is saccharose and 10% glucose and fructose. Milling extracts roughly 95% of the cane's sugar content leaving behind the solid cane fibre is known as "bagasse" (Wheals, 1999). The amount of bagasse produced in India is 67 million tons in 2006. Sugar cane bagasse is a low-cost and abundant biomass material containing about 27–54% cellulose, which can serve as a potent substrate for cellulase production. There have been several studies on the use of sugarcane bagasse as a substrate in cellulose production both under submerged and SSF (Aiello *et al.*, 1996; Pandey *et al.*, 2000). In order to obtain optimum yield of an enzyme, development of a suitable medium and cultural conditions is obligatory. Selection of appropirate carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic bioprocess. Statistical optimization not only allows quick screening of a large experimental domain, but also reflects the role of each of the components. Response surface methodology (RSM) is a powerful tool for the optimization of chemical reactions and industrial processes (Domingos *et al.*, 2008). Response surface methodology (RSM) is a set of useful models for studying the effects of several factors affecting the responses by

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varying them simultaneously and carrying out only a limited and fixed number of experiments. RSM is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables (Myers and Montgomery, 1995). Here the term ''optimum conditions'' means the operating conditions for maximizing the production of enzymes. Optimization studies help in understanding the interactions among the nutrients at varying concentrations and in calculating the optimal concentration of each nutrient for a given target, *i.e*., maximal enzyme production in less time and at lower cost (Khurana *et al.,* 2007).

This investigation deals with the optimization of the effective concentration of peptone, sugarcane bagasse and pH as variables using RSM for the production of endoglucanase enzyme from *A. heteromorphus* a local isolate.

MATERIALS AND METHODS

Microorganism, its maintenance and preparation of inoculum:

A locally isolated fungal strain identified as *Aspergillus heteromorphus* MTCC 8624 was used for SSF and it was maintained on PDA slants by monthly transfer and was stored at 4^0C. Slants were incubated at 30^0 C for 7 days until complete sporulation. The spores from the slants were suspended in sterile water. The suspention was used as inoculum (10^7 spores/ml).

Lignocellulosic substrate:

Sugarcane bagasse from the sugar industry was used as a carbon source, with bacteriological peptone as the nitrogen source and pH. Sugarcane bagasse was air dried, milled and size fractionated. Bagasse with a particle size 1mm was used as substrate for SSF without any pretreatment. The proportion of bagasse was 0.5 to 5 g/ L, peptone 0.5 to 3 g/L and pH 3.0 to 7.0 (pH adjusted by using 1 N NaOH and HCl) and varied for optimization process.

Endoglucanase enzyme production under SSF:

Erlenmeyer flasks (250 ml) containing lignocellulosic substrate moistened with Czapeck inorganic medium to attain 70% initial moisture content at pH 5.0 were sterilized by autoclaving at 121^0C for 15 min. The flasks were inoculated with 1 ml of 10^7 spores/ml. The inoculated flasks contents were mixed thoroughly and incubated under controlled conditions of temperature and humidity at 30^0C and 70 % moisture, respectively under static conditions for 120 hours. The flasks were gently tapped intermittently to mix the contents. At the end of incubation period, the enzyme was recovered by extraction with distilled water. The extract was centrifuged to remove wreckage at 7200 rpm for 10 min at 4^0C in refrigerated centrifuge (REMI), and the supernatant was used as the crude enzyme sample.

Enzyme assay:

Endoglucanase activity was measured according to IUPAC recommendations employing CMC (low viscosity) as substrate (Ghose, 1987). One unit of enzyme activity was defined as the amount of enzyme required for liberating 1 μM of glucose per milliliter per minute and was expressed as U/ml.

Experimental design for optimization of Endoglucanase enzyme:

A Box- Behnken factorial design with three factors and three levels, including three replicates at centre point, was used for the optimization of endoglucanase enzyme production and conditions were variable concentration of amount of peptone, bagasse and initial pH value. The levels of these variables were optimized for enhancing the endoglucanase yield using a response surface Box–Behnken experiment design (Box- Behnken, 1960). For response surface methodology based on the Box–Behnken design, 17 experimental were run with different combinations. Three different variables of peptone concentration (0.5, 1.75, 3 g/l), bagasse (0.5, 2.75, 5 g/l) and initial pH (3, 5, 7) were chosen as the critical variables and designated as A, B and C, respectively, as shown in Table 1.

