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Optimization of Endoglucanase Production in Liquid State Fermentation from Waterhyacinth by *Rhizopus oryzae* Using Response Surface Methodology

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Abstract: Statistically based experimental design were applied for the optimization of endoglucanase production in liquid state fermentation by *Rhizopus oryzae* MTCC 9642 using water hyacinth as sole carbon source. Effect of three critical culture parameters substrate concentration, cultivation temperature and pH on enzyme production, examined by Response Surface Methodology using Central Composite Design(CCD) indicated that although a substrate concentration of 1.25% was essential to maximize the production of the enzyme by the strain , but the combination of pH 6 and 40^0C temperature were required for highest production of endoglucanase. Under the optimized cultivation condition the strain synthesized 450 IU/ml for endoglucanase utilizing water hyacinth in the media. A verification experiment was accomplished and revealed approximately 95% model validity.

Key words: Endoglucanase, response surface methodology (RSM), *Rhizopus oryzae,* Water hyacinth

INTRODUCTION

Water Hyacinth *(Eichornia crassipes)* is a troublesome aquatic weed, the luxuriant growth of which in the water bodies interferes in the activities of mankind, which has caused great concern throughout the world. Being a microphyte and rich in cellulose content, it can be used as renewable source of energy by using water hyacinth cellulose as the sole carbon source in the culture medium (Ismail *et al.,*1995) and as feedstock for fermentable sugar production by enzymatic hydrolysis (Mukhopadhay *et al*., 2008). The bioconversion of lignocellulosic waste material to energy has gained much interest during the recent past (Baig *et al.* 2004).The bioconversion may be achieved by cellulase, a synergistic enzyme comprises of three component enzymes: endoglucanase (E.C.3.2.1.4), exoglucanase (E.C.3.2.1.9) and β-glucosidase or salicinase (E.C.3.2.1.21) that convert cellulose to glucose. Cellulase production by different organism in submerged state fermentation has received more attention and is found to be cost prohibitive because of the high cost of process engineering (Singh *et al.,*2009) .Although a number of reports are available on endoglucanase production by various fungal strains utilizing agrowastes (Pothirajm *et al*., 2006; Immanuel *et al.,*2007; Peciulyte, 2007; Acharya *et al*.,2008; Omosajola *et al*.,2008), but out current work comprises of production of endoglucanase from waterhyacinth wastes.

In order to check the potential of the *Rhizopus* strain to synthesize endoglucanase for biotechnological applications, determination of the cumulative effect of multiple parameters regulating the rate of enzyme production became warranted. Bioprocess technologies require effective problem solving methods as they involve multiple parameters to adjust. Response surface methodology is more satisfactory and effective than other methods such as classical one-at-a-time or mathematical methods because it can study many variables simultaneously with a low number of observations, saving time and costs (Bezerra *et al*.,2008, Deepak *et al*.,2008) It has been successfully utilized to optimize compositions of fermentation medium (Cui *et al*., 2006; Zhang *et.al*., 2006; Saval *et.al*., 1993; Lee and Chen, 1997; Li *et.al*.,2007; Vohra and Satyanarayana,2002;Berecka *et al.*,2006). The present paper describes the optimum conditions for endoglucanase production by *Rhizopus oryzae* applying response surface methodology (RSM).

MATERIALS AND METHODS

Micro Organism:

Rhizopus oryzae PR7 MTCC 9642 (Karmakar and Ray, 2010a), was isolated from the decaying vegetation enriched soil of India. The strain was identified by and deposited to Microbial Type Culture Collection, India.

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Chemicals:

All chemicals used were of analytical grade.

Cultivation of the Strain:

The pellet form of the strain was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed of (gl^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1;$MgSO_4.H_2O$ 0.1 and Dextrose 0.5 at pH 6 and temperature 37°C for 48 hrs in static condition.

Enzyme Extraction and Enzyme Assay:

The grown culture was filtered through filter paper (Whatman No1) and filtrate was used centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To measure the activity of endoglucanase tubes containing the assay mixture (1ml) each containing an 0.5 ml of enzyme diluted with 0.1(M) phosphate buffer (pH-6) was incubated with 1 %(w/v) CM-cellulose for 10 minutes (Karmakar and Ray,2010b) at 33°C . The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld, 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of endoglucanase was defined as that amount of enzyme that liberated 1µ mole of glucose per ml per minute of reaction.

