African Journal of Biotechnology Vol. 8 (16), pp. 3879-3886, 18 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Optimizing cellulase production of *Penicillium waksmanii* F10-2 with response surface methodology

L. Han#, J. Feng#, C. Zhu and X. Zhang*

R&D Center of Biorational Pesticides, Northwest A & F University, 22 Xinong Road, Yangling, Shaanxi 712100, China.

Accepted 21 July, 2009

Cellulase is an enzyme complex which breaks down cellulose to ß-glucose. It has been used widely in commercial food processing, textile industry and laundry detergents. In this article, medium composition and fermentation conditions for *Penicillium waksmanii* F10-2, an isolated cellulase production strain, are studied. Wheat straw and peptone are found to be the most promising and effective carbon and nitrogen sources for cellulase production by *P. waksmanii* F10-2. Response surface methodology (RSM) is employed to optimize the medium constituents (wheat straw, peptone and minerals) and fermentation conditions (pH, rev and temperature) for cellulase production. The predicted maximal cellulase activity is about 5.64 U/mL, which is achieved at the following condition: pH: 6.4, rev: 136.2 r/min, temperature: 26.5°C, fermentation condition: wheat straw 20.3 g/L, peptone 11.3 g/L, KH_2PO_4 2.7 g/L, NaCl 3.3 g/L, $MgSO_4.7H_2O$ 0.2 g/L. Under the proposed optimized conditions, the model predicted a cellulase activity of 5.64 U/mL, which is very closely matching the experimental activity of 5.9 U/mL.

Key words: *P. waksmanii* F10-2, cellulase, response surface methodology, optimization.

INTRODUCTION

Cellulose, the world's most abundant carbohydrate polymer, is mainly broken down by cellulases. Cellulases can be divided into 3 groups based on their activity on cellulose: endoglucanase (*endo*-1, 4-ß-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase (*exo*-1,4-ß-D-glucanase, CBH, EC 3.2.1.91) and ß-glucosidase (1,4-ß-D-glucosidase, BG, EC 3.2.1.21) (Hong et al., 2001; Li et al., 2006). Cellulases, mainly produced by fungi, bacteria and protozoans, not only play a very important role in recycling cellulose in biosphere (Beguin and Auber, 1994), but also have broad industrial and commercial applications (Bhat, 2000; Adsul et al., 2007; Kaur et al., 2007).

However, the cellulase production step has been shown to be the most expensive step in any of the processes using enzymatic hydrolysis. Thus, the economics of cellulase production needs to be improved by reducing production cost or increasing the enzyme activity. Several researches have shown that the production costs of cellulase are tightly associated with the productivity of enzyme-producing microbial strain, the final activity in the fermentation broth and the type of substrate used in fermentation production of the enzyme (Duff and Murray, 1996; Nieves et al., 1998; Chahal et al., 1992; Reczey et al., 1996). In addition, the media composition accounts for a significant amount of the overall cost of cellulase fermentation production. Research efforts have been undertaken to replace the expensive carbon and nitrogen sources with cheap raw materials in the media to bring down the production cost of cellulose.

There have been extensive efforts to find more efficient strains and to improve fermentation techniques. For example, a strain of *Bacillus* sp. VG1, which grows at pH 7.0-11.0 and produces a highly thermo-stable, alkaline and extra-cellular CMCase, was isolated (Singh et al., 2001); a *Scytalidium thermophilum* type culture *Humicola insolens* MTCC 4520 isolated from composting soil was optimized to produce cellulolytic and hemicellulolytic enzymes by solid-state fermentation (SSF) (Jatinder et al., 2006). Statistics-based experimental methods have also been designed for bioprocess optimization. Response surface methodology is one of the most practical optimization methods. This method enables us to identify the

*Corresponding author. E-mail: zhxing1952@126.com. Tel.: 86-29-87093344. Fax: 86-29-87093344.

