Research Article
Optimization of Cellulase Production by *Trichoderma reesei HY07* Using Response Surface Methodology

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Abstract: Response Surface Methodology (RSM) based on a three-level, three-variable Box and Behnken Factorial Design (BBFD) was used to evaluate the interactive effects of corn stalk: bran ratio, tween80 and temperature on the cellulase production by solid fermentation. The optimum conditions derived via RSM were: corn stalk: bran ratio 1.29:1, Tween80 11.05 μL and temperature 31 °C for carboxymethyl cellulase (CMCase) and corn stalk: bran ratio 0.77:1, Tween80 12.54μL and temperature 32 °C for Filter Paper Activity (FPA). The actual experimental yield was 406.42 U/g for CMCase and 93.62 U/g for FPA under optimum condition, which compared well to the maximum predicted value of 405.67 U/g and 91.29 U/g. The cellulase yield under optimal conditions was 1.45 fold for CMCase and 1.33 fold for FPA to the control.

Keywords: Cellulose, carboxymethyl cellulose, FPA, response surface methodology, solid state fermentation

INTRODUCTION

Cellulose is the most abundant renewable resource in the world; it can be degraded into glucose by cellulase, which in turn can be converted to other valuable chemicals, energy or single cell protein (Zhang and Lynd, 2004; Jarvis, 2003; Bhat, 2000). Cellulase system consists of cellobiohydrolases (EC 3.2.1.91), endo-1,4-β-D-glucanases (EC 3.2.1.4) and 1,4-β-D-glucosidase (EC 3.2.1.21) (Latifian *et al*., 2007) , it can be produced by a large diversity of microorganisms, among which Trichoderma reesei is known to be most powerful in cellulose hydrolysis (Cherry and Fidantsef, 2003; Esterbauer *et al*., 1991; Henning and Lisbeth, 2006), which has been widely studied for its cellulase production from various cellulosic materials such as wood, waste paper, corn cob, wheat bran, wheat straw, banana wastes, cassava waste Sugarcane bagasse and rice stalk (Das and Singh, 2004; Haight, 2005; Jacobus and van Wyk, 2001; Kirk *et al*., 2002; Chundakk, 1999; Adsul *et al*., 2001) many factors including the culture conditions, the type of strain used and media components affect cellulase production (Lynd *et al*., 2002), However, optimization of cellulase production by RSM are few.

In our previous study, a cellulase-producing strain *Trichoderma reesei HY07* was screened from decayed corn stalk (Chen and Shu, 2008). Some factors including ammonium sulphate, inoculum, Tween 80, temperature, solid: liquid, incubation time, pH and corn stalk: bran ratio were assessed for the production of cellulase by

Trichoderma reesei HY07 (Shu *et al*., 2011).The main factors including corn stalk: bran ratio, tween-80 and temperature were screened by Plackett–Burman design. The objectives of this study were to optimize and to study the effect of corn stalk: bran ratio, tween-80 and temperature on the cellulase (CMCase and FPA) production by *Trichoderma reesei HY07* using response surface methodology.

MATERIALS AND METHODS

**Microorganism*:* The strain *Trichoderma reesei HY07*, isolated from decayed corn stalk, was used for cellulase production. A spore suspension was obtained for each organism by growing them on potato dextrose agar at 28 °C for one week and harvesting the spores with sterile water containing 0.1% peptone, the final suspension contained 10^8 spores/mL.

Medium and culture conditions: The initial cultivation medium was composed of corn stalk 4g, wheat bran 6g, tween80 20μL, $(NH_4)_2SO_4$ 0.01g, K_2HPO_4 0.005g, $MgSO_4 \cdot 7H_2O$ 0.025g and 10 mL distilled water in 250 mL Erlenmeyer flasks. pH 5.0 adjusted by 1N HCl and 1N NaOH, The flasks were plugged with cotton and autoclaved at 121°C for 20 min, cooled and inoculated spore suspension to 106 spores g-1 medium, 30 °C for 5d. For the experiments of optimization, the cultivation medium was composed of different concentrations of corn stalk, bran, tween80, also varying the temperature of cultivation, according to the experimental design.

Extraction of cellulase: The mouldy substrates (koji) produced by solid state fermentation were mixed with 10 volumes of water to extract cellulase, stirred slowly at 30 °C for 1 h and filtered. The liquid portion was then used for the measurement of cellulase activity.

