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RESEARCH ARTICLE

CELLULASE PRODUCTION BY *TRICHODERMA ATROVIRIDE* UNDER SOLID STATE FERMENTATION: STATISTICAL OPTIMIZATION OF PROCESS PARAMETERS

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ABSTRACT

Response surface methodology (RSM) is a powerful and efficient mathematical approach widely applied in the optimization of process parameters. Box-Behnken design was applied to elucidate the process parameters that significantly affect cellulase production. The experiment established the optimum conditions of incubation time (5.5 days), temperature (32.50C), pH (5.5), spore suspension (1.75ml) for solid state fermentation that led to the maximum production of cellulase at a level of 86.34 IU/gds. Good correlation was observed between the actual and predicted results indicated that the present model was applicable to production of cellulase enzyme efficiently.

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INTRODUCTION

Lignocellulosic feedstock is among the most plentiful complex organic carbons in form of plant biomass. Lignocellulosic biomass includes forestry, agricultural and agro-industrial wastes. Lignocellulosic biomass is a great potential resource for the production of biofuels because it is largely abundant, inexpensive and production of such resources is environmentally sound. It mainly consists of three types of polymers cellulose, hemicelluloses and lignin that are strongly intermeshed and chemically bonded by non-covalent forces and by covalent cross linkage (Ali *et al.,* 2012). Cellulases are association of free enzymes, including endoglucanases, exoglucanases and cellobiohydrolases, which are found in many of the glycosyl hydrolase families. Cellulases catalyze the hydrolysis of cellulose, which is the principle component of the cell wall of most plants. Simple, efficient and economical processes for conversion of cellulose to glucose by enzymatic hydrolysis would aid in decreasing pollution. Microbial cellulases are used currently in food industries, paper pulp industry and animal feed industries (Guo *et al.,* 2008). The genus of *Trichoderma* has been especially famous for producing cellulolytic enzymes with relatively high enzymatic activity (Morton, 1987).

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Solid state fermentation (SSF) is a way of fermenting substrate in the presence of excessive moisture in growth medium in spite of large amount of water being provided. SSF is an environmental friendly (less waste water production), low energy required and economical technology in synthesizing cellulase enzyme in response to submerged fermentation (Pandey, 2003). Response surface methodology (RSM) is a statistical technique for the optimization of multiple variables, which determine optimum process conditions by combining experimental designs with interpolation by first or second polynomial equations in a sequential testing procedure. Response surface methodology (RSM) analyzes the effects of defined independent variables on the response without the need for prior knowledge of a predetermined relationship between the response function and the variables (Shankar and Isaiarasu, 2012). The aim of this study was to obtain a high yield of cheap cellulase by using *Trichoderma atroviride* through solid state fermentation and also exploiting waste like *Eicchornia crassipes* and Rice husk. This study will help in proper disposal of wastes resulting in resolution of the environmental problems.

MATERIALS AND METHODS

Preparation of substrate

Eicchornia crassipes is an aquatic plant collected from local pond near by Rohtak city and rice husk is collected from Rice

milling industries and processed to particle size of 1mm, mixed and washed with distilled water and dried at 60^0C for 8 h in the oven. Processed substrates were collected in the polythene covers and stored at room temperature.

Microorganisms

The microorganism *Trichoderma atroviride* was procured from Institute of microbial Technology (IMTECH) Chandigarh. The strain was well preserved in our laboratory. A spore suspension was obtained for each organism by growing them on potato dextrose agar at 30^0C for one week and harvesting the spores with sterile water containing 0.1% Tween-80.

Medium and culture conditions

Erlenmeyer conical flasks (250ml) containing substrate 5g and Mendel's media (10ml) of following composition (g/L) $(NH_4)_2SO_4$ (1.4gm), KH_2PO_4 (2gm), $CaCl_2.2H_2O$ (0.4gm), $MgSO_4.7H_2O$ (0.6gm), Urea (0.3gm), Protease peptone (0.75gm), Yeast extract (10g), $COCl_2.6H_2O$ (3.7mg), $FeSO_4.7H_2O$ (5mg), $MnSO_4.7H_2O$ (1mg), Zinc Sulphate (1.4gm), Tween 80 (2ml) were sterilized at 121^0C for 20 min. After cooling, each flask was seeded with the inoculums concentration (0.5-3ml), initial pH (3-8), temperature (25-40^0C), incubation period (3-8 days) defined by the experimental design and in a shaker at 200 rpm for throughout the incubation periods. Thereafter, the enzyme was extracted by adding 50ml of 50mM citrate buffer pH 4.8 under shaking at 200 rpm on a rotary shaker and at 30^0C for 60min. The resultant slurry was filtered through a muslin cloth and centrifuged at 10000 rpm for 15min at 4^0C, and the supernatant was used as crude enzyme source for cellulase.