#These authors contributed equally to this work.

effects of individual variables and to efficiently seek the optimum conditions for a multivariable system. With this methodology, the effect of interaction of various parameters can be understood, generally resulting in high production yields and simultaneously limiting the number of experiments. The response surface methodology has been successfully applied to optimize alcoholic fermentation and other fermentation media. This methodology is also widely used for optimization studies in several biotechnological and industrial processes (Burker et al., 2004; Kalil et al., 2000; Puri et al., 2002; Muralidhar et al., 2001; Vohra and Satyanarayana, 2002; Saxena and Saxena, 2004).

There have been several studies published recently concerning the cellulase production from lighocellulosic materials under submerged liquid fermentation. However, the production of cellulase by *P. waksmanii* under submerged liquid has not been reported. *P. waksmanii* F10-2 has been found to produce high levels of cellulase and hemicellulase (Han et al., 2008). In the current study, the central composite design (CCD) is used to evaluate the coefficients in a quadratic mathematical model. And also, a response surface methodology is applied to predict the optimum yield of cellulase (carboxy methyl cellulose, CMCase).

MATERIALS AND METHODS

Materials

The lignocellulosic materials, including wheat straw, corn straw, rice straw and sawdust were obtained from Yangling, China. The materials were dried, chopped into small pieces, milled into smaller particles and then separated by 425 μm (40 meshes) sieve. The flow-through materials was used for submerged fermentation as described below.

Microorganism and maintenance

The strain of *P. waksmanii*, provided by the Research and Development Center of Biorational Pesticides (Northwest A&F University, Yangling, (China) was isolated from decomposed plant samples collected in Qinling Mountains, Shanxi, China (Han et al., 2008). The *P. waksmanii* was maintained on agar slants (10.0% wheat bran extract, 1.5% agar, pH 5.5) and sub-cultured monthly.

Submerged fermentation

Fermentation mix was prepared as following: Three percent of each type of the 40 meshes was incorporated into the basal medium for fermentation with lignocellulosic materials as the sole carbon source. The basal medium contained 1.5% peptone, 2.0% wheat bran extract, 0.25% KH_2PO_4, 0.3% NaCl and 0.02% $MgSO_4$.$7H_2O$. The pH of each mixture was adjusted to 6.5.

P. waksmanii inoculum was prepared by washing slant cultures with 3 mL of sterilized water. Spore concentration was determined by hemocytometer. 2 mL of spore suspension (10^6 - 10^7 spores/mL) was inoculated into 50 mL freshly prepared fermentation mix in a 250 mL flask. The flask was incubated at 28°C for 120 h, shaking at 145 rev/min.

CMCase activity assay

CMCase activity assay was carried out according to the methods developed by Mandels et al. (1976). Reducing sugar was measured by the DNS method using glucose as the standard (Miller, 1959). In this study, one unit (U) of enzyme activity was defined as the amount of enzyme that liberates 1 μmol of glucose equivalent per minute under the specified conditions from the appropriate substrate.

Selection of the best carbon and nitrogen sources

To study the effect of carbon and nitrogen sources on the CMCase activity of *P. waksmanii* F10-2 for optimization studies, various of simple and complex carbon and nitrogen sources and minerals were examined individually (Table 2) for CMCase production as compared to the basal medium, while other components in the basal medium were kept unchanged. After selection, the ratio of carbon source and wheat bran was tested ranging from 5:0 to 0:5 for CMCase production.

Experimental design and optimization

The response surface methodology which employed a set of experimental design (central composite experimental design CCD) was used to optimize media constituents and conditions for cellulases in fermentation. Wheat straw (X_1, g/L), peptone (X_2, g/L), mineral (X_3, g/L), pH (X_1), rev (X_2, r/min) and temperature (X_3, °C) were chosen as the independent variables shown in Table 1. CMCase activity (*Yi*, U/mL) was defined as the dependent output variable. For statistical analysis, the variables *Xi* were coded as *xi* according to Equation (1):

$$x_i = (Xi - \overline{Xi}) / \Delta Xi \quad I = 1, 2, 3\ldots\ldots, k \tag{1}$$

Where x_i is the independent variable coded value, X_i was the independent variable real value, $\overline{Xi}$ is the independent variable real value on the centre point, and ΔXi is the step change value.

