Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 819–824
Carbohydrate RESEARCH

Quantitative estimate of the effect of cellulase components during degradation of cotton fibers

Lu-Shan Wang,[a] Yu-Zhong Zhang,[a] Hong Yang[b] and Pei-Ji Gao[a,*]

[a]*State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China*
[b]*Department of Mathematics, Shandong University, Jinan 250100, China*

Received 16 December 2002; accepted 13 January 2004

Abstract—A comprehensive mechanistic kinetic model for enzymatic degradation of cotton fibers has been established based on a complete factorial experiment in combination with multivariate stepwise regression analysis. The analysis of the statistical parameter value in the model suggests that the enzymatic degradation of cotton fiber is a progressive and heterogeneous process that includes, at least, two courses that occur sequentially and then progress in parallel. Cellulose fibers were first depolymerized or solubilized by the synergism between cellobiohydrolase I (CBHI) and endoglucanase I (EGI), and then the oligomers obtained were randomly hydrolyzed into glucose by EGI and β-glucosidase. The proposed model can be applied to the quantitative estimation of the effects of three cellulase components, CBHI, EGI, and β-glucosidase separately, or in combination during the entire process of cellulose degradation. The validity of the proposed model has been verified by a filter paper activity assay. Its other applicability was also discussed.

Keywords: Cellulose degradation; Cellulase; Multivariate regression analysis

1. Introduction

Cellulose is the most abundant renewable polysaccharide in nature. Its biodegradation by microorganisms is one of the major steps of the carbon cycle on Earth. The efficient utilization of this process could make a significant contribution to the solving of present environmental and ecological problems. However, current technologies make cellulose utilization through enzymatic hydrolysis uneconomical in the production of sugar syrups or alcohol fuels. Because a long incubation time under complex reaction conditions is necessary, the complete conversion of cellulosic substrate to glucose by cellulase could not be easily achieved.[1–3] Although in recent years, some significant technological advances have been achieved in this field, the costs are still barely affordable, and the enzyme is the most expensive part. A mechanistic kinetic model for enzymatic hydrolysis of cellulose is needed not only for the understanding of its mechanism, but also for the development of a practical process of optimal usage of cellulase. In general, to derive a mechanistic kinetic model, it is necessary to investigate in detail the structural features of the substrate and the mode of action of the reaction system. However, because of the complexity of the cellulose–cellulase system, it is difficult to carry out the investigation by using traditional kinetic techniques.[4–9] With this background, we then tried to seek a solution by following the approach of Solomon and Erickson,[10,11] which combines experimental design with computer analysis based on a statistical method. In our previous studies,[12–14] we have studied about the properties of cellulase of *Trichoderma pseudokoningii* S-38. In the present work, a series of experiments on cotton-fiber degradation by cellulase of *T. pseudokoningii* S-38 were performed based on the factorial experimental design that combines multivariate stepwise regression analysis. By applying the quantitative estimate of different effects

* Corresponding author. Tel.: +86-531-856-3756; fax: +86-531-856-5234; e-mail: gaopj@sdu.edu.cn

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.01.004

of three major cellulase components, CBHI, EGI, and one β-glucosidase during the hydrolysis of cotton fibers, Solomon and Erickson's approach is further developed, and a comprehensive mechanistic picture of enzymatic hydrolysis of cellulose is obtained.

2. Results and discussion

2.1. Enzymatic degradation of crystalline cellulose that includes two heterogeneous processes: cellulose solubilization and glucose formation

During cellulose biodegradation by cellulase, a series of structural changes occur in various parameters such as the crystallinity index (Cr I), the degree of polymerization (DP), and the surface area, among others.[4,8,15,16] Because it is difficult to continuously determine those structural changes, until now glucose formation has still been selected as the main index for this process.[1,3,4,17,18] It is well known that glucose formation only results from the hydrolysis of β-(1→4)-glycosidic bonds in cellulose. In previous studies[19] we observed that the decrease of turbidity of the cellulose suspension did not synchronize with the increase of glucose formation, and the latter lagged behind during hydrolysis of crystalline cellulose powder. These results suggest that the variation of cellulose depolymerization or solubilization may reflect certain structural changes during the biodegradation process by cellulase. These phenomena are similar to those reported by Srisodsuk et al.,[8] who observed that the hydrolysis of bacterial cellulose by EGI resulted in a significant decrease in its DP, and that smaller fragments of cellulose were produced.