Enzyme assay

The fortitude of Cellulase activity was examined according to the method of Ghose (Ghose, 1987). In test tubes, 0.5ml of 1% CMC was added with 0.5ml diluted enzyme was incubated at 50^0C for 30min. The reaction was terminated by addition of 2ml DNSA (Dinitrosalicyclic acid) reagent and tubes were kept at boiling water bath for 5min (Chang *et al.,* 2011). After cooling the tubes at room temperature, 10ml distilled water was added in each tube. The intensity of the colour was read at 540nm in UV-VIS spectrophotometer. Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1µ mol of glucose per minute under assay condition.

Experimental design

The experimental design and statistical analysis were performed on Box-Behnken design with quadratic model was employed to study the combined effect of four independent variables namely incubation period (A, days), Temperature (B, ^{0}C), pH (C), spore suspension (D, ml) for the dependent variables such as cellulase (U/gds) production using Design-Expert software (version 9, Stat-Ease Inc., Minneapolis, USA). A total no. of 29 experimental runs given by this design. The dependent variables was expressed individually as a function of the independent variable known as response function. This design is interpreted by a second-order polynomial regression model as follows:

$$Y = \beta_0 + \sum \beta_i A_i + \sum \beta_{ii} A_i^2 + \sum \beta_{ij} A_i A_j$$

Where, Y is the measured response (cellulase yield), β_0 the constant, β_i the linear coefficient, β_{ii} the quadratic coefficient, β_{ij} the cross product coefficient Ai and Aj are the levels of the independent variable.

Data analysis

Regression analysis and estimation of the coefficients were performed using Design-Expert software (version 9, Stat-Ease Inc., Minneapolis, USA). The quality of the fitted model was expressed by the coefficient of determination R^2, and its statistical significance was checked by F-test.

RESULTS AND DISCUSSION

Optimization of Cellulase production by *Trichoderma atroviride* using response surface methodology

RSM based on the Box-Wilson, which was used to optimize different parameters for cellulase production, 29 experimental runs with different combinations of four factors were carried out. The variables such as incubation time, Temperature, pH, spore suspension that influence cellulase production in solid state fermentation (SSF) using RSM (Table1). The effect of each factor and their interaction were analyzed using the analysis of variance (ANOVA) (Table2).The calculated regression equation for the optimization of parameters for production of cellulase i.e. CMCase (Y, U/gds) is a function of the incubation period (A, days), temperature (B, ^{0}C), pH (C), spore suspension (D, ml). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to explain the cellulase production as shown below:

Cellulase (U/gds) 82.06 - 0.39A – 0.32B - 1.37C + 0.80 D +3.03A*B + 0.70 A*C – 2.65A*D + 7.99 B*C – 0.44 B*D + 2.10 C*D – $15.61A^2$ – $13.22B^2$ – $14.95C^2$ – $11.75D^2$ .....Equation (1)

Cellulase production by microorganisms is greatly influenced by physical factors such as incubation time, temperature, pH, spore suspension. Hence effect of these factors on cellulase production was investigated. One of the important factor is incubation time, it can be seen that the cellulase production increases with increases in incubation time and found maximum on 6^{th} day after inoculation. Further increases in the incubation time led to decreases in the production of cellulase due to the depletion of the nutrients in the fermentation medium (Aishwarya *et al.,* 2011). Probably the most important factor among all the physical variables affecting the SSF performance is the incubation temperature, because both cell growth and the production of enzymes and metabolites are usually sensitive to temperature (Krishna, 2005). As shown in Fig. 2 the optimum temperature for maximal production of cellulase enzyme was 32.5^0C. As the temperature increased, there was a gradual decrease in the enzyme production.

