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Full Length Research Paper

High-yield cellulase production in solid-state fermentation by *Trichoderma reesei* SEMCC-3.217 using water hyacinth (*Eichhornia crassipes*)

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In this study, the strain *Trichoderma reesei* SEMCC-3.217 was used for producing cellulase in solid-state fermentation with water hyacinth (*Eichhornia crassipes*). The results of fractional factorial design showed that, the addition amount of wheat bran, $(NH4)_2SO_4$, $CaCl_2$ and Tween 80 had significant effect on the cellulase production. Then, these four factors were selected for further optimization by central composite design for the yield of cellulase. The statistical analysis of the results showed that, the optimum composition were: 5 g of substrate containing 3.9 g water hyacinth, 1% corn steep liquor, 1% soybean meal, 0.2% NH_4NO_3, 0.2% KH_2PO_4, 0.08% $MgSO_4{\cdot}7H_2O$, 2.8% $(NH_4)_2SO_4$, 1.5% urea, 13.9% wheat bran, 0.08% $ZnSO_4{\cdot}7H_2O$, 0.08% $FeCl_2$ 0.05% $CaCl_2$, 0.08% $NaNO_3$, 0.08% KCl and 0.27% (v/v) Tween-80. Under these conditions, the cellulase production was 4-fold increased (13.4 FPIU/g dry solid) compared with the initial level (3.4 FPIU/g dry solid) after 7 days of fermentation in a 250 ml Erlenmeyer flask.

Key words: Cellulase, solid-state fermentation, optimization, water hyacinth, *Trichoderma reesei.*

INTRODUCTION

Many fungi could produce cellulase enzyme when grown on the pure cellulose substrates or lignocellulosic substrates and *Trichoderma reesei* is a kind of excellent filamentous fungus for cellulose production, which has been industrially produced by fermentation at present. Cellulase is a group of enzymes which have the capability of hydrolyzing cellulose into fermentable sugars such as glucose (Ladisch et al., 1981), which can be used for producing many useful products such as ethanol, biofuel and other useful chemicals from the cellulosic feedstocks (Bhat, 2000; Peng and Chen, 2008; Tomás-Pejó et al., 2009). Cellulase could be produced by many lignocelluloytic feed stocks such as straws, bagasse, wheat bran, corn stover, corncob, etc (Xia and Cen, 1999; Romero et al., 1999; Camassola and Dillon, 2009). Water hyacinth was known as one of the fastest growing plants and a kind of unwanted species in China and the large-scale outbreak of it leads to many environmental problems in South China. In order to resolve the problems, water hyacinth has been used for producing biogas (Singhal and Rai, 2003), ethanol (Mishima et al., 2008) and high caloric fuel (Lu et al., 2009). However, no data about cellulase production fromwater hyacinth has been reported at present. Water hyacinth have high content of hemicellulose (35 to 55% of dry mass) (Nigam, 2002; Kumar et al., 2009), cellulose (18% of the dry mass (Nigam, 2002) and protein (13% of the dry mass (Nigam, 2002), which can provide enough nutrients for cellulase production by the strain SEMCC-3.217. In this study, water hyacinth was firstly used as the main substrate for cellulase production in solid-state fermentation by the strain *T. reesei* SEMCC-3.217. Four factors were selected by fractional factorial design and further optimized by response surface methodology. The production of cellulase was up to 13.2 PFIU/g dry solid, which was almost 4-fold of the initial yield.

MATERIALS AND METHODS

Microorganism and inoculum preparation

The mutant strain *T. reesei* SEMCC-3.217 was maintained on potato dextrose agar (PDA) slants, stored at 4℃. For preparing

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Table 1. Values for the fractional factorial design.

Variable	Medium component	+Values (%, w/w)	-Value (%, w/w)
X_1	Corn steep liquor	1.5	0.5
X_2	Soybean meal	1.5	0.5
X_3	NH_4NO_3	0.3	0.1
X_4	KH_2PO_4	0.3	0.1
X_5	$MgSO_4 \cdot 7H_2O$	0.1	0.05
X_6	$(NH_4)_2SO_4$	2	1
X_7	Urea	2	1
X_8	Wheat bran	10	30
X_9	$ZnSO_4 \cdot 7H_2O$	0.1	0.05
X_{10}	$FeCl_2$	0.1	0.05
X_{11}	$CaCl_2$	0.1	0.05
X_{12}	$NaNO_3$	1	0.5
X_{13}	KCl	0.1	0.05
X_{14}	Tween 80	0.3	0.1

spore suspension, strain SEMCC-3.217 was cultured on PDA slants in the dark at 30℃ for 10 days. The spores were eluted with sterile distilled water containing 0.1% of Tween 80 and the spore suspension (1×10^7/ml) was used as inoculum.