As shown in Figure 1A, an important degradation pattern of cellulose hydrolysis is that glucose formation lags behind the cellulose hydrolysis that was determined by a decrease of turbidity of the cellulose suspension, which is inversely described as an increase in cellulose solubilization. It is well known that the short-fiber fragments and higher-molecular-weight oligoses (DP > 6) are all insoluble and turbidity changes can be used for determining them. On the contrary, glucose and lower oligose (DP < 6) are water soluble, and they have no contribution to the turbidity. In the present study, the glucose concentration was specifically determined by glucose oxidase. Thus, the differential value between cellulose solubilization and glucose formation would be reflected by the presence of smaller oligoses. The percent of cellulose solubilization or glucose formation shown in Figure 1A are all a function of time (defined t). Polynomial regression fits the data to the equation $Y = f(t)$, that is, $Y = a + a_1 t + a_2 t^2 + a_3 t^3 + \cdots$, and a best-fit was obtained ($R^2 > 0.95$). The derivative of Y with respect to

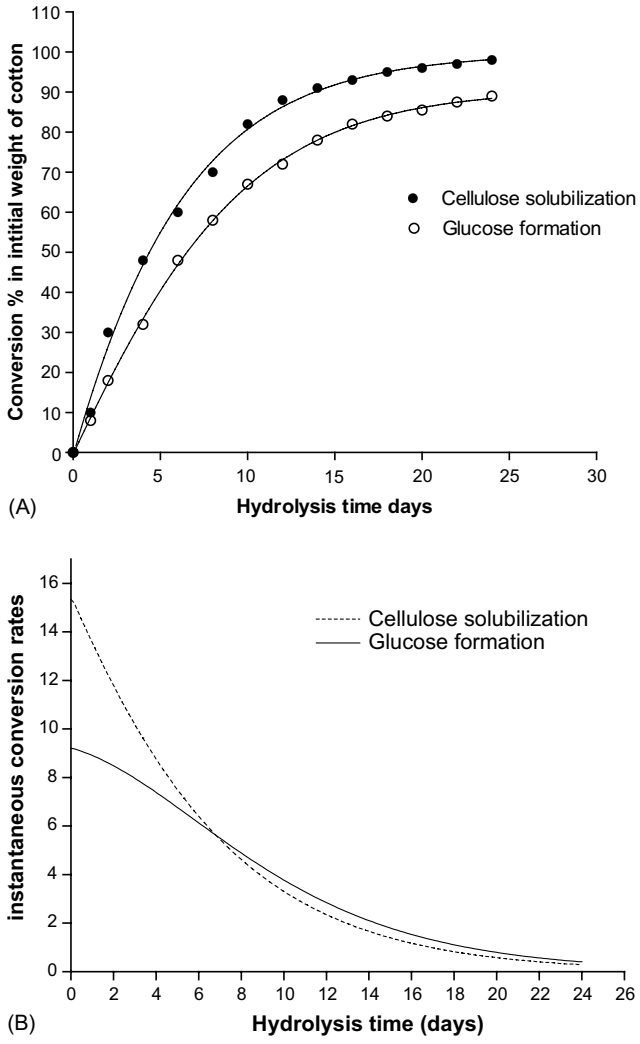

Figure 1. A. Hydrolysis progress of cotton fibers (10 mg/mL) by crude cellulase. cellulose solubilization (●) and glucose formation (○). All tests were determined in triplicate, and the SD is about 5%. B. Comparison of instantaneous conversion rates of cellulose solubilization (—) and glucose formation (- - -) during the enzymatic degradation of cotton fibers.

t, $\mathrm{d}Y/\mathrm{d}t$, is the instantaneous conversion rate. The kinetics processes of instantaneous conversion rates of cellulose solubilization and glucose formation are presented in Figure 1B. Up to 6 days, the instantaneous conversion rate of glucose formation was behind cellulose solubilization, and then it moved slightly higher. After 6 days, the hydrolysis rate of cotton fibers continuously decreased, while the accumulative oligose was also hydrolyzed by β-glucosidase that leads to some increase in instantaneous conversion rate of glucose. These results clearly suggest that the enzymatic hydrolysis of cotton fibers includes two heterogeneous processes that occur sequentially and then progress in parallel. Furthermore, cellulose solubilization might be considered as a rate-limiting factor.