To optimize media constituents for fermentation, a total number of 20 experiments including 2^3-factorial central composite experiments along with 6 axial points and 6 replications of center points were employed. The experimental results of the CCD were fitted with a second-order polynomial equation (2).

$$Y_i = b_o + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 \tag{2}$$

Where Y_i was the predicted response, b_0 was the intercept term, b_1, b_2, and b_3 were linear effects b_{11}, b_{22}, b_{33} were squared effects, b_{12}, b_{23}, b_{13} were interaction terms and X_1, X_2, X_3 were independent variables, respectively.

RESULTS AND DISCUSSION

Selection for best carbon and nitrogen sources

Since biotechnological processes involving enzymes were most likely based on crude enzymes, optimizing carbon and nitrogen sources and concentrations became important for optimizing enzyme activities in the culture supernatants (Gomes et al., 2000). We first tested

Table 1. Experimental range and levels of the independent variables

Variable	Rang and levels				
	-1.682	**-1**	**0**	**1**	**1.682**
Equation 3					
Rice straw (g/l), X_1	13.2	20.0	30.0	40.0	46.8
Peptone (g/l), X_2	10.0	12.0	15.0	18.0	20.1
Mineral (g/l), X_3	2.3	3.7	5.7	7.7	9.1
Equation 4					
pH, X_1	3.6	5.0	7.0	9.0	10.4
Rev (r/min), X_2	94.5	115.0	145.0	175.0	195.5
Temperature (℃), X_3	23.0	25.0	28.0	31.0	33.1

x_i = coded value of the variable X_i.
Equaton (3): x_1 = (Rice straw-30)/10; x_2 = (Peptone-15)/3; x_3 = (Mineral-5.7)/2.
Equaton (4): x_1 = (pH -7)/2; x_2 = (rev -145)/30; x_3 = (Temperature -28)/3.

Table 2. Effect of various carbon and nitrogen sources and mineral on CMCase of strain F10-2.

Carbon source	CMCase (U/mL)	Nitrogen source	CMCase (U/mL)	Mineral	CMCase (U/mL)
Rice straw	1.26 ± 0.02a	Peptone	1.17 ± 0.03a	KH_2PO_4	1.31 ± 0.03a
Corn straw	0.90 ± 0.03b	Soyptone	0.68 ± 0.03c	NaCl	1.26 ± 0.03a
Wheat straw	1.02 ± 0.09 b	Yeast extract	0.66 ± 0.05c	$MgCl_2$	0.99 ± 0.05b
Sawdust	0.58 ± 0.05c	Beef extract	0.97 ± 0.08b	KNO_3	0.76 ± 0.06cd
		UREA	0.58 ± 0.03c	$FeSO_4{\cdot}7H_2O$	0.55 ± 0.03e
		NH_4NO_3	0.45 ± 0.02d	$CuSO_4{\cdot}5H_2O$	0.66 ± 0.06de
		$NH_4H_2PO_4$	0.58 ± 0.02c	$CaCl_2.2H_2O$	0.87 ± 0.01bc
		$(NH_4)_2SO_4$	0.62 ± 0.04d	$MgSO_4{\cdot}7H_2O$	1.21 ± 0.02a

various lignocellulose carbon sources on cellulose production by *P. waksmanii* F10-2. Table 2 shows the CMCase activities of *P. waksmanii* F10-2 with different carbon sources. CMCase activity was at the highest (1.26 U/mL) when wheat straw was used as substrate (1.26 U/mL), followed by rice straw (1.02 U/mL), corn straw (0.9 U/mL) and sawdust (0.58 U/mL). In terms of the effect of nitrogen sources on the CMCase activities of *P. waksmanii* F10-2, organic nitrogen is better than inorganic nitrogen. The highest CMCase activity (1.17 U/mL) was achieved when peptone was used as the nitrogen source, while lower CMCase activities were observed when NH_4NO_3 (0.45 U/mL), $NH_4H_2PO_4$ (0.58 U/mL) and Urea urea (0.62 U/mL) were used as the nitrogen source. Minerals were also examined for CMCase production. Minerals, KH_2PO_4 (1.31 U/mL), NaCl (1.26 U/mL) and $MgSO_4$.7 H2O (1.21 U/mL) favored CMCase production and there were no significant differences observed for the CMCase units.