Growth medium and culture conditions

Water hyacinth was locally obtained from Jinan city, dried, milled and was utilized as the substrate for solid-state fermentation. The average particles size of the substrates were 0.9 to 1.2 mm. 5 g of substrates (the composition was based on the experimental design) was weighted into a 250 ml Erlenmeyer flask and to this, the distilled water was added; to the 5 g of the well mixed substrate, containing 70% (w/w) of moisture. After sterilization for 30 min at 121℃, 1 ml of the spore suspension, suspended in distilled water (containing 0.1% of Tween 80) was aseptically added to the substrate and mixed thoroughly, then was incubated in the dark at 32℃ for 7 days. Experiments were conducted based on the statistical design; variations of the parameters were conducted according to the design.

Enzyme extraction and assay

Cellulase was extracted by suspending the fermented substrate with 5-fold of distilled water and mixing it for 1 h at 300 rpm. And then, the crude enzyme was further extracted by centrifugation (10,000 *g*, 20 min). The total cellulase activity (filter paper unit, FPU) was measured by the standard filter paper assay (Afolabi, 1997). The filter paper enzyme activity (FPA) was expressed as FPIU/g of dry substrate (FPIU/gds). One international unit (IU) of enzyme activity was defined as the amount of enzyme required to liberated 1 μmol of product per min at 50℃.

Experimental design and data analysis

Fractional factorial design

The influences of 14 factors on cellulase production were investigated using a fractional factorial design, which could evaluate the main effects of factors (Cochran and Cox, 1957). A 2^{14-10} fractional factorial design with 14 factors at two levels (Table 1) was required in the experiment. The factors, which were significant at 95% of confidence level ($P < 0.05$) from the regression analysis were considered to have greater effects on cellulase production and were further optimized by a central composite design. The first-order model was established to fit the results of the fractional factorial design and was represented as:

$$Y = \beta_0 + \sum \beta_i x_i \quad (1)$$

Where, Y was the predicted value; β_0 was the intercept; β_i was the linear coefficient and x_i was the coded independent factor.

Steepest ascent

The direction of steepest ascent was the direction in which Y increased most rapidly. The direction of the tests was parallel to the normal contour line of the response curve of model 1 and passed through the center point of the fractional factorial design. The steps along the path were according to the regression coefficients β_i. Experiments were conducted along the path until the response did not increase any longer. This point was near the optimal point and could be selected as the center point for optimization (Tang et al., 2004).

Central composite design and response surface methodology

Central composite design and response surface methodology was used to optimize the four most significant factors (wheat bran, $(NH_4)_2SO_4$, $CaCl_2$ and Tween 80) for enhancing cellulase production which were screened by fractional factorial design. The four independent factors were studied at five different levels (-2, -1, 0, 1 and 2) (Table 2). The factors were coded according to the following equation:

$$X_i = \frac{(X_i - X_o)}{\Delta X}, i = 1, 2, ..., k \quad (2)$$

Where, X_i was the coded independent factor, X_i was the real independent factor; X_0 was the value of X_i at the center point; ΔX

table 2. Coded and concentration values of the variables studied in central composite design.

Coded value (level)	Real value of variable			
	***A*(X_6)**	***B*(X_8)**	***C*(X_{11})**	***D*(X_{14})**
-2	1.7	10	0.02	0.20
-1	2.2	11	0.03	0.24
0	2.7	12	0.04	0.28
1	3.2	13	0.05	0.32
2	3.7	14	0.06	0.36

was the step change value.
The behavior of the system was explained by the following second-order polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j, i = 1, 2, ..., k \quad (3)$$

Where, Y was the predicted response, β_0 was the intercept, x_i and x_j were the coded independent factors, β_i was the linear coefficient, β_{ii} was the quadratic coefficient and β_{ij} was the interaction coefficient.

Statistical analysis

Design expert (version 7.0, STATEASE Inc., Minneapolis, USA) was used for the experimental designs and regression analysis of the data obtained from the experiments. Statistical analysis of the model was carried out to estimate the analysis of variance (ANOVA). The quality of the polynomial model equation was determined by the determination coefficient R^2 and the statistical significance was judged by an *F*-test. The significance of the regression coefficients was evaluated by a *t*-test.

Validation of the optimized condition

In order to validate the optimization of medium composition, three tests were carried out using the optimized from the analysis of the response surface.

RESULTS AND DISCUSSION

Fractional factorial design

The fractional factorial design is a powerful method for screening significant factors. 20 runs were conducted to analyze the effect of 14 variables (including four center points) on cellulase production; the results are listed in Table 3.