Statistical Analysis:

An evaluation copy of the statistical software, Design-Expert version 7.1.6, from Stat-Ease, Inc., Minneapolis, USA was used for analysis of experimental data and to plot response surface. ANOVA was used to estimate the statistical parameters.

RESULTS AND DISCUSSION

Detection of the Substrate Concentration, Effects of Temp and pH for Optimum Enzyme Production by RSM:

To detect the effect of three major key factors responsible for enzyme production, each factor in the design was studied at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$) (Table 1) by central composite design. The minimum and maximum ranges of variables were investigated and the full experimental plan with respect to their values in actual form is listed in Table 1. Upon completion of experiments, the average maximum enzymatic activity was taken as the dependent variable or response(Y). A second order polynomial equation was then fitted to the data by a multiple regression procedure. This resulted in an empirical model that related the response measured to the independent variables of the experiment. For a three factor system the model equation is,

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{23}BC+\beta_{13}AC \quad (1)$$

Where Y, predicted response; β_0, intercept; β_1, β_2, β_3, linear coefficients, β_{11}, β_{22}, β_{33}, squared coefficients; β_{12}, β_{23}, $\beta_{13,}$ interaction coefficients and A substrate concentration (% w/v), B is the pH and C is the temperature (°C). The responses of the CCD design were fitted with a second-order polynomial equation.

The CCD contained a total of 20 experimental trials that include 14 trials for not centre points and 6 trials for replications of the central points. Concentration ranges of the three components used in Central Composite Design is shown in (Table 1).

Optimization of Parameters:

Various factors like waste(water hyacinth) substrate concentration(A), pH(B) and cultivation temperature(C) tested by varying the determining factor.Each experiment was carried out in triplicate and their values were averaged.

$$\text{Endoglucanase (X)} = +300.23+94.03*A-83.03*B+10.29*C+12.48*A*B+2.02*A*C-22.60*B*C-10.16*A2-50.70*B2+22.07*C2 \quad (2)$$

Where (X) is the predicted response is the endoglucanase activity,A is substrate concentration(w/v %), B is the p^H ,C is the temperature in degree Centigrade.

RSM was employed to characterize the individual and interactive effects of substrate concentration, pH and temperature on the endoglucanase production. The second-order model was hypothesized and statistically evaluated by analysis of variance (ANOVA). ANOVA result showed that the value of R2 for the response was in reasonable agreement with the adjusted R^2. This ensured a satisfactory adjustment of a quadratic model to the experimental data. The values for predicted sum of squares (PRESS), which indicate how a particular model fits each point in design, 8386.24 for X. Values of ''prob > F" obtained was <0.001, indicating that the mathematical models generated were highly significant. The mathematical models' signal to noise ratio was well in control, as assessed by the values of adequate precision (59.828), which were quite higher than the standard value of 4 (Design expert guide). Therefore, this model can be used to navigate the design space. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. Usually, the higher the value of CV, the lower the reliability of experiment is. Here, a lower value of CV (3.83) indicated a better precision and reliability of the experiments (Box *et al*., 1978). The three-dimensional response surfaces for the three variables (Figs. 1, 2,3) showed non-linear relationship between the independent variables. 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 give poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy (Myers and Montgomery, 2002). By constructing a normal probability plot of the residuals, a check was made for the normality assumption, as given in Fig. 4. The normality assumption was satisfied as the residual plot approximated along a straight line. The plot (Fig. 4) is satisfactory, so we conclude that the empirical model is adequate to describe the endoglucanase activity by response surface. Normally, a regression model having an *R*2 value higher than 0.9 is considered to have a very high correlation (Haaland ,1989) The closer the value of *R* (correlation coefficient) to 1, the better the correlation between the experimental and predicted values. Here, the value of R^2 (0.9959) for Eq.(2) indicates a close agreement between the experimental results and the theoretical values predicted by the model equation(Fig 5). The natural logarithm (ln) of the residual SS (sum of square) against lambda is one, dip suddenly with a minimum in the region of the best optimum value 0.88 (Fig.6). The data do not require a transformation, as current value of confidence interval it contains (lambda) is very close to the optimum value (Box and Cox, 1964) The model shows the minimum and maximum confidence interval value is 0.69 and 1.06 respectively (Fig. 6).