Enzyme assays: Carboxymethyl cellulase (CMCase) and Filter Paper Activity (FPA) assay were carried out by mixing 0.5 mL enzyme sample with 0.5 mL of 1% Carboxymethylcellulose (CMC) in 0.05M sodium citrate buffer (pH 4.8) at 50 °C for 30 min, or 50 mg of Whatman No. 1 filter paper suspended in 0.5 mL of the same buffer, and followed by incubation for 30 min. by shaking at 50 °C. Reducing sugar was determined using 3, 5-dinitrosalicylic acid (DNS) reagent with glucose as a standard (Miller, 1959). The CMCase and FPA were both expressed as U/g of koji. One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate 1μmol of product per 30 min.

Experimental design: After identifying the variables affecting cellulase production by 'one factor-at-a-time' approach, the three most important variables, and viz. stalk: bran ratio(X_1), tween80 (X_2) and temperature(X_3) were selected. RSM using Box and Behnken factorial design (BBFD) (Caroline *et al.*, 2011) was adopted for improving cellulase production using the software Design-Expert Version 7.1.3 (Stat-Ease Inc., Minneapolis, USA) to find the interactive effects of three variables. Box and Behnken design at the given range of the above parameters in terms of coded and true values is presented in Table 1.

The average CMCase activity (U/g) and FPA activity (U/g) were taken as dependent variables or responses Y1 and Y2. Regression analysis was performed on the data obtained. The regression model between dependent variables (Y) and independent variables was:

$$Y = b_0 + \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} x_i^2 + \sum \sum_{i \neq j=1}^{3} b_{ij} x_i x_j \tag{1}$$

where,

b_0 = The constant
b_i = The linear coefficient
b_{ii} = The quadratic coefficient
b_{ij} = The cross product coefficient xi and xj are the levels of the independent variable

Data analysis: The data from the experiments performed were analyzed using design expert 7.1.3 version obtain the coefficients of the quadratic polynomial model. 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 the screened variables: The results obtained by BBFD were analyzed by standard analysis of variance (ANOVA), and the mean predicted and observed responses were presented in Table 1. The second order regression equation provided the levels of CMCase and FPA production as a function of initial values of corn stalk: bran ratio, tween80 and temperature, which can be predicted by the following equation:

$$Y_1 = 396.93 + 11.26x_1 + 5.83x_2 + 21.54x_3 + 0.92x_1x_2 - 17.21x_1x_3 - 2.48x_2x_3 - 3.24x_1^2 - 27.27x_2^2 - 9.94x_3^2 \tag{2}$$

$$Y_2 = 89.70 + 12.02x_1 + 1.99x_2 + 10.64x_3 - 3.70x_1x_2 \\ 9.10x_1x_3 + 1.93x_2x_3 + 1.66x_1^2 - 12.36x_2^2 - 8.66x_3^2 \tag{3}$$

According to the model above mentioned, x_1, x_2, x_3, x_1x_3, x_2^2 and x_3^2 were significant model terms for Y1 and x_1,x_3, x_1x_3, x_2^2 and x_3^2 were significant model terms

Table 1: Results of BBFD showing observed and predicted response for CMCase and FPA activity, x_1= Corn stalk:bran ratio (w/w), x_2 =Tween80(Ml), x_3 = temperature (°C)

Std	Run	x_1	x_2	x_3	CMCase activity(U/g)		FPA activity(U/g)	
					Observed[a]	Predicted	Observed[a]	Predicted
1	1	0.65:1(-1)	10(-1)	30(0)	350.62	350.26	64.53	61.29
2	8	1.35:1 (1)	10(-1)	30(0)	369.51	370.94	93.07	92.74
3	7	0.65:1(-1)	30(1)	30(0)	361.53	360.06	72.41	72.66
4	4	1.35:1 (1)	30(1)	30(0)	384.13	384.44	86.12	89.31
5	3	0.65:1(-1)	20(0)	28(-1)	335.76	333.75	51.25	50.94
6	15	1.35:1 (1)	20(0)	28(-1)	394.42	390.67	96.22	93.19
7	14	0.65:1(-1)	20(0)	32(1)	407.54	411.23	87.49	90.41
8	6	1.35:1 (1)	20(0)	32(1)	397.49	399.35	96.28	96.26
9	10	1:1(0)	10(-1)	28(-1)	327.61	329.89	54.74	57.98
10	11	1:1(0)	30(1)	28(-1)	343.14	346.49	58.33	58.10
11	2	1:1(0)	10(-1)	32(1)	381.32	377.91	75.21	75.40
12	9	1:1(0)	30(1)	32(1)	386.93	384.61	86.55	83.23
13	13	1:1(0)	20(0)	30(0)	397.59	396.93	89.61	89.70
14	12	1:1(0)	20(0)	30(0)	396.51	396.93	91.18	89.70
15	5	1:1(0)	20(0)	30(0)	396.87	396.93	88.56	89.70