A first-order model was fitted to the results obtained from the twenty tests:

$$Y(\text{IU/gds}) = 3.44 + 0.22X_1 - 0.32X_2 - 0.09X_3 - 0.08X_4 + 0.18X_5 + 0.47X_6 + 0.006X_7 - 0.78X_8 + 0.08X_9 - 0.28X_{10} - 0.69X_{11} - 0.08X_{12} + 0.16X_{13} + 0.49X_{14} \quad (4)$$

The coefficient R^2 of the first-order model was 0.9604, indicating that nearly 96% of the variability in the response could be explained by the model. Table 4 shows that wheat bran, $(NH_4)_2SO_4$, Tween 80 and $CaCl_2$ were the most significant factors ($P < 0.05$). And then, these four factors were selected for further optimization in order to obtain a maximum yield.

Steepest ascent

Based on the results of the fractional factorial design, wheat bran, $(NH_4)_2SO_4$, Tween 80 and $CaCl_2$ were significantly influenced by the cellulase production. The path of steepest ascent was used to find the proper direction of changing variables by increasing or decreasing the value of the main factors. The results according to the model of fractional factorial design and the experimental design are shown in Table 5. It is shown that, the highest response was 13.2 FPIU/gds when the selected factors were: wheat bran 12%, $(NH_4)_2SO_4$ 2.7%, Tween 80 0.28% and $CaCl_2$ 0.04%. It suggested that, this point was near the optimal point and was chosen for central composite design optimization.

Central composite designs and response surface analysis

Based on the fractional factorial design and steepest ascent experiments, response surface methodology using central composite designed was applied to find the optimal levels of these four variables (wheat bran, $(NH_4)_2SO_4$, $CaCl_2$ and Tween 80), the coded levels for the four variables are defined in Table 6.

A total of 27 run with different combination of wheat bran (X_4, *A*), $(NH_4)_2SO_4$ (X_6, *B*), $CaCl_2$ (X_{11}, *C*) and Tween 80 (X_{14}, *D*) was designed in Table 7.

The experimental results were analyzed by ANOVA and central composite design was fitted with the second-order polynomial equation:

$$Y(\text{IU/gds}) = 13.13 + 1.03A + 0.43B + 0.57C - 0.20D - 0.61AB + 0.84AC + 0.91BC - 1.14AD - 0.41AD + 0.04CD - 1.63A^2 - 0.53B^2 - 1.13C^2 - 1.56D^2 \quad (5)$$

Table 3. Fractional factorial design matrix with cellulase production.

Run number	Variable														Cellulase production (FPIU/gds)
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2.5±0.3
2	1	1	1	-1	-1	-1	-1	-1	-1	-1	-1	1	1	1	4.7±0.9
3	1	1	-1	1	-1	-1	-1	-1	1	1	1	-1	-1	-1	1.8±0.2
4	1	1	-1	-1	1	1	1	1	-1	-1	-1	-1	-1	-1	3.5±0.1
5	1	-1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	1	2.6±0.6
6	1	-1	1	-1	1	1	-1	-1	1	1	-1	-1	-1	1	6.6±1.2
7	1	-1	-1	1	1	1	-1	-1	-1	-1	1	1	1	-1	4.8±0.7
8	1	-1	-1	-1	-1	-1	1	1	1	1	-1	1	1	-1	3.1±0.2
9	-1	1	1	1	-1	1	-1	1	-1	1	-1	-1	1	-1	2.6±0.4
10	-1	1	1	-1	1	-1	1	-1	1	-1	1	-1	1	-1	3.1±0.6
11	-1	1	-1	1	1	-1	1	-1	-1	1	-1	1	-1	1	4.3±0.7
12	-1	1	-1	-1	-1	1	-1	1	1	-1	1	1	-1	1	2.8±0.3
13	-1	-1	1	1	1	-1	-1	1	1	-1	-1	1	-1	-1	2.4±0.7
14	-1	-1	1	-1	-1	1	1	-1	-1	1	1	1	-1	-1	2.6±0.1
15	-1	-1	-1	1	-1	1	1	-1	1	-1	-1	-1	1	1	6.2±1.1
16	-1	-1	-1	-1	1	-1	-1	1	-1	1	1	-1	1	1	2.1±0.2
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.3±0.5
18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.1±0.6
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.4±0.3
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.3±0.7

Table 4. Parameter estimates for cellulase production from the result of fractional factorial design.