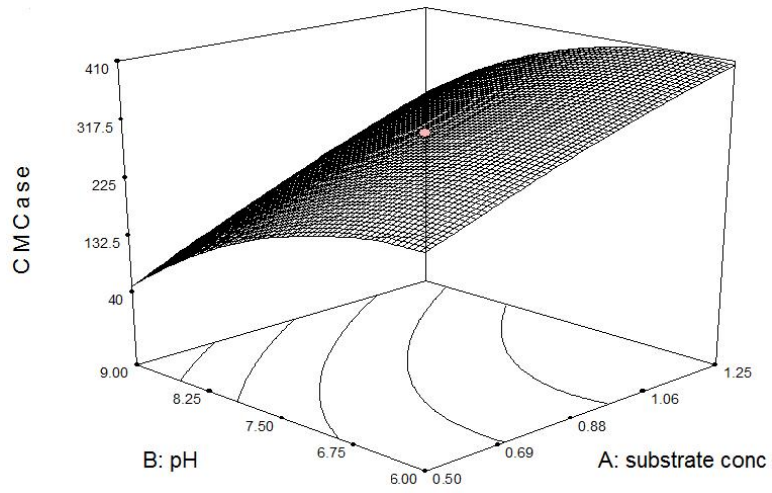

Fig. 1: Response surface plots showing the effect of substrate concentration and pH on Endoglucanase production with other variable constant at middle level.

Validation of the Model:

The statistical optimal values of variables were obtained .The central composite design and the response at the different points yielded maximum endoglucanase production. A repeat fermentation of water hyacinth for highest production of endoglucanase by Rhizopus oryzae MTCC 9642 under optimal conditions was carried out for verification of the optimization. The maximal endoglucanase production found under optimal conditions were 4501 IU/ml which was 2.17% less than the predicted values.

This discrepancy might be due to the slight variation in experimental conditions. The optimization resulted in 4.91 fold increase of endoglucanase production, compared with the lowest enzyme production at run 3 in Central composite design.

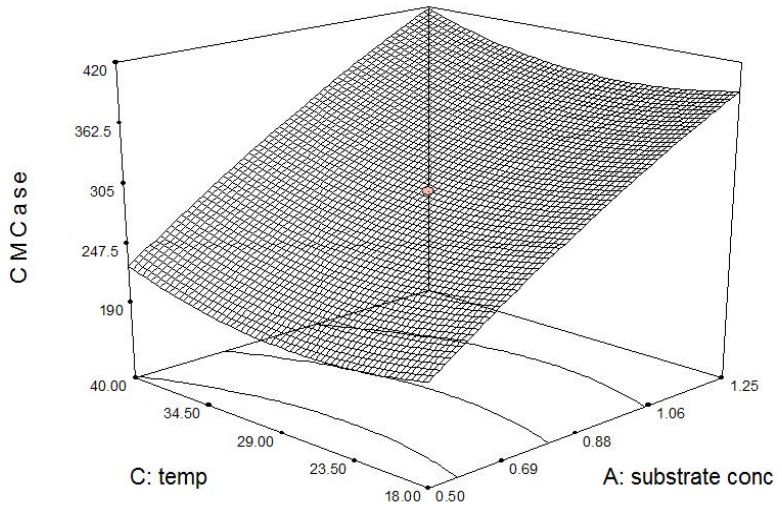

Fig. 2: Response surface plots showing the effect of temperature and substrate concentration on Endoglucanase production with other variable constant at middle level.

