J. Microbiol. Biotechnol. (2001), **11**(2), 229–233

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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Pilot-Scale Production of Cellulase Using *Trichoderma reesei* Rut C-30 in Fed-Batch Mode

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Received: September 9, 2000
Accepted: March 2, 2001

Abstract *Trichoderma reesei* Rut C-30 produced high levels of β-glucosidase, endo-β-1,4-glucanase, and exo-β-1,4-glucanase. In pilot-scale production (50-l fermentor), productivity and yield of CMCase (carborymethyl cellulose) and FPase (filter paper activity) were 273 U/ml and 35 U/ml, and 162 FPU/l · h and 437 FPU/g, respectively. The fed-batch techniques were used to improve enzyme activities with constant cell concentration. The acidity was an important parameter and controlled at pH 3.9 and 5.0 by automatic addition of ammonium hydroxide. Cellulase powder was prepared by ammonium sulfate precipitation and its CMCase and FPase activities were 3,631 U/g and 407 U/g, respectively.

Key words: Cellulase, *Trichoderma reesei*, fed-batch, pilot-scale

Cellulase is known for many industrial usages, with its major application in the hydrolysis of cellulose to produce glucose, which is used for fuel, food, and chemical production [8, 15, 19]. Cellulose is the most abundant renewable carbon source with a great potential for an energy source, if it can be easily hydrolyzed into glucose. Cellulase is one of the most extensively studied enzyme system, since it can decompose cellulosic materials into glucose. One of the recent applications of cellulase in the pulp and paper industry has been in the enzymatic deinking process of waste paper [6].

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials. Most of the studies conducted on the production of cellulase have been carried out using fungal cellulolytic systems. The Rutgers C-30 strain of the fungus *Trichoderma reesei* is one of the best strains developed as of this time. *Trichoderma reesei* Rut C-30 produces large amounts of crude cellulases: β-glucosidase, endo-β-1,4-glucanase, and exo-β-1,4-glucanase (cellobiohydrolase). Compared with other microorganisms, it possesses an ability to produce significantly higher quantities of cellulase that is strongly resistant to catabolite repression [12].

The production of cellulolytic enzymes has been studied in both batch and continuous modes of cultures. For the Rut C-30 system, various temperature and pH levels were attempted with no significant improvement in performance. In the batch system, an initial concentration of cellulose higher than 5% did not improve cellulase activity, which eventually led to lower productivity [4, 17]. This is due to the combined effects of aeration, mixing, and foaming. This implies the occurrence of oxygen transfer limitation and/or denaturation as a result of excessive shear on the enzymes [1, 9]. A continuous system provides high productivity, though it yields a significantly lower enzyme activity [1, 4]. As reported by many studies, the greatest potential for improving cellulase production appears to be with the use of fed-batch fermentation [2, 5, 18, 19].

In a fed-batch production of cellulase using *T. reesei* Rut C-30, the effects of the volume of the feed substrate, the time interval among feeds, and the feeding rate were all examined [10, 11]. For higher cellulase activity and productivity, high cell concentration was kept during the cellulase production. Relatively low cellulase activity and productivity were reported with continuous culture of recycled mycelials [2]. McLean *et al.* [10] claimed that a cell concentration of 12 g/l is an optimal concentration level for cellulase production during fed-batch culture.

In this study, cellulase activities, yields, and productivities were compared between laboratory and pilot-scale fermentations with varying concentrations of ammonium hydroxide. The effect of high cell concentration during cellulase production on cellulase activity and productivity were also examined.

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Table 1. Compositions of media for *Trichoderma reesei* Rut C-30 culture.

Contents	Seed culture medium	Cellulase production medium
Solka Floc	10 g/l	50 g/l
Wheat bran	10 g/l	10 g/l
$(NH_4)_2SO_4$	2 g/l	2 g/l
Yeast extract	0.5 g/l	0.5 g/l
Protease peptone	3 g/l	3 g/l
KH_2PO_4	4 g/l	4 g/l
$MgSO_4 \cdot 7H_2O$	0.3 g/l	0.3 g/l
Tween-80	0.2 ml/l	0.2 ml/l
$CaCl_2 \cdot 2H_2O$	0.3 g/l	0.3 g/l

MATERIALS AND METHODS

Microorganism and Medium Compositions

Trichoderma reesei Rut C-30 (ATCC 56765) was used for the production of cellulase. The subcultures were made on slants of potato dextrose agar (Difco Lab., U.S.A.) at 30°C for 3 days and then stored at 4°C until they were ready to be used.