Table 1. Result of BBD showing observed and predicted response for Cellulase

Run	Factor A Incubation Time	Factor B Temperature	Factor C pH	Factor D Spore suspension	Response Cellulase	Predicted value Cellulase
	(Days)	(^{0}C)		(ml)	(IU/gds)	(IU/gds)
1	3	25	5.5	1.75	57.23	56.98
2	8	32.5	3	1.75	52.34	51.78
3	5.5	32.5	5.5	1.75	85.78	82.06
4	5.5	25	5.5	3	65.32	57.77
5	5.5	32.5	5.5	1.75	82.34	82.06
6	5.5	40	8	1.75	57.55	60.18
7	5.5	32.5	8	3	59.89	56.89
8	5.5	32.5	3	3	48.56	55.43
9	8	32.5	8	1.75	45.65	50.44
10	3	32.5	8	1.75	51.67	49.83
11	3	32.5	5.5	3	55.65	58.55
12	5.5	25	3	1.75	58.34	63.57
13	8	25	5.5	1.75	53.67	50.13
14	5.5	40	5.5	3	56.77	58.00
15	5.5	25	8	1.75	35.11	44.85
16	5.5	32.5	8	0.5	63.41	51.08
17	5.5	32.5	5.5	1.75	82.12	82.06
18	5.5	32.5	5.5	1.75	86.34	82.06
19	5.5	32.5	3	0.5	60.48	58.02
20	5.5	40	5.5	0.5	50.37	55.51
21	3	40	5.5	1.75	52.18	50.26
22	5.5	40	3	1.75	48.82	46.94
23	3	32.5	5.5	0.5	43.32	51.63
24	3	32.5	3	1.75	61.16	53.96
25	8	32.5	5.5	3	52.90	52.45
26	8	40	5.5	1.75	60.76	55.55
27	5.5	32.5	5.5	1.75	73.70	82.06
28	5.5	25	5.5	0.5	60.67	57.04
29	8	32.5	5.5	0.5	51.19	56.16

This is because higher temperature (above 32.5°C) alters the cell membrane composition and stimulates protein catabolism, thus causes the cell death (Sherief *et al.,* 2010). The effect of pH was studied by varying the pH from 3 to 8 using appropriate buffers. It was found that enzyme has got activity over a broad range of pH (Fig. 2). Enzymes have an optimum pH, at which their activity is maximum; at higher or lower pH values, their activity decreases (Jahangeer *et al.,* 2005). Inoculum size (or spore suspension) certainly has an effect on the cellulase production. In the present study, effect of the size of inoculum was also explored (Fig.2).

The maximum enzyme production was at 1.75mL of spore suspension of *Trichoderma atroviride*. The decrease in cellulase production with further increase in inoculums might be due to depletion of nutrients by the enhanced biomass, which resulted dwindle in metabolic activity (Kashyap *et al.,* 2002). A balance between the increasing biomass and accessible nutrient would yield an optimal production of enzyme (Ramachandran *et al.,* 2004). The predicted levels of cellulase production in substrate using the above equations are given in Table 1 along with the experimental data. Several indicators were used to evaluate the adequacy of the fitted model i.e., Determination coefficient (R^2), Coefficient of variation (CV), Model significance (F-value). The quadratic regression model illustrate that Equation 1 are highly significant statistical models for Cellulase production responses in substrate, as it was evident from the Fisher's F-test with a very low probability value [(P model >F) (Table 2). The p-value is a tool for evaluating the significance and contribution of each parameter.

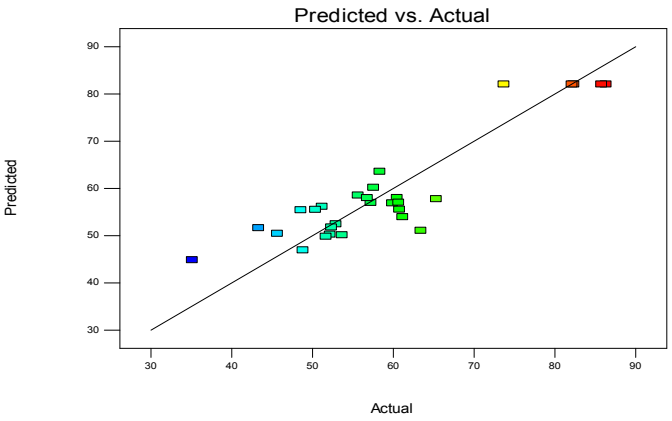

Fig. 1. Predicted VS Actual values for Cellulase

Table 2. ANOVA for the experiment

Term	Response Y, Cellulase
F-value	4.71
P>F	0.0032
R^2	0.825
Mean	59.08
Adjusted R^2	0.65
Adequate precision	6.96
Coefficient of variance (%)	12.56
Lack of fit	18.30

Model term having values of Prob > F less than 0.05 are considered significant, whereas those greater than 0.10 are insignificant (Zahangir *et al.,* 2008). ANOVA indicated the R^2-value of 0.825 respectively, for responses Y.