Table 1 : Experimental range and levels of independent variables studied using Box-Behnken design in terms of actual and coded factors

	Symbol		Level	
	Coded	Uncoded	Coded	Uncoded
Peptone (g/l)	X_1	A	1	3
			0	1.5
			-1	0.5
Sugarcane bagasse (g/l)	X_2	B	1	5
			0	2.5
			-1	0.5
pH	X_3	C	1	3
			0	5
			-1	7

$$Y = {}_0 + {}_1A + {}_2B + {}_3C + {}_{11}A^2 + {}_{22}B^2 + {}_{33}C^2 + {}_{12}AB + {}_{13}AC + {}_{23}BC$$

where, Y is the predicted response; β_0 is a constant;

β_1, β_2, β_3 are the linear coefficients; β_{12}, β_{23}, β_{13} are the cross-coefficients; β_{11}, β_{22}, β_{33} are the quadratic coefficients. This response is preferred because a relatively few experimental combinations of the variables are adequate to estimate potentially complex response function. Data were analyzed using Design Expert 6.0 programme including ANOVA to find out the interaction between the variables and the response. The quality of the fit of this model was expressed by the coefficient of determination (R^2) in the same programme.

RESULTS AND DISCUSSION

Data from Box-Behnken design for the optimization of endoglucanase enzyme were subjected to a second order multiple regression analysis using the least squares regression methodology to obtain the parameter estimates of the mathematical model. The experimental conditions and results are shown in Table 2.

The final response equation that represented a suitable model for endoglucanase production is given below:

Endoglucanase activity = +60.45+9.11 * A+3.15 * B+1.03 * C-14.34 * A^2-11.56 * B^2-13.19 * C^2+8.90 * A * B+0.26 * A * C-4.05 * B * C

The highest endoglucanase activity was observed in run 14, 61.70 U/ml while the least activity was observed in run 5, 23.20 U/ml (Table 2). The independent variables were fitted to the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model. R^2 represents the proportion of variation in the response data that can be explained by the fitted model. The regression equation indicated that coefficient of determination (R^2) was 0.9953 (a value of $R^2 > 0.75$ indicates the aptness of the model) for endoglucanase production and thus the model could explain more than 99.53 % of variability in the response (Table 3). Moreover, R^2 is in reasonable agreement with adjusted R^2 of 0.9893. The adjusted R^2 corrects the R^2 value for the sample size and number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than predicted R^2. The "Pred R-Squared" of 0.9379 is in reasonable agreement with the "Adj R-Squared" of 0.9893. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 34.467 indicates an adequate signal. This model can be used to navigate the design space. The lack-of-fit term in the residual indicates the variation due to model inadequacy. The computed F-value (137.37), which is the ratio of mean square due to regression to the mean square due to error and indicates the influence

Table 2 : Box-Behnken experimental desogn matrix with observed responses for different trials

Std.	Peptone (g/l)	Sugarcane bagasse (g/l)	pH	CM Case(U/ml)	
1.	-1.000	- 1.000	0.000	29.80	31.19
2.	1.000	- 1.000	0.000	32.10	31.61
3.	-1.000	1.000	0.000	19.20	19.69
4.	1.000	1.000	0.000	57.10	55.71
5.	- 1.000	0.000	-1.000	23.20	23.04
6.	1.000	0.000	-1.000	39.02	40.74
7.	- 1.000	0.000	1.000	26.29	24.57
8.	1.000	0.000	1.000	43.16	43.32
9.	0.000	-1.000	–1.000	28.70	27.47
10.	0.000	1.000	-1.000	42.20	41.87
11.	0.000	-1.000	1.000	37.30	37.63
12.	0.000	1.000	1.000	34.60	35.83
13.	0.000	0.000	0.000	60.23	60.45
14.	0.000	0.000	0.000	61.70	60.45
15.	0.000	0.000	0.000	60.90	60.45
16.	0.000	0.000	0.000	59.40	60.45
17.	0.000	0.000	0.000	60.0	60.45

Table 3 : Analysis of variance (ANOVA) for response surface quadratic model for total cellulase production

Sources	Sum of squares	DF	Mean square	F value	Prob > F	
		9	394.07	165.78	< 0.0001	Significant
Residual	16.64	7	2.38			
Lack of Fit	13.52	3	4.51	5.78	0.0616	Non significant
Pure error	3.12	4	0.78			
Cor Total	3563.30	16				
R.-squared	0.9953					
Adj R-squared	0.9893					
Pre R-squared	0.9379					
Adeq precision	34.467					
C.V.	3.67					