Variable	Estimate	S. E.	*t*-value	P > |t/
Intercept	3.44	0.11	30.96	<0.0001*
X_1	0.22	0.12	1.76	0.1385
X_2	-0.32	0.12	-2.57	0.0503
X_3	-0.09	0.12	-0.75	0.4844
X_4	-0.08	0.12	-0.65	0.5419
X_5	0.18	0.12	1.46	0.2043
X_6	0.47	0.12	3.77	0.0130*
X_7	0.01	0.12	0.05	0.9618
X_8	-0.78	0.12	-6.29	0.0015*
X_9	0.08	0.12	0.65	0.5419
X_{10}	-0.28	0.12	-2.26	0.0730
X_{11}	-0.69	0.12	-5.59	0.0025*
X_{12}	-0.08	0.12	-0.65	0.5419
X_{13}	0.16	0.12	1.26	0.2640
X_{14}	0.49	0.12	3.98	0.0106*

*Statistically significant at 95% of confidence level.

Where, *A*, *B*, *C* and *D* are the coded factors of wheat bran, $(NH_4)_2SO_4$, $CaCl_2$ and Tween 80, respectively. The analysis of variance and regression analysis for the cellulase production are listed in Table 8. The model *F*-value of 8.65 implied that the model was significant. There is only a 0.03% chance that a "Model *F*-value"

Table 5. Experimental design of steepest ascent and corresponding responses.

Run number	Coded value				Experimental value				Cellulase production (FPIU/gds)
	X_6 0.6	X_8 -0.9	X_{11} -1	X_{14} 0.6	X_6 0.3	X_8 2	X_{11} 0.01	X_{14} 0.2	
1	0	0	0	0	1.5	20	0.08	0.2	3.4±0.5
2	0.6	-0.9	-1	0.6	1.8	18	0.07	0.22	5.6±0.4
3	1.2	-1.8	-2	1.2	2.1	16	0.06	0.24	8.7±1.3
4	1.8	-2.7	-3	1.8	2.4	14	0.05	0.26	11.8±1.9
5	2.4	-3.6	-4	2.4	2.7	12	0.04	0.28	13.2±1.4
6	3.0	-4.5	-5	3.0	3.0	10	0.03	0.3	10.9±1.8
7	3.6	-5.4	-6	3.6	3.3	8	0.02	0.32	8.7±0.9

Table 6. Central composite design with experimental and predicted values of cellulase production.

Run number	Variable				Cellulase production (FPIU/gds)	
	***A*: wheat bran (X_6)**	***B*: $(NH_4)_2SO_4$(X_8)**	***C*: $CaCl_2$ (X_{11})**	***D*: Tween 80(X_{14})**	**Experiment**	**Predicted**
1	1	1	1	1	9.6±1.3	9.7
2	1	1	1	-1	13.6±2.6	13.2
3	1	1	-1	1	4.9±0.8	5.0
4	1	1	-1	-1	7.6±1.2	8.6
5	1	-1	1	1	9.3±2.1	9.1
6	1	-1	1	-1	10.6±1.3	10.9
7	1	-1	-1	1	6.8±0.4	8.0
8	1	-1	-1	-1	9.6±0.8	10.0
9	-1	1	1	1	10.7±2.2	9.5
10	-1	1	1	-1	8.1±1.9	8.4
11	-1	1	-1	1	6.9±0.7	8.1
12	-1	1	-1	-1	7.8±1.6	7.2
13	-1	-1	1	1	5.9±1.3	6.4
14	-1	-1	1	-1	4.6±0.8	3.6
15	-1	-1	-1	1	9.1±1.1	8.7
16	-1	-1	-1	-1	4.7±0.4	6.1
17	0	0	0	0	13.4±1.7	13.1
18	0	0	0	0	12.1±0.9	13.1
19	0	0	0	0	13.9±2.4	13.1
20	0	0	0	-2	7.6±1.0	7.3
21	0	0	0	2	6.9±1.6	6.5
22	0	0	-2	0	9.3±1.3	7.5
23	0	0	2	0	8.6±0.6	9.7
24	0	-2	0	0	10.9±1.2	10.1
25	0	2	0	0	11.8±2.3	11.9
26	-2	0	0	0	4.3±0.5	4.5
27	2	0	0	0	9.6±1.7	8.7

which is this large could occur due to noise. The fit of the model was checked by the coefficient of determination R^2, which was calculated to be 0.9099, indicating that about 91% of the variability in the response could be explained by this model. It was considered as very high correlation when the R^2-value was higher than 0.9 (Li et al., 2005). The statistical significance of the model equation was evaluated by the *F*-test for ANOVA. The *P*-value was also very low ($P < 0.0003$), indicating the significance of the model. In this case, eight model terms(A^2, C^2, D^2, *A*, *C*, *AD*, *BC* and *AC*) were significant. The coefficient of variation (*CV*) indicates the degree of

Table 7. Parameter estimates for cellulase production from the result of central composite design.