Different ratios of wheat straw and wheat bran, ranging from 5:0 to 0:5, were tested for their effect on CMCase production. The ration of 3:2 was determined as the optimal ratio for wheat straw and wheat bran, yielding a CMCase activity of 1.42 U/mL which was markedly higher than the conditions when wheat straw was used alone (0.94 U/mL) or wheat bran was used alone (0.43 U/mL).

Optimization of medium constituents

Based on the results obtained below, wheat straw was chosed as the carbon source, peptone as the nitrogen source, KH_2PO_4, NaCl and $MgSO_4.7H_2O$ as the minerals for the future experiments to determine the effects of the concentrations of medium constituents on CMCase activity in *P. waksmanii* F10-2. The different combinations of wheat straw, peptone and minerals were designed using CCD. Total of 20 experiments were analyzed using the analysis of variance (ANOVA). The derived regression equation for the optimization of medium constituents indicated that the CMCase activity (*Yi*, U/mL) is a function of the concentration of wheat straw (X_1, g/L), peptone (X_2, g/L) and mineral (X_3, g/L). By applying multiple regression analysis on the experimental data, the following second order polynomial equation was derived to explain the CMCase activity:

Table 3. CCD plan in coded value, the observed and predicted response.

Run	X_1	X_2	X_3	Observed value (U/mL)	Predicted value (U/mL)	Residual
1	1	1	1	1.39	1.3761	0.0159
2	1	1	-1	1.40	1.3991	-0.0011
3	1	-1	1	1.51	1.4678	0.0372
4	1	-1	-1	1.34	1.3428	-0.0018
5	-1	1	1	1.51	1.5032	0.0108
6	-1	1	-1	1.44	1.4693	-0.0283
7	-1	-1	1	1.91	1.8979	0.0101
8	-1	-1	-1	1.71	1.7159	-0.0069
9	-1.682	0	0	1.79	1.7782	0.0128
10	1.682	0	0	1.33	1.3575	-0.0255
11	0	-1.682	0	1.82	1.8406	-0.0186
12	0	1.682	0	1.56	1.5561	0.0059
13	0	0	-1.682	1.32	1.2950	0.0270
14	0	0	1.682	1.39	1.4287	-0.0397
15	0	0	0	1.97	1.8910	0.0800
16	0	0	0	1.87	1.8910	-0.0180
17	0	0	0	1.90	1.8910	0.0090
18	0	0	0	1.82	1.8910	-0.0680
19	0	0	0	1.93	1.8910	0.0340
20	0	0	0	1.86	1.8910	-0.0350

Table 4. Analysis of variance (ANOVA) for the quadratic model

Source o variations	DF	SS	MS	F-value	P- value
Model	9	1.0535	0.1171	58.4900	0.0001**
Residual	10	0.0200	0.0020		
Lack of fit	5	0.0062	0.0012		
Pure error	5	0.0138	0.0028		
Total	19	1.0735			

R = Coefficient of correlation = 0.9906; R^2 = Coefficient of determination = 0.9814.
**Significant at 1% level.

$$Y=1.89-0.13x_1-0.085x_2+0.04x_3-0.11x_1^2-0.07x_2^2-0.19x_3^2+0.08x_1x_2-0.01x_1x_3-0.04x_2x_3 \quad (3)$$

The results for three independent variables (wheat straw, peptone and mineral) from CCD experiments were presented in the Table 3 along with the mean predicted and observed response. The results of the second-order response surface model in the form of analysis of variance (ANOVA) were given in Table 4. The goodness of the model can be checked by different criteria.

The R^2 (coefficient of determination) value provides a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. The closer the R^2 value is to 1.00, the stronger the model is and the better it predicts the response (Kaushik et al., 2006). In this case, the value of R^2 was equal to 0.9814, suggesting that the sample variation of 98.14% for CMCase activity was attributed to the given independent variables. This indicated a good correlation between the experimental and predicted values. Thus, the analysis of the response trend using the model was considered to be reasonable. ANOVA of the quadratic regression model demonstrated that the model was highly significant [(P_{model}>F) = 0.0001]. The computed *F-value* was much greater than the tabulated F-value [(F_{model} = 58.49) > ($F_{0.01}$ (9,5) = 10.15)], indicating that the treatment differences were highly significant. This suggested that the model equation as expressed in Equation (3) provided a suitable model to describe the response of the experiment pertaining to CMCase activity.