[a]: Results are mean of three determinations

Table 2: Regression coefficient and their P values for CMCase and FPA activity

Factor	CMCase activity(U/g)				FPA activity(U/g)			
	Regression coefficient	Standard error	F-value	Prob > F	Regression coefficient	Standard error	F-value	Prob > F
Intercept	396.93	2.212629			89.70	2.059247		
x_1	11.26	1.354953	69.04	0.0004	12.03	1.261026	90.93	0.0002
x_2	5.83	1.354953	18.48	0.0077	1.99	1.261026	2.48	0.1758
x_3	21.54	1.354953	252.75	<0.0001	10.64	1.261026	71.16	0.0004
x_1x_2	0.92	1.916193	0.23	0.6497	-3.70	1.783361	4.30	0.0927
x_1x_3	-17.21	1.916193	80.64	0.0003	-9.10	1.783361	26.04	0.0038
x_2x_3	-2.48	1.916193	1.67	0.2530	1.93	1.783361	1.17	0.3297
x_1^2	-3.24	1.994437	2.64	0.1654	1.66	1.856181	0.80	0.4115
x_2^2	-27.27	1.994437	186.96	<0.0001	-12.36	1.856181	44.36	0.0012
x_3^2	-9.94	1.994437	24.83	0.0042	-8.66	1.856181	21.78	0.0055

Table 3: ANOVA for the experiments

Term	Response Y_1 CMCase activity(U/g)	Response Y_2 FPA activity(U/g)
F-value	69.49	28.97
P > F	0.0001	0.0009
R^2	0.9921	0.9812
Mean	375.36	79.37
Adjusted R^2	0.9778	0.9473
Adequate precision	26.002	15.564

for Y2 (Table 2). Table 4 gives the ANOVA values for the two responses viz. CMCase and FPA activity from the RSM experiments. ANOVA for CMCase production (Y1, U/g) indicated the 'F-value' to be 69.49, which implied the model to be significant. Model terms having values of 'Prob > F' less than 0.05 are considered significant, whereas those greater than 0.10 are insignificant. Correspondingly ANOVA for FPA activity (Y2, U/g) indicated the 'F-value' to be 28.97, which implied that the model was significant.

ANOVA indicated the R^2-value of 0.9921 and 0.9812, respectively, for responses Y1 and Y2. This again ensured a satisfactory adjustment of the quadratic model to the experimental data, and indicated that the model could explain 90-95% of the variability in the response. The adequate precision which measures the signal to noise ratio was 26.00 and 15.56 (Table 3) for responses Y1 and Y2, respectively, which indicates an adequate signal.

The parameter combination resulting in optimal CMCase and FPA yield were obtained by solving the system of partial derivatives for the different independent variables. This resulted in the following optimal values for CMCase yield, respectively, corn stalk: bran ratio 1.29:1, Tween80 11.05μL and temperature 30.69 °C; optimal values for FPA yield, respectively, corn stalk: bran ratio 0.77:1, Tween80 12.54 μL and temperature 31.96 °C.

The response surface curves were plotted to understand the interaction of the variables and to determine the optimum level of each variable for maximum response (Fig. 1 to 6).

Validation of the model: The suitability of the model equation for predicting the optimum response values
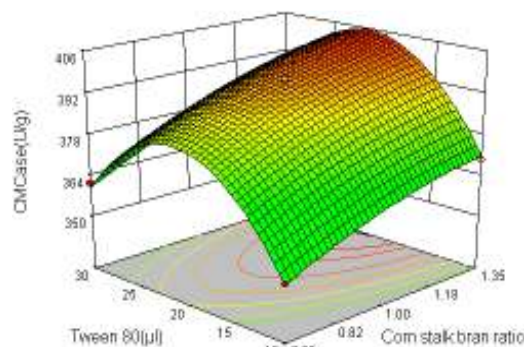

Fig. 1: Response surface graph representing the interaction between corn stalk: Bran ratio and Tween 80
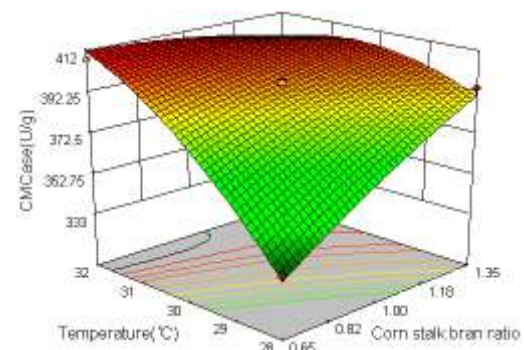