2.2. Quantitative estimate of the effects of cellulase components during degradation of cotton fibers by applying multivariate regression analysis

Since the total enzymatic-degradation process of cotton cellulose includes at least two sequential and heterogeneous reactions, the kinetic-analysis expression obtained from any one of them cannot be adapted in predicting the total progress. As shown in Figure 1, about 60% of cotton fibers have been solubilized and about 47% of the glucose has been formed after 6 days of hydrolysis. Thus, which process can be used as a foundation remains to be resolved for evaluating the main effects of cellulase components in degradation of cotton fibers. In order to find the determinative factor, cellulose solubilization or glucose formation, we designed a $(3\times3\times3)$ factorial experimental design,[20] in which there were three factors, each on three levels. The values of cellulose solubilization and glucose formation were selected as objective functions, respectively, and three cellulase component factors, each alone or in combination, were selected as independent variables. Therefore, each of the 27 experiments was conducted in triplicate, and then the data obtained were analyzed to calculate the parameters based on the following model:

$$\widehat{Y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + b_7X_7,$$

where $\widehat{Y}$ is the predictor of dependent variable.

In the present study, $\widehat{Y}$ is the predicted value of the conversion percent of reduced sugar formation or the cellulose solubilization, and b_0 is the regression constant, b_{1-7} is the standard regression coefficient. X_1, X_2, and X_3 represent the effects of CBHI, EGI, and one β-glucosidase, respectively, and X_4, X_5, X_6, X_7 represent the synergistic effects between them.

When glucose formation is selected as the objective function:

$$\widehat{Y} = b_0 + 0.018\text{EGI} + 0.176(\text{EGI} + \beta\text{-glucosidase}) + 0.667(\text{CBHI} + \text{EGI}) + 1.106(\text{CBHI} + \text{EGI}) \times \beta\text{-glucosidase}.$$

Similarly, as cellulose solubilization is selected as the objective function:

$$\widehat{Y} = b_0 + 0.781(\text{CBHI} + \text{EGI}) + 0.814(\text{CBHI} + \text{EGI}) \times \beta\text{-glucosidase}.$$

Since the standard regression coefficient is a dimensionless term, its absolute value is normally used as an index for quantitative evaluation of the effect of the factor term for the objective function.[20] Consequently, here it can be used in the quantitative evaluation of the effect of each cellulase component, alone or in combination, both in cellulose solubilization and in glucose formation during the entire enzymatic-degradation process of cellulose. As shown in Table 1, according to the statistical analysis for glucose formation, the effect of synergism between CBHI and EGI is the main factor, and adding β-glucosidase can significantly enhance this effect. The effects of EGI alone and of EGI together with the presence of β-glucosidase are small. However, for cellulose solubilization, the main effect comes only from the synergism between CBHI and EGI. The effect of β-glucosidase is weaker. The results clearly demonstrate that the effects of each of the three cellulase components alone or in combination in these two heterogeneous processes could be quantitatively distinguished by this analysis (Table 1, Fig. 2). Table 1 shows the summary of multiple regression analysis for identifying the effects of the three cellulase components, each alone or in combination, in cellulose solubilization, and glucose formation during cotton-fiber degradation 6 days after the start of reaction.

Usually, reduced sugars have been selected as an index for kinetic analysis. They are continuously produced during enzymatic degradation of cellulose, but as mentioned above, they are only related to the hydrolysis of β-(1→4)-glycosidic bonds in the degradation progress. Thus, the supplement of cellulose solubilization as another index will provide more information about the degradation progress.

2.3. Estimating the effect of cellulase components in the filter paper unit (FPU) assay

The filter paper unit assay is a widely used method recommended by the International Union of Pure and Applied Chemistry for evaluation of potential saccharifying capacity of a cellulase system.[21] Usually, different

Table 1. Summary of multivariate regression analysis of three cellulase components, alone or in combination during cotton-fiber degradation

Objective function	Variable entered	Standard regression coefficient	*t*-Value
Glucose formation	EGI	0.118	1.43
	CBHI + EGI	0.667	7.30[a]
	EGI + β-glucosidase	0.176	2.01
	(CBHI + EGI) × β-glucosidase	1.106	10.21[b]
Cellulose solubilization	CBHI + EGI	0.781	5.24[a]
	(CBHI + EGI) × β-glucosidase	0.814	7.10[b]

[a] Extremely significant difference: $t > t_{(30)0.01} = 2.843$.
[b] Significant difference: $t > t_{(30)0.05} = 2.086$.