The compositions of seed culture medium and cellulase production medium were summarized in Table 1. Culture media were made from Solka Floc (Fiber Sales and Development Co., U.S.A.), wheat bran (Donga Mill Co., Korea), $(NH_4)_2SO_4$ (Sigma Chemical Co., U.S.A.), yeast extract (Difco Lab.), protease peptone No. 2 (Difco Lab.), KH_2PO_4 (Shinyo Pure Chemicals Co., Japan), $CaCl_2 \cdot 2H_2O$ (Sigma Chemical Co.), $MgSO_4 \cdot 7H_2O$ (Shinyo Pure Chemicals Co.), and Tween-80 (Shinyo Pure Chemicals Co.). Antifoam 204 (Sigma Chemical Co.) of 0.2 ml/l, containing silicone and non-silicone deformers, was added to the fermentation broth. Solka Floc of 10 g/l was intermittently fed in the fed-batch culture.

Fermentation Conditions

Seed cultures of laboratory and pilot-scale fermentations were prepared for two and three days, respectively. The production media for laboratory-scale fermentation were sterilized in the fermentor using pressurized steam of 1.5 kgf/cm^2 for 30 min, and then cooled to room temperature. The agitation speed was in the range of 150-300 rpm. Media for pilot-scale fermentation were sterilized with steam of 1.5 kgf/cm^2 for 60 min, and cooled to room temperature. The agitation speed in pilot-scale was in the range of 150-330 rpm. Agitation speed must be varied, since the fermentor geometries of 5-l and 50-l are quite different. For the fermentation of filamentous fungi, the agitation speed must be very low [7]. To prevent the cell degradation until 3 days of fermentation, agitation speed was kept at 150 rpm, and then varied depending on the dissolved oxygen (DO) and the viscosity of fermentation broth. The volume of inoculum was made up of 10% of the media. Cultivations were carried out in a 5-l fermentor (Korea Fermentor Co.) with working volume of 3-l, and in a 50-l fermentor (Korea Fermentor Co.) with working volume of 30-l, for laboratory and pilot-scale, respectively. The working pressure was 0.2 kgf/Cm^2 and the aeration was maintained at 1 vvm in 50 l fermentor. DO-value was normally kept above 20% saturation by maintaining the agitation speed.

Fermentation was carried out at 28°C for the first 48 h and at 25°C for the rest of the fermentation process for the prolonged period of slow growth and enzyme production [14, 21]. Ammonium hydroxide solution was used for pH control and as a nitrogen source. In laboratory experiments (5 l fermentor), pH was maintained between 3.9 and 5.0 by adding 1 N of NH_4OH. It was controlled by adding 1 N or 2.5 N NH_4OH in the pilot-scale (50 l fermentor).

Analytical Methods

Samples were analyzed for cell concentration, filter paper (FPase) activity, and carboxymethyl cellulose (CMCase) activity. CMCase and FPase activities were measured by following the method of the International Union of Pure and Applied Chemistry (IUPAC) [3, 20]. The filter paper activity as described by Mandels and Andreotti was measured by the amount of released reducing sugar produced from a mixture of 1 ml of diluted enzyme, 1 ml of acetate buffer, and 50 mg of Whatman No. 1 filter paper in 60 min at 50°C [9]. Carboxymethyl cellulase was determined by the amount of reducing sugar increased in 10 min from a mixture of 1 ml diluted enzyme and 1 ml of solution of 1% CMC (in sodium acetate buffer), incubated at 50°C [9]. The amount of reducing sugar liberated was determined by the dinitrosalicylic acid (DNS) method. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugar per minute.

The dry cell weight (DCW) of fungi was estimated from the amount of DNA in the whole culture broth treated with 1 N aqueous perchloric acid solution and measuring absorbance at 260 nm (GENESYS 5, Spectronic Instruments, Inc.) [13, 22].

Preparation of Cellulase Powder

Fungal mycelium and solid particles were removed from the culture broth by filtration through four layers of gauze. Ammonium sulfate 60% (w/v) was added to the cell extract and placed in a 4°C refrigerator [14]. The precipitate was collected by filtration using filter paper (Whatman No. 1) and dried in a vacuum drying oven at 40°C for 2-3 days. The final cellulase powder was obtained by grinding at 4°C.

RESULTS AND DISCUSSION

Comparisons of Laboratory and Pilot-Scale Fermentation

Cellulase productions by fermentation of *Trichoderma reesei* were carried out in 5-l and 50-l fermentors, and the

Table 2. Effects of scale of fermentor on cellulase production in fed-batch fermentation.