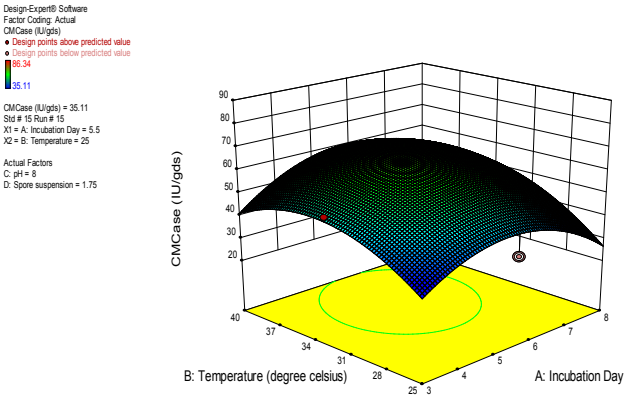

Figure 2A.

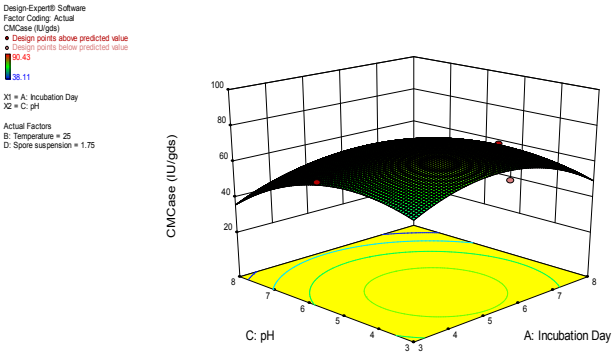

Figure 2B.

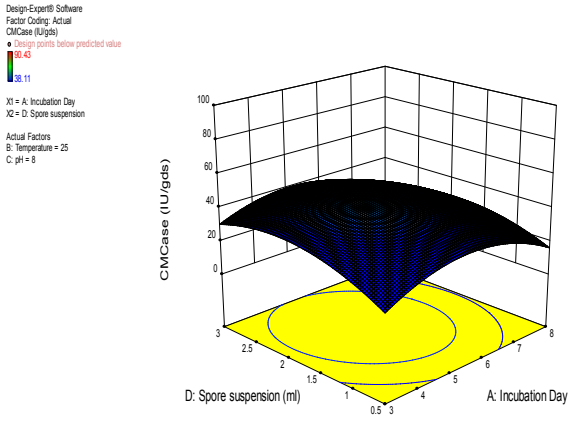

Figure 2C.

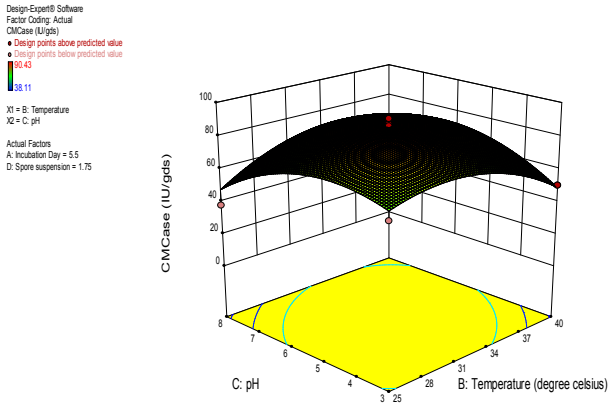

Figure 2D.