The P-values are used as a tool to check the significance of each coefficient, and also indicate the interaction strength between each independent variable.

Table 5. Model coefficients estimated by multiples linear regression.

Model coefficient	Coefficient value	Computed t-value	P-value
B_0	1.8910		
B_1	0.8751	5.7175	0.0001**
B_2	0.2605	0.8534	0.4116
B_3	0.9464	9.2661	0.0001**
B_{11}	-0.9507	9.6976	0.0001**
B_{22}	-0.8774	5.7820	0.0001**
B_{33}	-0.9807	15.8786	0.0001**
B_{12}	0.8345	4.7900	0.0006**
B_{13}	-0.2740	0.9011	0.3868
B_{23}	-0.5948	2.3397	0.0392*

**Significant at 1% level; *Significant at 5% level.

Table 6. CCD plan in coded value, the observed and predicted response.

Run	X_1	X_2	X_3	Observed value (U/mL)	Predicted value (U/mL)	Residual
1	1	1	1	1.59	1.7264	-0.1364
2	1	1	-1	3.28	3.0608	0.2192
3	1	-1	1	2.06	2.0455	0.0145
4	1	-1	-1	4.52	4.2199	0.3001
5	-1	1	1	1.82	1.8789	-0.0589
6	-1	1	-1	3.93	3.7033	0.2267
7	-1	-1	1	2.33	2.3081	0.0219
8	-1	-1	-1	5.35	4.9724	0.3776
9	1.682	0	0	4.27	4.4910	-0.2210
10	-1.682	0	0	3.61	3.7300	-0.1200
11	0	1.682	0	3.32	3.6283	-0.3083
12	0	-1.682	0	2.26	2.2927	-0.0327
13	0	0	1.682	3.42	3.9718	-0.5518
14	0	0	-1.682	0.82	0.6093	0.2107
15	0	0	0	5.32	5.2819	0.0381
16	0	0	0	5.31	5.2819	0.0281
17	0	0	0	5.28	5.2819	-0.0019
18	0	0	0	5.39	5.2819	0.1081
19	0	0	0	5.19	5.2819	-0.0919
20	0	0	0	5.26	5.2819	-0.0219

The smaller the P-values are, the bigger the significance of the corresponding coefficient is (Beguin and Auber, 1998). It can be seen from the degree of significance (Table 5) that the regression coefficients of linear and quadratic coefficients of X_1^2, X_2^2 and X_3^2 were significant at 1% level, one cross-product (X_1X_2) was significant at 1% level, another cross-product (X_2X_3) was significant at 5% level.

Optimization of fermentation conditions

Statistical CCD was used to optimize the fermentative production of CMCase. The experimental design matrix was given in Table 1. Total of twenty experiments were performed using different combinations of variables (Table 6). According to the results of these experiments, the following second order polynomial equation of the

Table 7. Analysis of variance (ANOVA) for the quadratic mode

Source of variations	DF	SS	MS	F-value	P- value
Model	9	41.4323	4.6036	51.9610	0.0001**
Residual	10	0.8860	0.0886		
Lack of fit	5	0.8637	0.1727		
Pure error	5	0.0223	0.0045		
Total	19	42.3183			

R = Coefficient of correlation = 0.9717; R^2 = Coefficient of determination = 0.9442.
**Significant at 1% level.

Table 8. Model coefficients estimated by multiples linear regression.