Variable	Estimate	S.E.	*t*-value	P > |t/
Intercept	13.13	0.70	18.66	<0.0001*
A	1.03	0.25	4.15	0.0013*
B	0.43	0.25	1.74	0.1071
C	0.57	0.25	2.28	0.0419*
D	-0.20	0.25	-0.80	0.4371
AB	-0.61	0.30	-2.01	0.0675
AC	0.84	0.30	2.75	0.0177*
BC	0.91	0.30	2.99	0.0111*
AD	-1.14	0.30	-3.73	0.0029*
BD	-0.41	0.30	-1.35	0.2008
CD	0.04	0.30	0.12	0.9041
A^2	-1.63	0.26	-6.18	<0.0001*
B^2	-0.53	0.26	-2.01	0.0671
C^2	-1.13	0.26	-4.29	0.0011*
D^2	-1.56	0.26	-5.90	<0.0001*

*Statistically significant at 95% of confidence level.

Table 8. Analysis of variance and regression analysis for the cellulase production.

Source	Sum of square	Degree of freedom	Mean square	*F*-value	*P* > *F*
Model	179.98	14	12.86	8.65	0.0003
Residual	17.83	12	1.49		
Lack of fit	16.10	10	1.61	1.87	0.3991
Pure error	1.73	2	0.86		
Corrected total	197.81	26			

R^2= 0.9099; R^2_{adj}= 0.8047;C.V.%= 13.82.

precision with which the treatments are compared. A lower *CV* means a higher reliability of the experiment (Box et al., 1978). The relatively lower value of *CV* (13.82%) demonstrated that, the performed experiments were reliable. The lack of fit *P*-value of 0.3991 implied that, the lack of fit was not significantly relative to the pure error.

The response surface curves were plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The response surface contour curves are shown in Figures 1 to 6. Each figure demonstrates the effect of two factors, while the other factors were fixed at zero level. The model predicted the optimal values (coded) of the four most significant variables and were *A*=0.47, *B*=0.97, *C*=0.46 and *D*=-0.13. Correspondingly, the values of the four values were 2.8, 13.9, 0.05 and 0.27%, respectively. The maximum predicted cellulase production was 13.8 FPIU/gds. By optimization of culture conditions, cellulose production of the strain was enhanced from 3.4 to 13.8 FPIU/gds.

Validation of the optimized condition

In order to confirm the optimized culture conditions, three additional experiments in the Erlenmeyer flasks were performed using the predicted medium composition. The mean value of the cellulase production was 13.4±2.1 FPIU/gds, which agrees well with the predicted value. This result demonstrated the validity of the response model.

Conclusions

Fractional factorial design and central composite design have been proved to be effective in optimizing cellulase production using water hyacinth as the main substrate. The final composition of the optimized medium was as follows: 5 g of substrate containing 3.9 g water hyacinth, 1% corn steep liquor, 1% soybean meal, 0.2% NH_4NO_3, 0.2% KH_2PO_4, 0.08% $MgSO_4 \cdot 7H_2O$, 2.8% $(NH_4)_2SO_4$, 1.5% urea, 13.9% wheat bran, 0.08% $ZnSO_4 \cdot 7H_2O$,