Fig. 2: Response surface graph representing the interaction between corn stalk: Bran ratio and temperature
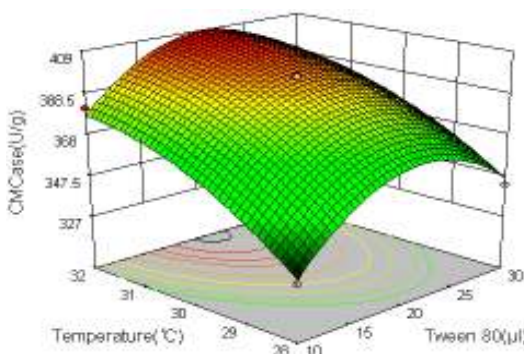

Fig. 3: Response surface graph representing the interaction between Tween 80 and temperature

Table 4: Predicted and experimental value of response under optimal and control conditions

Optimum condition	CMCase(U/g)			FPA(U/g)		
	Coded	Ture value	The control	Coded	Ture value	The control
Corn stalk:bran ratio (w/w)	0.84	1.30	0.65	-0.65	0.80	0.65
Tween80 (µL)	0.11	11	20	0.25	13	20
Temperature (°C)	0.34	30	30	0.98	32	30
Responses	Predicted	Experimental		Predicted	Experimental	
Yield	405.67	406.42 [b]	279.6	91.29	93.36	70.20

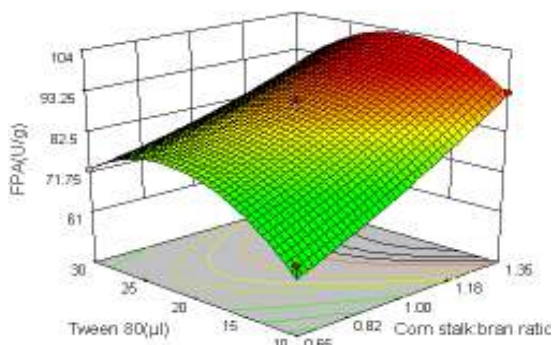[b]: Results are mean of three determinations

Fig. 4: Response surface graph representing the interaction between Corn stalk: Bran ratio and Tween 80
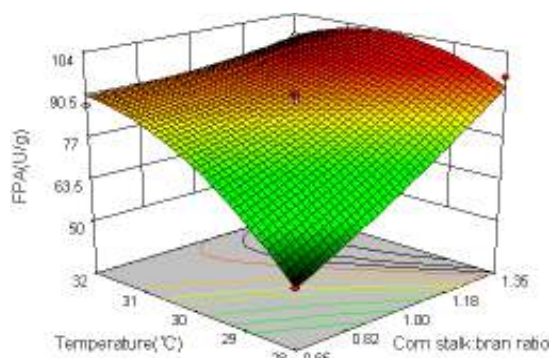

Fig. 5: Response surface graph representing the interaction between corn stalk: Bran ratio and temperature
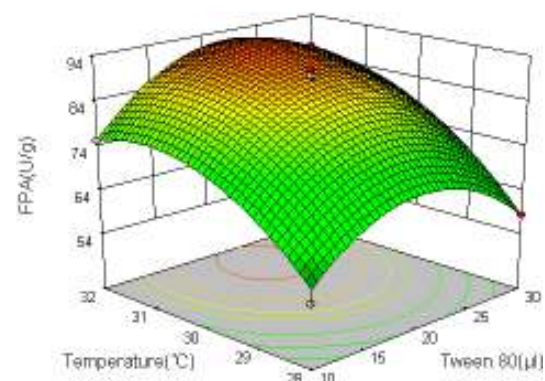

Fig. 6: Response surface graph representing the interaction between Tween 80 and temperature

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 4). The CMCase and FPA activity reached 406.42U/g and 93.36U/g under the optimal conditions, respectively; there are a 1.45 fold increase in CMCase yield and 1.33 fold increase in FPA activity to the control (Table 4).

CONCLUSION

Comparison of predicted and experimental values revealed good correspondence between them, implying that empirical models derived from RSM can be used to adequately describe the relationship between the factors and response in cellulase production by *Trichoderma reesei HY07*. These models can then be used to predict CMCase and FPA production under any given conditions within the experimental range. We have demonstrated that optimum conditions of cellulase production can be successfully predicted by RSM.

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