Model coefficient	Coefficient value	Computed t-value	P-value
B_0	5.2819		
B_1	-0.6641	2.8091	0.0170*
B_2	-0.8417	4.9298	0.0004**
B_3	-0.9690	12.4118	0.0001**
B_{11}	-0.8580	5.2820	0.0003**
B_{22}	-0.9573	10.4676	0.0001**
B_{33}	-0.9736	13.4887	0.0001**
B_{12}	0.0824	0.2613	0.7987
B_{13}	0.3454	1.1641	0.2690
B_{23}	0.5337	1.9955	0.0713

**Significant at 1% level; *Significant at 5% level.

CMCase activity as the function of pH (X_1), rev (X_2, r/min) and Temperature (X_3, ℃) was obtained.

$$Y = 5.2495 - 0.21x_1 - 0.38x_2 - 0.99x_3 - 0.41x_1^2 - 0.81x_2^2 - 1.05x_3^2 + 0.143x_1x_3 + 0.196x_2x_3 \quad (4)$$

The results of the second-order response surface model in the form of analysis of variance (ANOVA) were given in Table 7. In this case, the coefficient of determination (R^2) was 0.9895, indicating that the sample variation of 98.95% for CMCase activity was attributed to given independent variables. These results suggested that the polynomial model had a good prediction accuracy and it was an appropriate choice to use this model for the analysis of the responses. The ANOVA analysis demonstrated that the quadratic regression model was highly significant [(P_{model}>F) = 0.0001]. The computed F-value was much greater than the tabulated F-value [(F_{model} = 51.96) > ($F_{0.01}$ (9,5) = 10.15)], indicating that the treatment differences were highly significant. These facts indicated that the model equation as expressed in Equation (4) provided a suitable model to describe the response of the experiment pertaining to CMCase activity. It can be noticed from the degree of significance (Table 8) that the regression coefficients of linear and quadratic coefficients of X_1^2, X_2^2 and X_3^2 were significant at 1% level.

The 3D response surface based on independent variables pH (X_1), rev (X_2, r/min) and temperature (X_3, ℃) was shown in Figure 1. The canonical analysis revealed a maximum activity of CMCase of 5.64 U/mL, which could be achieved at the point when pH 6.3, rev 135.6 r/min, temperature 26.4℃, respectively.

Validation of the models

Shake-flask experiments were carried out to verify the results with the optimized medium and fermentation condition. The availability of the regression model (Equation (3)) of the CMCase activity by *F. waksmanii* F10-2 was tested using the optimized medium composition in triplicate. The mean value of the CMCase activity was 2.1 U/mL. It agreed well with the predicted value (2.0 U/mL). The triplicate experiments were also carried out to verify the availability and accuracy of the model (Equation (4)). Under the optimized fermentation conditions, pH 6.3, rev 135.6 r/min, temperature 26.4℃, the mean value of the cellulase activity was 5.9 U/mL, which was in agreement with the predicted value (5.64 U/mL). The close agreement of our observed results with the model prediction indicated that our model was accurate and reliable for predicting the production of CMCase by *P. waskmanii* F10-2.