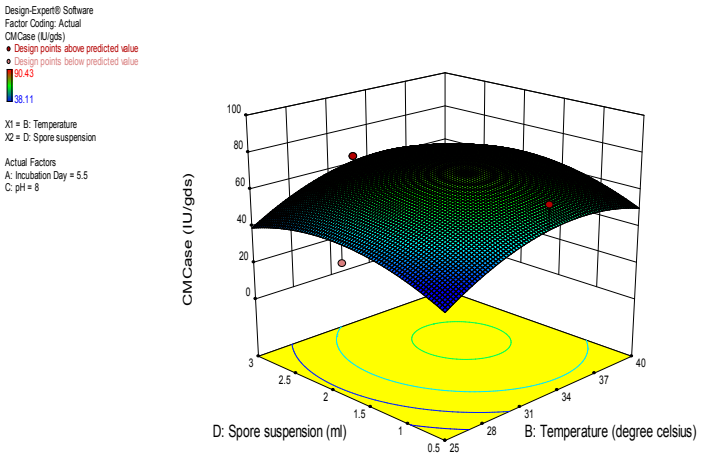

Figure 2E.

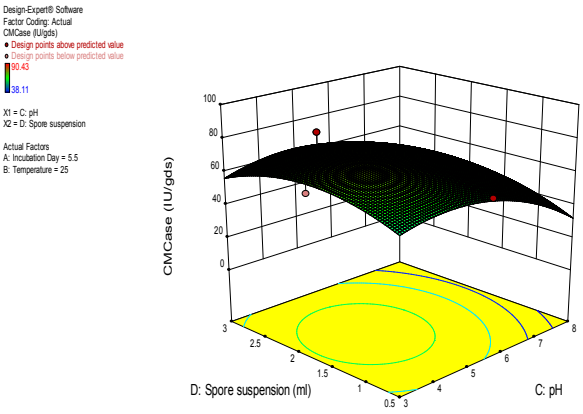

Figure 2F.

Figure 2. A-F. Three dimensional response plots for Cellulase activity showing interactive effects of variables

For good statistical model, the R^2 value should be in the range of 0-1.0, and the values are obtained in the data analysis indicates the model is good. This again ensured a satisfactory adjustment of the quadratic model to the experimental data, and indicated that the model could explain 80-85% of the variability in the response. The coefficient of variation (CV) indicate the degree of precision with which the experiments are compared. Generally, the higher the value of the CV is, the lower the reliability of the experiment.

Here the value of coefficient of variation is 12.56 for responses Y indicate good reliability of the experiment performed (Box *et al.,* 1978). The adequate precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The adequate precision was 6.96 for Y responses that indicates an adequate signal. The lack of fit, F-value of 18.3% for responses Y which is the ratio of mean square due to regression to the mean square due to error and indicates the influence of each controlled factor on tested model, was significant at high confidence level. Non significant lack of fit is good. (Fig.1) shows that the actual response values agree well with the predicted response values of cellulase. The 3D response surface plot described the regression model was drawn to illustrate the combined effects of the independent variables and combined effects of each independent variable upon the response variable. The optimum conditions for maximum production of enzyme production was determined by response surface analysis and also estimated by regression equation. The optimum conditions are namely: Incubation time (5.5 days), Temperature (32.5°), pH (5.5) and spore suspension (1.75 ml). The optimal values for variables as predicted are found to be within design region. This shows that the model correctly explains the influence of the chosen variables on enzyme production. The response surface curves were plotted to understand the interaction of the variables and to determine the optimum levels of each variables for maximum response (Fig 2).

Validation of the model

The suitability of the model equation for predicting the optimum response values was tested using the optimum conditions mentioned above. This set of conditions was determined to be optimum by a RSM optimization approach, which was also used to experimentally validate and predict the value of the responses using model equations. The experimental values were found to be in accord with the predicted ones (Table 1). The cellulase activity reached 86.34 IU/gds for cellulase under the optimal conditions.

Conclusion

In the present work, the applied response surface methodology (RSM) proved to be efficient in optimizing process parameters for cellulase production using a very cheap substrate *Eicchornia crassipes* and rice husk. From the optimization studies, the optimum experimental condition are incubation period (5.5 days), temperature (32.5^0C), pH (5.5) and spore suspension (1.75ml). Using the optimized conditions the maximum cellulase production of 86.34 IU/gds was obtained. Comparison of predicted and experimental values revealed good correlation between them, implying that Box-Behnken models derived from RSM can be used to adequately describe the relationship between the dependent and independent variables in cellulase production.

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Conflict of Interest

The author declare no conflict of interest.

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