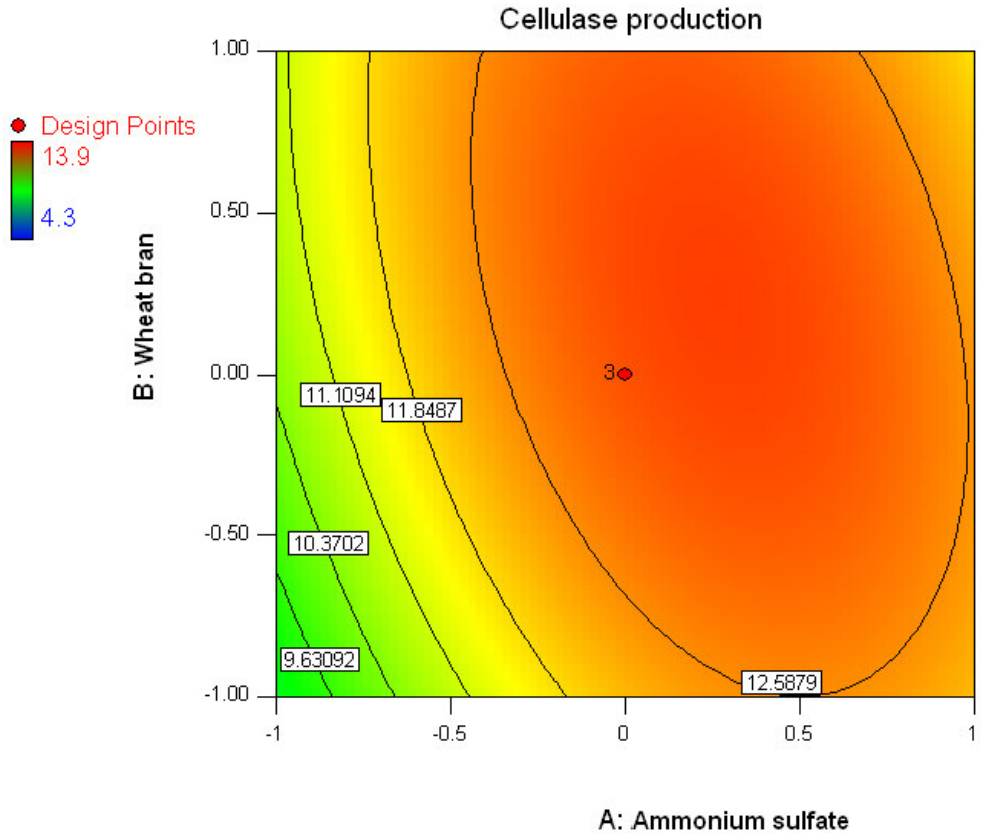

Figure 1. Contour plot of cellulase production (FPIU/gds): the effect of ammonium sulfate and wheat bran and their mutual interaction on cellulase production.

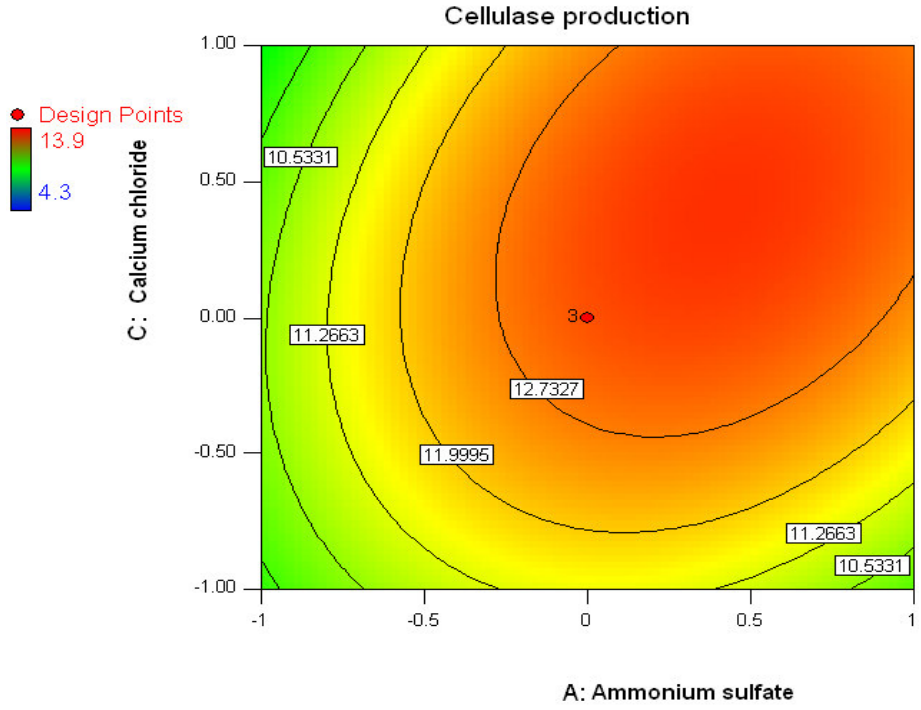

Figure 2. Contour plot of cellulase production (FPIU/gds): the effect of ammonium sulfate and calcium chloride and their mutual interaction on cellulase production.