Solka floc conc. (g/l)	FPase (U/ml)	CMCase (U/ml)	Productivity (FPU/l · h)	Yield (FPU/g)	Cell conc. max (g/l)	Cell conc. mean (g/l)
80 (5-l)	28.2	184	96.9	352	9.54	8.29
70 (50-l)	23.2	237	145.9	331	10.27	9.55
80 (50-l)	34.9	273	161.8	437	13.73	10.84

enzyme productivities of CMCase and FPase activities were compared. The experimental results are shown in Table 2, Fig. 1 and Fig. 2. The CMCase activity and FPase activity were 183 U/ml and 28.2 U/ml, and 237 U/ml and 23.2 U/ml in 5-l and 50-l fermentors, respectively. The higher CMCase activity in the 50-l fermentor was considered to have resulted from an efficient oxygen mass transfer due to the better designs of aerator and mixer in the fermentor. The dissolved oxygen-value during the exponential growth phase was about twice the level in the small fermentor, while the aeration rate and the agitation speed were the same. For the purpose of scale-up cellulase fermentation, more controlled experimental studies on dissolved oxygen and fluidics are needed, depending on geometric similarity of fermentors.

The lower activity and yield of FPase in the 50-l fermentor were believed to be caused from shorter fermentation time due to excessive foaming. However, CMCase activity and yield were higher than that in the 5-l fermentor, because endo-β-1,4-glucanase was thought to be more stable than exo-β-1,4-glucanase.

Effect of NH_4OH Concentration on Enzyme Activities

Cell growth and cellulases production are shown in Fig. 2. Using 1 N of NH_4OH, the volume was increased in the 50-l fermentor, because too much solution was added to control the pH. To avoid volume increase, NH_4OH concentration was increased from 1 to 2.5 N and the results are shown in Fig. 3. In the pilot-scale using 1 N NH_4OH, the maximum dry cell weight was 10.27 g/l after 3 days and the stationary phase was maintained for 96 h after 72 h of fed-batch fermentation. The CMCase and FPase activities were 273 U/ml and 34.9 U/ml after 9 days of fermentation, respectively. Maximum dry cell weight was 13.73 g/l, after the stationary phase of 96 h. Although the patterns of stationary phase were similar, cell concentration obtained with 2.5 N NH_4OH was higher than that obtained when 1 N NH_4OH was used. With higher NH_4OH concentration, cell concentration, yield, and productivity of enzyme activity all increased. In the pilot-scale fermentation controlled by 2.5 N ammonium hydroxide, CMCase activity and FPase activity were increased by 49.2% and 23.8%, respectively, compared with the laboratory-scale fermentation. Yield and productivity of the pilot-scale experiment when controlled by 2.5 N NH_4OH were 161.8 FPU/l · h and 437 FPU/g cellulose fed, respectively.

Effect of Initial Cell Concentration and Cell Condition

To study the effect of initial cell concentration on cellulase production in a fed-batch culture, *Trichoderma reesei* Rut C-30 was grown at different initial cell concentrations. The results are shown in Figs. 1 to 3 and summarized in Table 3. For the laboratory-scale and pilot-scale fermentation, inocula were cultured for 2 days in flask and for 3 days in 5-l of fermentor, respectively. Initial cell concentrations of the laboratory fermentor and pilot-scale fermentor were 0.49 g/l, 0.81 g/l, and 1.01 g/l DCW. However, the initial enzyme

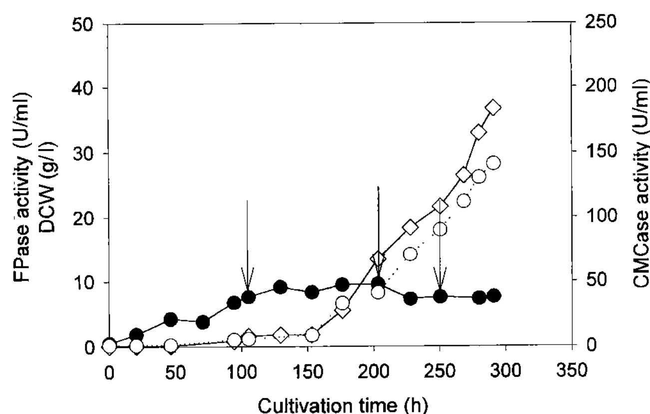

Fig. 1. Fed-batch production of cellulases by intermittent feedings (↓) in a 5-l fermentor (pH control by 1 N NH_4OH).
◇: CMCase activity, ○: FPase activity, ●: Cell concentration.