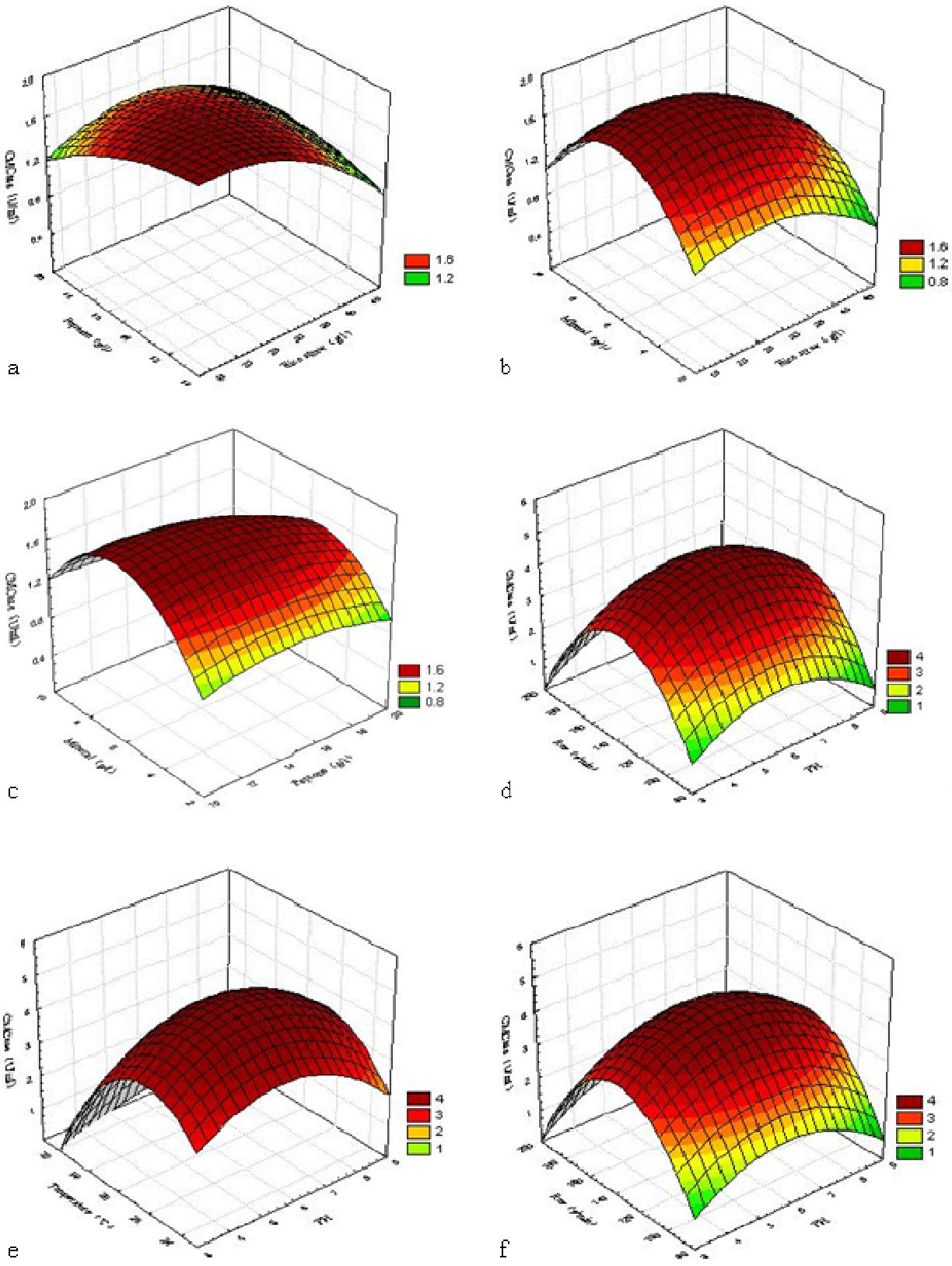

Figure 1. Surface plot of CMCase activity of *P. waksmanii* F10-2. (a) the effect of wheat straw and peptone on CMCase activity; (b) the effect of wheat straw and mineral on CMCase activity; (c) the effect of peptone and mineral on CMCase activity; (d) the effect of pH and rev on CMCase activity; (e) the effect of pH and temperature on CMCase activity; (f) the effect of pH and rev on CMCase activity.

Conclusion

In this study, we successfully developed a model using RSM for optimization of cellulase production from *P. waskmanii* F10-2 under submerged fermentation using ligno-celluloses waste. Using this model, CMCase activity was predicted at 5.64 U/mL, when *P. waskmanii* F10-2 was cultivated at 26.4℃ with a shaking speed of 135.6 r/min in a media containing wheat straw 21.0 g/l, wheat bran 14.0 g/l, peptone 11.8 g/l, KH_2PO_4 2.7 g/l, NaCl 3.3 g/l and $MgSO_4.7H_2O$ 0.2 g/l with pH adjusted to 6.3. These predictions were validated by experiments. These results indicated that the model we have developed is reliable for maximizing cullulase production by *P. waksmanii.*

ACKNOWLEDGEMENTS

The authors wish to acknowledge with thanks to the advice and critical reviews provided by Dr. Wen Zhimou. The authors also want to acknowledge financial support from the Key Scientific and Technological Innovation Special Projects of Shaanxi“13115” (No.2007ZDKG-04).

REFERENCES

Adsul MG, Bastawde KB, Varma AJ, Gokhale DV (2007). Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. Bioresour. Technol. 98: 1467-1473.