DF = Degree of freedom, CV= Coefficient of variation

(significant or not) of each controlled factor on tested model was significant at high confidence level. The low probability p-value (<0.05) indicated that model terms are significant. For endoglucanase production, in this case A, B, A^2, B^2, C^2, AB, BC are significant model terms. The three dimensional response surfaces were plotted to study the interaction among the various factors selected and to determine the optimum concentration for attaining maximum endoglucanase enzyme production. The plots were generated by plotting the response using the z-axis against two independent variables while keeping the other independent variables at their O-level. The coordinates of the central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components. Fig.1 depicts the three- dimensional plot showing the effects of pH and peptone on endoglucanase enzyme production, while the baggase conc. was fixed at middle level. It could be seen from the figure, enzyme production increases gradually with the pH and peptone conc. but only upto middle level after that enzyme production decreased with further increase of pH and peptone conc. The Fig. 1 shows that optimal pH is 5 and peptone conc. is 1.75g/l for endoglucanse enzyme production. Most of the cellulase secreting fungi are acidophilic and secreted cellulase function of optimally at around pH 5.0. Tryptophan aminoacid at the binding site showed change in polarity in active site at varible pH. This feature of the isolates is similar to *T. reesei* cellulases which have a maximum activity at pH 5 and 50^0C (Boer *et al.*, 2000). The present research is also supported by primary findings. Increase in peptone concentration increases the production of the endoglucanase activity (Levin *et al.*, 2008). Kachlishvili *et al.* (2005) also found that peptone is the most effective

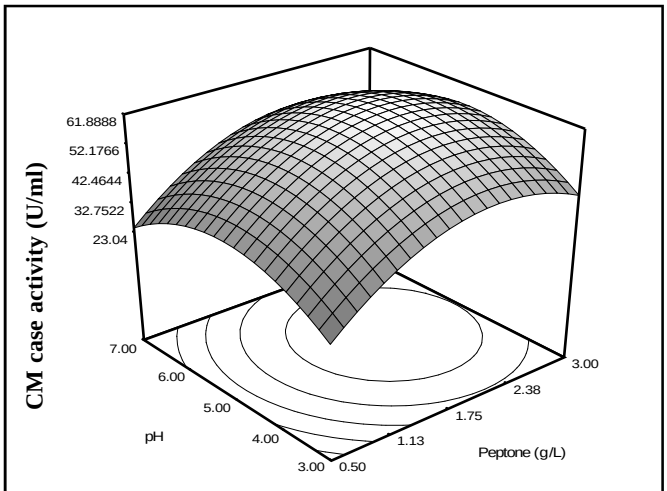

Fig. 1 : Response surface plot showing the effect of pH and peptone on Endoglucanase activity

nitrogen source among various nitrogen sources like ammonium nitrate, ammonium sulphate, potassium nitrate and peptone on endoglucanase activity and found that peptone was more effective among all nitrogen sources used. On the other hand Narasimha *et al.* (2006) found that urea was more effective as nitrogen source for the production of endoglucanase by *Aspergillus niger. Aspergillus niger* synthesized exoglucanases during growth on corn steep liquor associated to different nitrogen sources, like ammonium nitrate, ammonium sulfate, sodium glutamate, sodium nitrate, and urea (Hanif *et al.*, 2004). Fig. 2 shows the effect of substrate (baggase) and peptone on endoglucanase enzyme production while the third

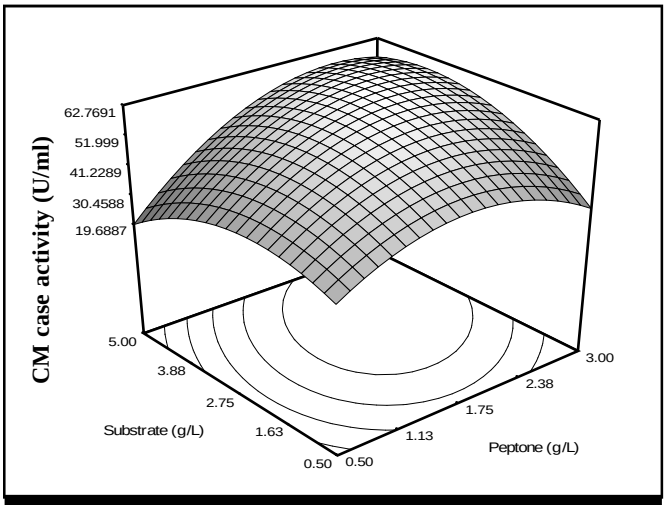

Fig. 2 : Response surface plot showing the effect of substrate and peptone on Endoglucanase activity

variable pH was fixed at middle level. It was evident that at low concentration of peptone and substrate, enzyme production was low, but with the increase in the amount of peptone and substrate incrases the enzyme production. But this increase exists upto certain limit after that further increase decreases the enzyme production. Fig.3 shows the effect of pH and substrate on enzyme production while peptone conc. was fixed at middle level. Similar trend was observed with pH and subsrate.