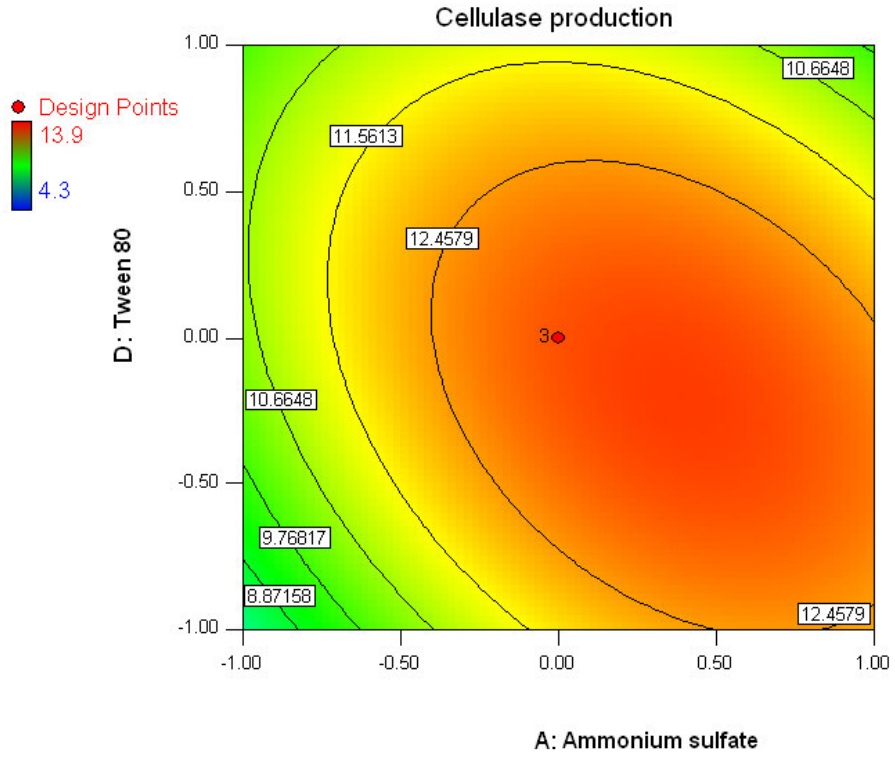

Figure 3. Contour plot of cellulase production (FPIU/gds): the effect of ammonium sulfate and Tween 80 and their mutual interaction on cellulase production.

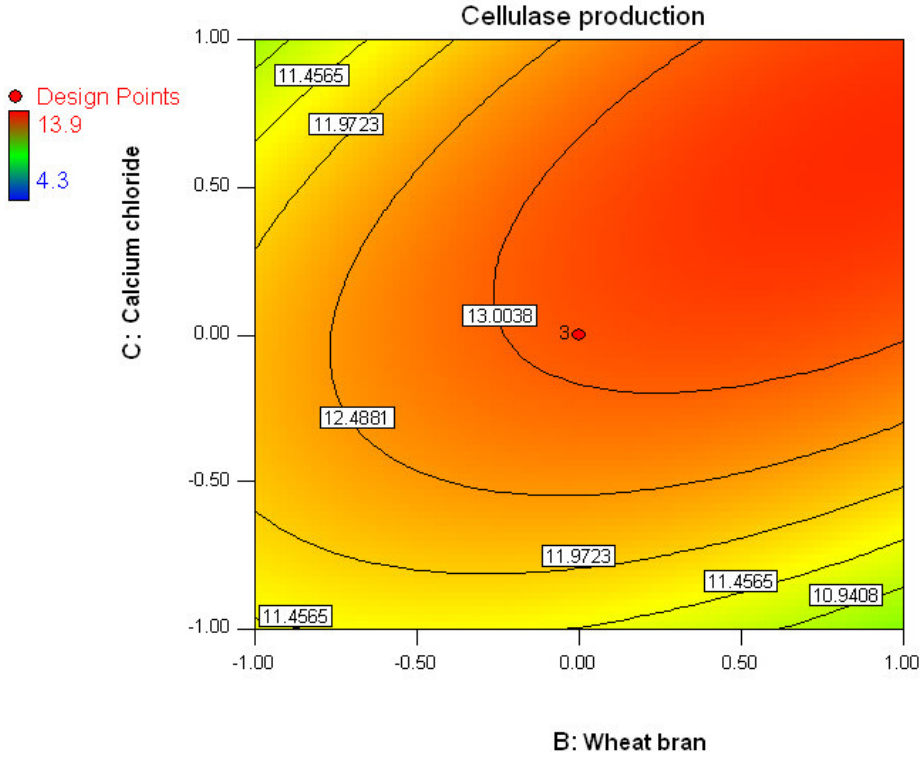

Figure 4. Contour plot of cellulase production (FPIU/gds): the effect of wheat bran and calcium chloride and their mutual interaction on cellulase production.