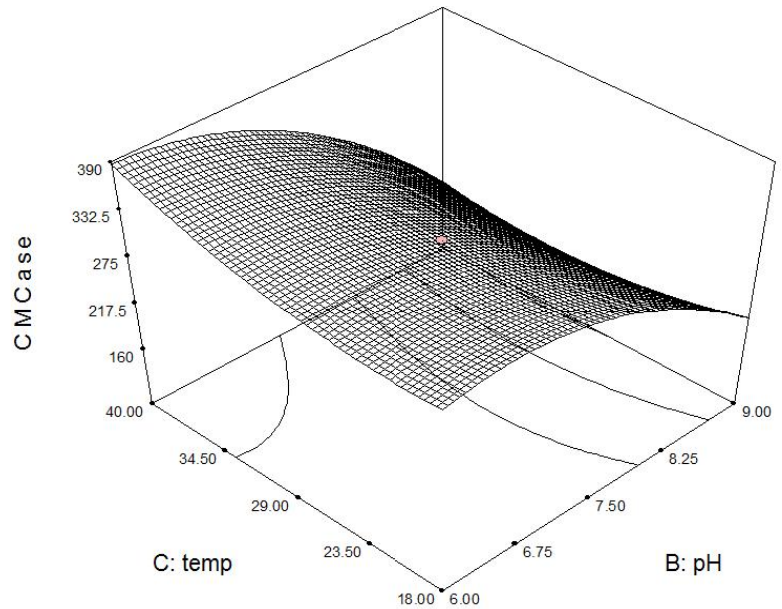

Fig. 3: Response surface plots showing the effect of temperature and pH on Endoglucanase production with other variable constant at middle level

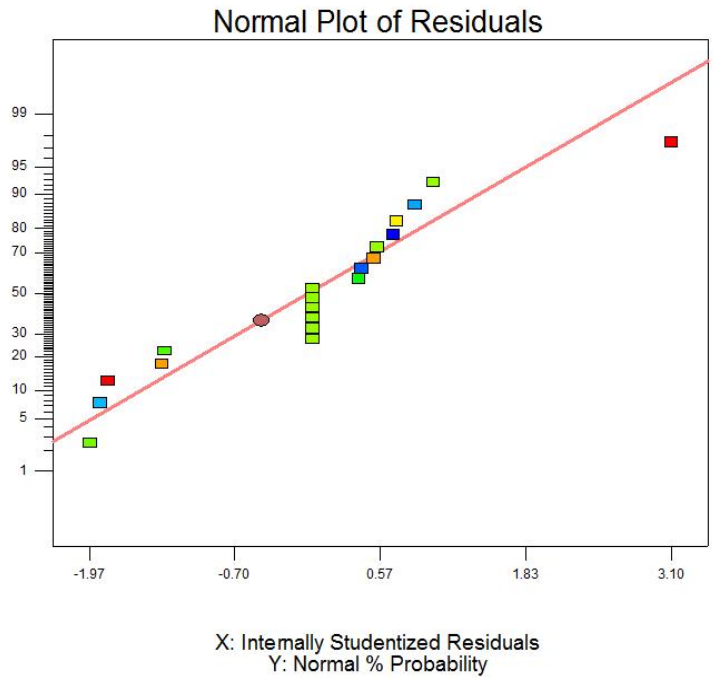

Fig. 4: Plot of internally studentized residuals vs. predicted response.

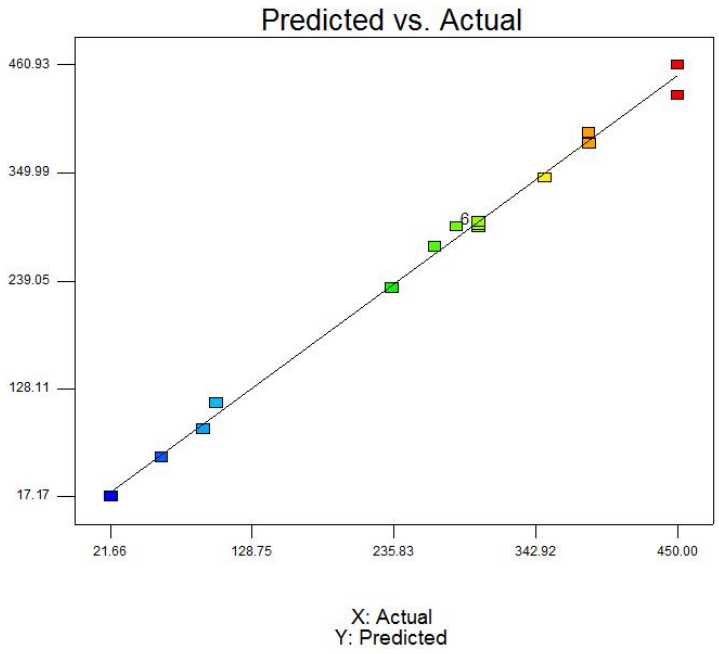

Fig. 5: Actual vs. predicted values.