Model adequacy checking and validation of model:

It is necessary to check the fitted model to ensure that it provides an adequate approximation to the real system. Unless the model shows an adequate fit, proceeding with the investigation and optimization of the fitted response surface likely gave poor or misleading results (Cao *et al.*, 2008). Fig. 4 presents a plot of residuals versus the predicted response. The general

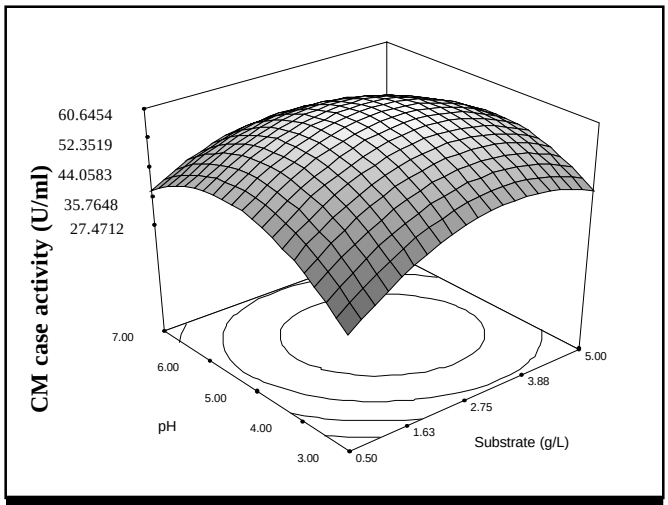

Fig. 3 : Response surface plot showing the effect of substrate and pH on Endoglucanase activity

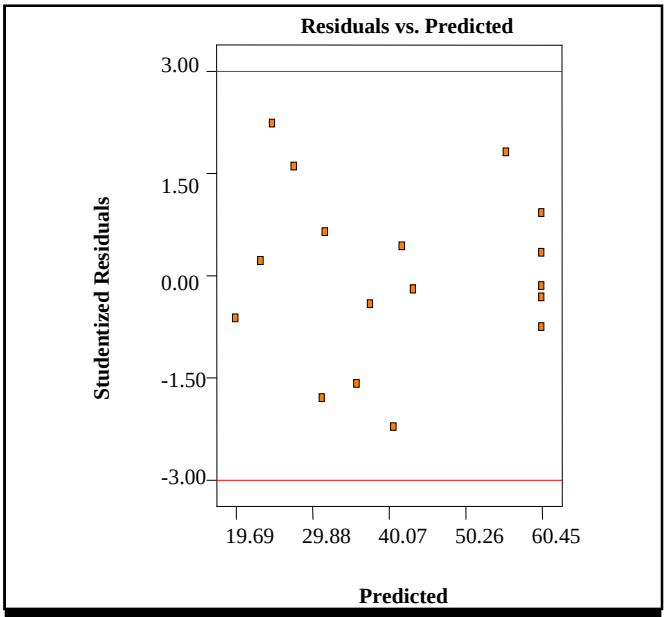

Fig. 4 : Plot of residuals versus predicted respons

impression is that the residuals scatter randomly on the display, suggesting that the variance of Y. To validate the optimum combination of the variables, confirmatory experiments were carried out. Verification experiments were performed at the predicted conditions, indicating the validity of the predicted models. Validation of the experment was repeated three times under optimal conditions in order to confirm the mathematical model, the maximal of which was 61.7 IU/ml. This value was found to have a marked increase compared with a lowest value of 19.2 IU/ml at run 3 in experiments according to Box-Behnken design.

Conclusion:

Response surface methadology was proved to be a powerful tool for optimization of culture conditions and culture medium components. In the present work, endoglucanase enzyme production from *A. heteromorphus* growing on bagasse was studied by BBD and response surface methodology. The enzyme production could be improved by controlling various nutritional factors, and a statistical approach has proved to be a useful and powerful tool for rapid identification of the signal parameters and development of optimal culture conditions with a minimum number of experimental trials. The use of cheap substrate like sugarcane bagasse could provide a robust solution for production of enzymes and give a solution in developing a suitable SSF process.