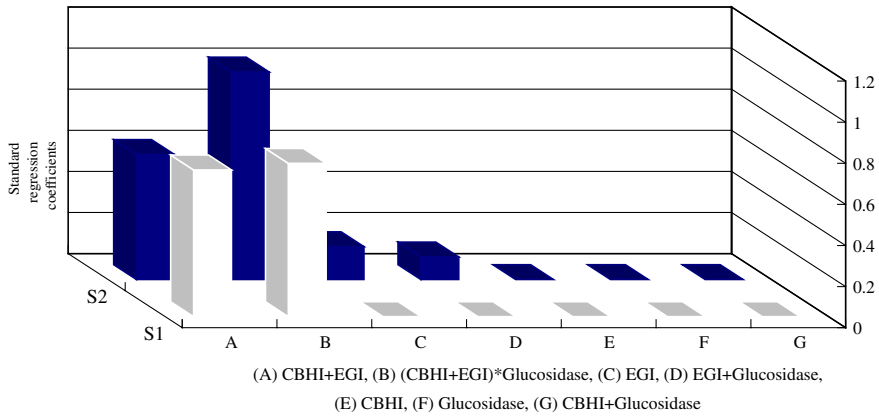

Figure 2. Comparison of standard regression coefficients in two regression equations: cellulose solubilization (front) and glucose formation (backside).

cellulase systems may have same activities in the FPU assay but show different saccharifying capacities for different cellulosic substrates due to the different ratios of crystalline and amorphous fractions in the cellulosic materials.[1,22] So estimating the effects of each cellulase component, alone or in combination, that contribute to the total FPU assay activity is required for accurately evaluating the saccharifying capacity of a cellulase system.

We selected this problem to check the validity of the present regression equation model. A series of experiments were performed under the three factors for a complete combinatorial design ($3\times3\times3$), and the results were analyzed by using multivariate regression analysis as aforesaid. Experimental conditions and assay procedures are the same as in the report by Ghose.[21]

As shown in Table 2, the synergism of CBHI and EGI is still the main force to produce reduced sugar in the FPU assay, but a new variable synergism of EGI + β-glucosidase has entered the regression equation. This reflects that a certain amorphous fraction in the filter paper was hydrolyzed by the synergism of EGI + β-glucosidase under the conditions of the FPU assay. Those results also suggest that the activity of the β-glucosidase has a significant role in this assay, which is so different from that in the cotton fibers (Table 1).

The results in Table 3 indicate that similar results of reduced sugar produced could be obtained under different combinations of three cellulase components in the FPU assays, such as for tests 1, 3, and 5 or for tests 2, 4, and 6. But the saccharifying capacities of these enzymes would be different when they are hydrolyzing cotton fibers. For example, cotton fibers cannot be effectively hydrolyzed by the enzyme combinations of tests 1 and 2 because of the absence of CBHI. On the other hand, test 4 can do this well.

Table 3 shows comparison of the results of reduced sugar production in different ratios of three cellulase components estimated by the DNS method or predicted by multivariate regression analysis.

3. Conclusions

A method that can quantitatively estimate the synergism of cellulase components was proposed. This method can be used, not only in establishing mechanistic kinetic model, but also in designing a practical protocol for the enzymatic treatment of cellulosic substrate.

For cellulase application, besides total hydrolysis of cellulose into glucose, several new areas are being developed such as in textiles and in paper pulp processing.[6,23] These applications are based on the modification of cellulose fibers by certain cellulase components. The main obstacle in this process is how to quantitatively estimate the effects of cellulase compo-

Table 2. Comparison of standard coefficient of three cellulase components in the filter paper unit (FPU) assay[a]

Objective function	Variable entered regression coefficient	Standard regression coefficient	*t*-Value
Reducing sugar formation	EGI	0.118	1.43
	EGI + β-glucosidase	0.305	2.75[b]
	CBHI + EGI	0.7368	6.50[c]
	(CBHI + EGI) × β-glucosidase	0.8409	7.42[c]

[a] Selected reduced sugars as dependent variables, determined by the DNS method and glucose as standard.
[b] Significant difference: $t > t_{0.05} = 2.086$.
[c] Extremely significant difference: $t > t_{0.01} = 2.843$.