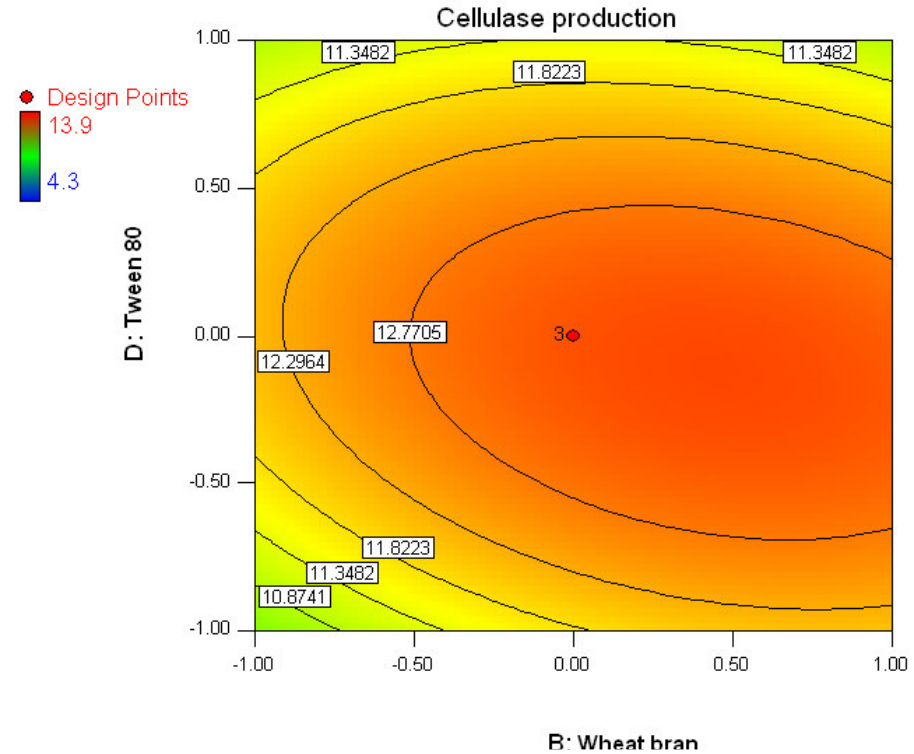

Figure 5. Contour plot of cellulase production (FPIU/gds): the effect of wheat bran and Tween 80 and their mutual interaction on cellulase production.

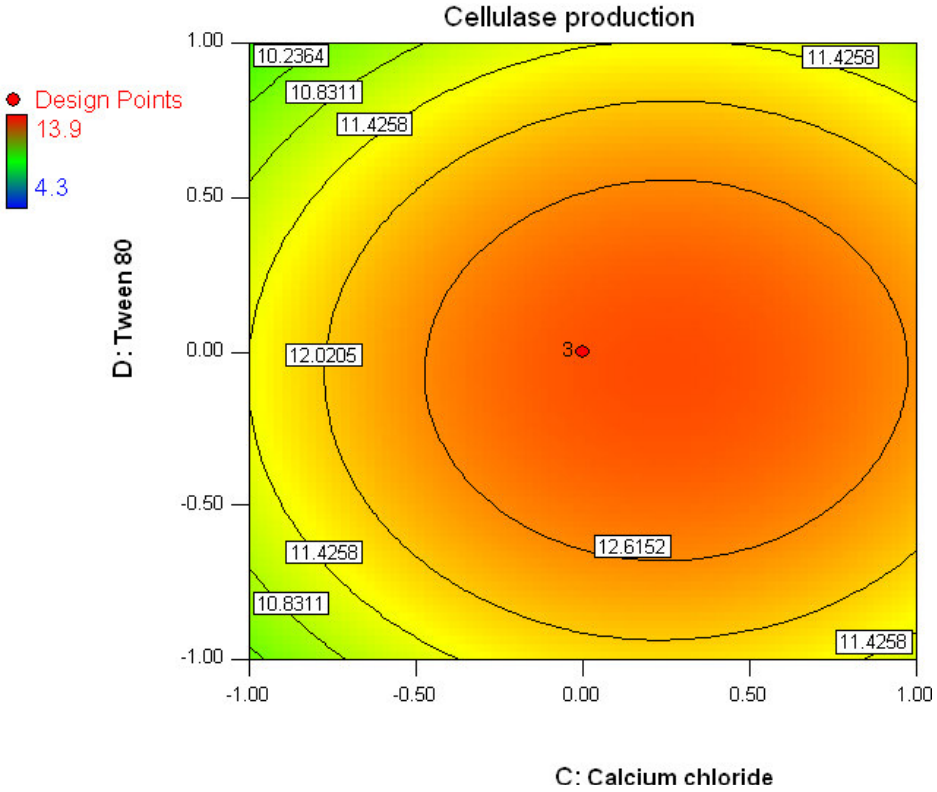

Figure 6. Contour plot of cellulase production (FPIU/gds): the effect of calcium chloride and Tween 80 and their mutual interaction on cellulase production.

0.08% $FeCl_2$, 0.05% $CaCl_2$, 0.08% $NaNO_3$, 0.08% KCl and 0.27% (v/v) Tween-80, which resulted in an around 4-fold increase compared with that using the original conditions in the flask cultivation. Thus, water hyacinth might be a suitable substrate for cellulase production using the strain *T. reesei* SEMCC-3.217.

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