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REFERENCES

Aiello, C., Ferrer, A. and Ledesma, A. (1996). Effect of alkaline treatments at various temperatures on cellulase and biomass production using submerged sugarcane bagasse fermentation with *Trichoderma reesei* QM9414. *Bioresource Technol.*, **57** : 13–18.

Boer, H., Teeri, T.T. and Koivula A. (2000) Characterization of *Trichoderma reesei* cellobiohydrolase Cel7A secreted from *Pichia pastoris* using two different promoters. *Biotechnol. Bioengg.*, **69** : 486–494 .

Box, G.E.P. and Behnken, D.W. (1960) Some new three level design for the study of quantitative variables. *Tecnometrics*, **2** : 455-475.

Cao, Y., Meng, D.J., Lu, J. and Long, J. (2008). Statistical optimization of xylanase production by *Aspergillus niger* AN-13 under submerged fermentation using response surface methodology. *African J. Biotechnol.*, **7** (5) : 631-638.

Domingos, A.K., Saad, E.B., Wilhelm, H.M. and Ramos, L.P. (2008). Optimization of the ethanolysis of *Raphanus sativus* L. crude oil applying the response surface methodology. *Biores. Technol.*, **99** : 1837–1845

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure & Applied Chem.*, **59** : 257–268.

Hanif, A., Yasmeen, A. and Rajoka, M.I. (2004). Induction, production, repression, and de-repression of exoglucanase synthesis in *Aspergillus niger. Biores. Technol.*, **94** : 311–319

Kachlishvili, E., Penninckx, M.J., Tsiklauri, N., Elisashvili, V. (2005). Effect of nitrogen source on lignocellulolytic enzyme production by white-rot basidiomycetes under solid-state cultivation. *World J. Microbiol. & Biotechnol*.

Khurana, S., Kapoor, M., Gupta, S. and Kuhad, R.C. (2007). Statistical optimization of alkaline xylanase production from *Streptomyces violaceoruber* under submerged fermentation using response surface methodology. *Indian J. Microbiol*., **47** : 144–152

Levin, L., Herrmann, C. and Papinutti, V.L. (2008). Optimization of lignocellulolytic enzyme production by the white-rot fungus *Trametes trogii* in solid-state fermentation using response surface methodology. *Biochem. Engg. J.*, **39** : 207–214

Lynd, L.R., Wyman, C.E. and Gerngross, T.U. (1999). Biocommodity Engineering. *Biotechnol. Progress*, **15** : 777–793.

Myers, R.H. and Montgomery, D.C. (1995). *Response surface methodology: Process and product optimization using designed experiments*, John Wiley & Sons, New York, NY.

Narasimha, G., Sridevi, A., Buddolla, V., Subhosh, C.M. and Rajsekhar, R.B. (2006). Nutrient effect on production of cellulolytic enzymes by *Aspergillus niger*. *African J. Biotechnol.*, **5** (5) : 472-476.

Pandey, A., Soccol, C.R., Nigam, P. And Soccol, V. T. (2000) Biotechnological potential of agroindustrial residues I: Sugarcane bagasse. *Bioresource Technology*, **74**, 69–80.

Reith, J. H., den Uil, H., van Veen H., de Laat W.T.A.M., Niessen, J. J. and de Jong, E. (2002). Coproduction of bioethanol, electricity and heat from biomass residues. 12th European Conference and Technology Exhibition on Biomass from Energy, Industry and Climate Protection, Amsterdam, The Netherlands, 17–21 June, 2002.

Singh,A., Singh,N., Singh, R., Bishnoi,K. and Bishnoi, N.R. (2008). Production of cellulases by *Aspergillus heteromorphus* from wheat straw. Proceeding International Conference on Molecular Biology and Biotechnology held at Vanasthali Vidhyapeeth-Rajasthan (India), 19-21 Oct.-2008, pp 52-53.

Singh, R., Singh, N., Parkash, A. and Poonia, S. (2006). Saccharification studies by a thermophilic fungus *Sporotricum thermophile* isolated from agriculture waste of Bhopal, *Indian J. Environ. & Ecoplan*, **12** (1) : 97-104.

Wheals, A.E., Basso, L.C., Alves, D. and Amorim, H. (1999). Fuel ethanol after 25 years. *Focus*, **17** : 482-487.