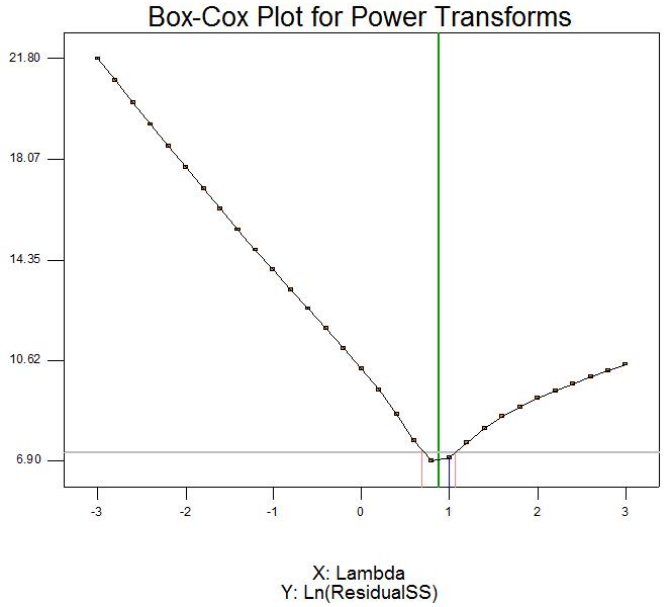

Fig. 6: Box-Cox plot for power transforms.

Table 1: The range of the variables in central composite design for optimizing the parameters in Endoglucanase production In liquid state fermentation

Independent variables	Symbol	Code levels			
		-1	+1	$-\alpha$	$+\alpha$
Substrate conc (%)	A	0.5	1.25	0.244328	1.50567
pH	B	6	9	4.97731	10.0227
Temp	C	18	40	10.5003	47.4997

Table 2: Experimental design of central composite for three factors and the production of Endoglucanase.

Serial no.	Sub. Conc.(A)	pH(B)	Temp ^{0}C (C)	Endoglucanase IU/ml
1	0.5	6	18	234.33
2	1.25	6	18	383
3	0.5	9	18	91.47
4	1.25	9	18	283.33
5	0.5	6	40	300
6	1.25	6	40	450
7	0.5	9	40	60
8	1.25	9	40	266.66
9	0.24	7.5	29	101
10	1.5	7.5	29	450
11	0.87	4.9	29	300
12	0.87	10	29	21.66
13	0.87	7.5	10.5	350
14	0.87	7.5	47.49	383.33
15	0.87	7.5	29	300
16	0.87	7.5	29	300
17	0.87	7.5	29	300
18	0.87	7.5	29	300
19	0.87	7.5	29	300
20	0.87	7.5	29	300

The response (endoglucanase) of the CCD design were fitted with a quadratic equation.

Table 3: Analysis of variance (ANOVA) for quadratic model for Endoglucanase activity.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	270300.7	9	30033.41	272.9625	< 0.0001
A-substrate conc	120745.6	1	120745.6	1097.411	< 0.0001
B-pH	94158.66	1	94158.66	855.7729	< 0.0001
C-temp	1447.178	1	1447.178	13.15286	0.0046
AB	1246.253	1	1246.253	11.32673	0.0072
AC	32.52211	1	32.52211	0.295581	0.5986
BC	4086.532	1	4086.532	37.14096	0.0001
A^2	1488.007	1	1488.007	13.52394	0.0043
B^2	37048.92	1	37048.92	336.7237	< 0.0001
C^2	7019.746	1	7019.746	63.79985	< 0.0001

R^2-0.9959, Adj $R^2$0.9923, Pred R^2-0.9691, C.V. %-3.83.

***Conclusion*:**

The present study demonstrated that R. oryzae offered options for the over production of endoglucanolytic enzymes for industrial applications. The endoglucanase production was influenced by different pH, temperature and substrate concentration and these parameters were successfully optimized using statistical based model response surface method. The optimum response (endoglucanase activity) 450 IU/ml was observed at substrate concentration 1.25%, temperature 40°C and pH 6 against the predicted 459.76 IU/ml indicated model accuracy. The enzymatic production at higher temperature could make the strain an attractive and alternative source for thermostable enzyme for paper and food industries improving the effectiveness of conventional bleaching and reducing the chances of food contamination respectively at higher temperature.Moreover utilization of water hyacinth as sole carbon source would reduce the cost of enzyme production with effective waste utilization.

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