Table 3. Comparison of reduced sugar production between using the DNS method and that predicted by the multiple regression model in the filter paper unit (FPU) assay

Test	Cellulases added/mg			Reduced sugar formation/mg[a]		Variation $X\pm$
	CBHI	EGI	β-Glucosidase	Determined by FPU assay	Predicted by regression equation	
1	0	100	2.5	0.30	0.33	0.03
2	0	100	5.0	0.56	0.52	0.08
3	30	70	2.5	0.26	0.31	0.05
4	30	70	5.0	0.58	0.52	0.06
5	15	35	2.5	0.20	0.15	0.05
6	15	35	5.0	0.29	0.25	0.04
$\sum X$						0.31

[a] $S_X = 0.162$, $t = 0.25 < t(5)_{0.01}$ $P > 0.01$.

nents, each alone or in combination. This problem seems to have been resolved, as mentioned above, by using the factorial experimental design plus multivariate regression analysis.

4. Experimental

4.1. Strains and crude cellulase preparation

Crude cellulase was the culture filtrate from the fungus, *T. pseudokoningii* S-38,[24] which was grown on stationary-layer culture containing mixed wheat straw and bran.[25,26]

4.2. Materials

The dewaxed cotton fibers were selected as substrate. These fibers were simply cut and selectively passed through a 100 ASTM mesh but retained by a 120 ASTM mesh. A cotton-fiber suspension (1%) was used to obtain uniformly sized particles according to a floatation technique suggested by Rautela and King.[27] The uniform fraction was obtained at a given flow rate. The average fiber length and width were 100 ± 25 and 15–20 μm, respectively, as measured with a fiber quality analyzer (Kajanii FS-100, Finland).

4.3. Enzymatic hydrolysis of cotton fibers by crude cellulase

To 0.5 g of cotton fiber suspended in 50 mL of acetate buffer, pH 4.8, 50 mM was added crude cellulase solution (0.005 FPU/per mg cotton fibers). A solution of 0.001% NaN_3 (w/v) was added to prevent contamination. Hydrolysis was performed at 45 °C in a shaking bath at 15 rpm. After hydrolysis, the hydrolyzates were separated by centrifuging at 5000*g* for 10 min, and the glucose in the supernatant was determined by glucose oxidase[28] every 2 days. The cellulose solubilization was measured by the turbidity as reported before.[29]

4.4. Measurement of cellulase activity

A filter paper strip (Whatman No. 1) and CMC-Na (carboxymethycellulose-Na, Sigma) was used in the assays of filter paper activity and of β-(1→4)-glucan endoglucanase activity (EG), respectively.[21] The relative activities were calculated in units defined by the release of glucose ($1\ \mu g\ min^{-1}$). *p*-Nitrophenyl cellobiose (*p*-NPC) and *p*-nitrophenyl glucose (*p*-NPG) were used in assays of β-(1→4)-glucan (CBH) and of β-(1→4)-glucosidase activity, respectively. The relative activities were calculated in units defined as 1 μmol of *p*-nitrophenol released per minute.[17]

4.5. Multivariate regression analysis

The statistical method used at present was that reported by Solomon and Erickson,[10] and it also combined the approach of Priore and Rosenthal.[11] Then a predictive regression equation with the interaction of three independent variables was selected to be imposed in deriving an appropriate nonlinear multivariate regression model:[12,20]

$$\widehat{Y} = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_6X_6 + b_7X_7,$$

where $\widehat{Y}$ is a predictor of the dependent variable. In the present case, it is the value of the conversion percent of reduced sugar formation or cellulose solubilization, and X_1, X_2, and X_3 represent CBHI, EGI, and one β-glucosidase, respectively. The X_4, X_5, X_6, X_7 represent the synergistic effects between them, which are computed as

$$X_4 = X_1 + X_2(\text{CBHI} + \text{EGI}),$$
$$X_5 = X_1 + X_3(\text{CBHI} + \beta\text{-glucosidase}),$$
$$X_6 = X_2 + X_3(\text{EGI} + \beta\text{-glucosidase}), \text{ and}$$
$$X_7 = (X_1 + X_2) \times X_3((\text{CBHI} + \text{EGI}) \times \beta\text{-glucosidase}),$$

where b_0 is the regression constant, and $b_{1\text{–}7}$ is the standard regression coefficient used as an effect index of cellulase components, each alone or in combination.

A three-factor complete combination design ($3 \times 3 \times 3$) was applied with the consideration that each combination is corresponding to the three cellulase components on three levels of each, CBHI (0, 5.0, and 10.0 μg protein added/mg cellulose), EGI (0, 1.0, 2.0 μg protein added/mg cellulose), and β-glucosidase (0, 0.5, 1.0 μg protein added/mg cellulose). Each of the 27 experiments was conducted for $\widehat{Y}$. The conversion percent of the cellulose solubilization and glucose formation were each selected as objective functions, respectively. The multivariate regression analysis was performed by the statistics package of ANALYST/REGERS command (Fujitsu, Co. Japan) on a M-340 S computer.