Beguin P, Aubert JP (1994). The biological degration of cellulose FEMS Microbiol. Rev. 13: 25-58.

Bhat MK (2000). Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18: 355-383.

Burker JF, Maureri MF, Rodrigues MI (2004). Optimization of extracellular lipase production by *Geotricum sp*. using factorial design. Bioresour. Technol. 91: 77-84.

Chahal PS, Chahal DS, Andre G (1992). Cellulase production profile of *Trichoderma reesei* on different cellulosic substrates at various pH levels. J. Ferment. Bioeng. 74: 126-128.

Duff SJB, Murray WD (1996). Bioconversion of forest products industry waste cellulosics to fuel ethanol: a review. Bioresour. Technol. 55: 1-33.

Gomes I, Gomes J, Gomes DJ, Steiner W (2000). Simultaneous production of high activities of thermostable endoglucanase and ß-glucosidase by the wild thermophilic fungus *Thermoascus aurantiacus*. Appl. Microbiol. Biotechnol. 53: 461-468.

Han LR, Zhang SX, Zhu CS, Zhang X (2008). Screening and identification of superior fungus degraded cellulose. J. Northwest A & F University, Nat. Sci. Ed. 36: 169-174.

Jatinder K, Chadha BS, Saini HS (2006). Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using Response Surface Methodology. W. J. Microbiol. Biotechnol. 22: 169-176.

Kaur J, Chadha BS, Kumar BA, Saini HS (2007). Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922. Bioresour. Technol. 98: 74-81

Kalil SJ, Mavgeri F, Rodrigues MI (2000). Response surface analysis and simulation as a tool for bioprocess design and optimization. Process Biochem. 35: 539-550.

Kaushik R, Saran S, Isar J, Saxena RK (2006). Statistical optimization of medium components and growth conditions by response surface methodology to enhance lipase production by *Aspergillus carneus*. J. Mol. Catal B-Enzyme, 40: 121-126.

Hong J, Tamaki H, Akiba S, Yamamoto K, Kumagai H (2001). Cloning of a gene encoding a highly stable *endo*-β-1, 4-glucanase from *Aspergillus niger* and its expression in yeast. J. Biosci. Bioeng. 92: 434-441.

Li YH, Ding M, Wang J, Xu GJ, Zhao F (2006). A novel thermoacidophilic endoglucanase, Ba-EGA, from a new cellulose-degrading bacterium, *Bacillus* sp. AC-1. Appl. Microbiol. Biotechnol. 70: 430-436.

Muralidhar RV, Chirumanila RR, Marchant R, Nigam P (2001). A response surface approach for the comparison of lipase production by *Candida cylindracea* using two different carbon sources. Biochem. Eng. 9: 17-23.

Mandels M, Andreotti R, Roche C (1976). Measurement of saccharifying cellulose. Biotechnol. Bioeng. Symp. 6: 21-33.

Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Biochem. 31:426-428

Nieves RA, Ehrman CL, Adney WS, Elander RT, Himmel ME (1998). Technical communication: survey and analysis of commercial cellulase preparations suitable for biomass conversion to ethanol. W. J. Microbiol. Biotechnol. 14: 301-304.

Puri S, Beg QK, Gupta R, Curr G (2002). Optimization of Alkaline Protease Production from *Bacillus* sp. by Response Surface Methodology. Microbiology, 44: 286-290.

Reczey K, Szengyel Z, Eklund R, Zacchi G (1996). Cellulase production by *T. reesei*. Bioresour. Technol. 57: 25-30.

Singh J, Batra N, Sobti RC (2001). A highly thermostable, alkaline CMCase produced by a newly isolated *Bacillus* sp. VG1 W. J. Microbiol. Biotechnol. 17: 561-565.

Saxena S, Saxena RK (2004) Statistical optimization of tannase production from Penicillium variable using fruits (*chebulic myrobalan*) of *Terminalia chebula*. Biotechnol. Appl. Biochem. 29: 99-106.

Vohra A, Satyanarayana T (2002). Statistical optimization of the medium components by response surface methodology to enhance phytase production by *Pichia anomala*. Process Biochem. 37: 999-1004.