Acknowledgements

This work was supported by a grant from the National Natural Science Foundation of China, No. 394300020.

References

1. Klyosov, A. A. *Biochemistry* **1990**, *29*, 10577–10585.
2. Tsao, G. T.; Ladisch, M. R.; Bungay, H. R. Biomass Refining. In *Advanced Biochemical Engineering*; Sungay, H. R., Ed.; John Wiley & Sons Inc: New York, 1978; p 79.
3. Wood, T. M.; McCrae, S. I.; Bhat, K. M. *Biochem. J.* **1989**, *260*, 37–43.
4. Fan, L. T.; Lee, Y. H.; David, H. *Biotechnol. Bioeng.* **1980**, *22*, 177–199.
5. Paice, M. G.; Jurasek, L. *Adv. Chem. Ser.* **1979**, *181*, 361–374.
6. Cao, Y.; Tan, H. *Carbohydr. Res.* **2002**, *337*, 1291–1296.
7. Eriksson, T.; Karlsson, J.; Tjerneld, F. *Appl. Biochem. Biotechnol.* **2002**, *101*, 41–60.
8. Srisodsuk, M.; Leyer, K. K.; Keranen, S.; Kirk, T. K.; Treeri, T. T. *Eur. J. Biochem.* **1998**, *151*, 885–892.
9. Valjamae, P.; Sild, V.; Pettersson, G.; Johansson, G. *Eur. J. Biochem.* **1998**, *253*, 469–475.
10. Solomon, B. O.; Erickson, L. E. *Biotechnol. Bioeng.* **1983**, *26*, 2683–2705.
11. Priore, R. L.; Rosenthal, H. E. *Anal. Biochem.* **1976**, *70*, 231–240.
12. Gao, P. J.; Qu, Y. B.; Wang, Z. N. *J. Biometr. (China)* **1989**, *4*, 144–150.
13. Gao, P. J.; Chen, G. J.; Wang, T. H.; Zhang, Y. S.; Liu, J. *Acta Biochim. Biophys. Sin.* **2001**, *44*, 13–18.
14. Xiao, Z. H.; Gao, P. J.; Qu, Y. B.; Wang, T. H. *Biotechnol. Lett.* **2001**, *23*, 711–715.
15. White, A. R.; Brown, R. M. *Proc. Natl. Acad. Sci. USA* **1981**, *78*, 1047–1051.
16. Lee, S. B.; Kim, I. H.; Ryu, D. D. Y.; Taguchi, H. *Biotechnol. Bioeng.* **1983**, *25*, 33–51.
17. Wood, T. M.; Bhat, K. M. *Methods Enzymol.* **1988**, *160A*, 87–112.
18. Zhang, S.; Wolfgang, D.; Wilson, D. *Biotechnol. Bioeng.* **1999**, *66*, 35–41.
19. Gao, P. J. *Chin. J. Biotechnol.* **1988**, *4*, 321–326.
20. Gomes, K. A.; Gomes, A. A. *Statistical Procedures for Agriculture Research*. 2nd ed.; John Wiley & Sons: New York, 1984.
21. Ghose, T. K. *Pure Appl. Chem.* **1987**, *59*, 257–268.
22. Halliwell, G. Microbial Glucanases. In *Progress in Industrial Microbiology*; Bull, M. J., Ed.; 1979; Vol. 15, pp 1–58.
23. Beguin, P.; Aubert, J. P. *FEMS Microbiol. Rev.* **1994**, *13*, 25–58.
24. Ma, D. B.; Gao, P. J.; Wang, Z. N. *Enzyme Microb. Technol.* **1990**, *12*, 631–635.
25. Yan, B. X.; Sun, Y. Q. *J. Protein Chem.* **1997**, *16*, 59–66.
26. Yan, B. X.; Sun, Y. Q.; Gao, P. J. *J. Protein Chem.* **1997**, *18*, 681–688.
27. Rautela, G. S.; King, K. W. *Arch. Biochem. Biophys.* **1968**, *123*, 589–601.
28. Parente, A. H. *Appl. Biochem. Biotechnol.* **1992**, *37*, 267–273.
29. Liu, J.; Gao, P. J. *ACS Symp. Ser.* **1996**, *655*, 166–174.