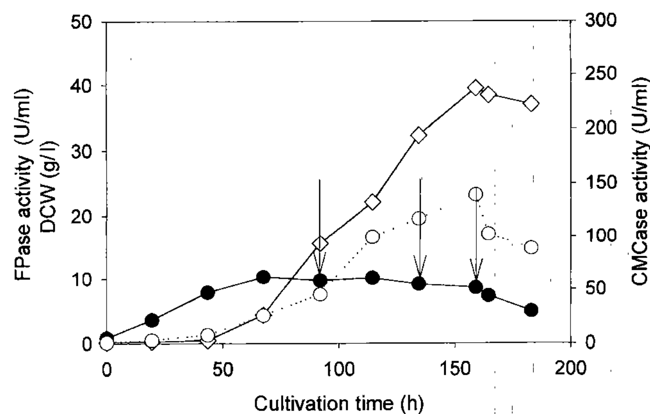

Fig. 2. Fed-batch production of cellulases by intermittent feedings (↓) in a 50-l fermentor (pH control by 1 N NH_4OH).
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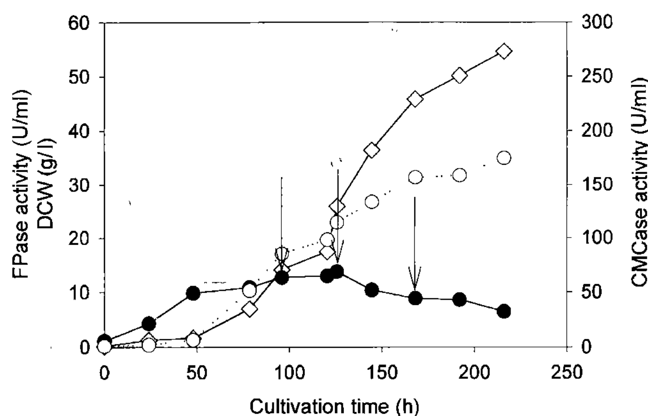

Fig. 3. Fed-batch production of cellulases by intermittent feedings (↓) in a 50-l fermentor (pH control by 2.5 N NH_4OH). ◇: CMCase activity, ○: FPase activity, ●: Cell concentration.

activity showed a similar pattern, being low during the exponential phase. Although patterns of similar inoculum enzyme activity were different, cell concentration using the fermentor for inoculum was higher than cells for the flask. With initial higher cell concentration, the cell concentration, yield, productivity, and enzyme activity were increased. A fermentor used for inoculum maintained better culture conditions due to aeration and agitation.

Feeding Strategy During Fed-Batch Culture

The C/N ratio of cellulase production media is usually set high because cellulase is synthesized under nitrogen limited condition [16]. Cellulase production was directly related to the consumption of carbon source and cell growth. In the present study, ammonium hydroxide was used for pH control and a sufficient nitrogen supply was also ensured. Consequently, the reduction rate of pH was decreased with the consumption of Solka Floc in the medium, and the large reduction rate of pH indicated an existence of large amounts of Solka Floc. Solka Floc was intermittently fed when the reduction rate of pH became slower.

Changing the feeding interval depending on the pH reduction rate is considered proper, as the cell growth and enzyme activity are influenced by many factors. In this study, feeding interval was varied from 30 to 48.5 h.

Recovery of Cellulase

Ammonium sulfate precipitation method was used to prepare dry cellulase powder from the culture broth. The precipitation of enzyme with ammonium sulfate was followed by

Table 3. Effects of initial cell concentrations on enzyme activities.

Scale	Dry cell weight (g/l)	CMCase (U/ml)	FPase (U/ml)
5-l Fermentor	0.494	0.388	0.213
50-l Fermentor (pH control by 1 N NH_4OH)	0.806	0.785	0.125
50-l Fermentor (pH control by 2.5 N NH_4OH)	1.01	0.758	0.186

centrifugation and solubilization in the sodium acetate buffer. This resulted in the recoveries of 70.7% and 80.6% FPase and CMCase, respectively. The higher recovery of CMCase was assumed to be caused by the fact that less endo-β-1,4-glucanase was adsorbed on the filter paper than other enzymes during the cellulase powder preparation [21]. The higher stability of CMCase in the culture broth might be counted as another reaction. The SDS-protein gel analysis for the CMCase should be studied in the future.

Conclusions

The effect of ammonium hydroxide concentration was studied in laboratory-scale and pilot-scale fed-batch fermentations. High cell mass is a critical factor for high cellulase production, and we obtained maximum cellulase production with a prolonged stationary phase. Yield and productivity of cellulase were higher with 2.5 N ammonium hydroxide in controlling pH of the pilot-scale culture. In the pilot-scale fermentation controlled by 2.5 N ammonium hydroxide, CMCase activity and FPase activity were increased by 49.2% and 23.8%, respectively, compared with the laboratory-scale fermentation. Dry cell weight was also increased. Large amounts of cellulase was recovered from the broth by the ammonium sulfate precipitation method. For the purpose of scale-up of cellulase fermentation, more controlled experimental studies based upon geometric similarity are needed.

Acknowledgments

This research work was supported by the Project Developments for the Clean Production Technology of the Korea Ministry of Commerce, Industry and Energy, and the Brain Korea 21 Project.

Table 4. Recovery of cellulase from 50-l fermentation (pH control by 2.5 N NH_4OH) using ammonium sulfate precipitation.

Culture vol. (ml)	CMCase (U/ml)	FPase (U/ml)	Weight of cellulase powder (g)	Activity (U/g)		Recovery (%)	
				FPase	CMCase	FPase	CMCase
1000	273	34.9	60.6	407	3631	70.7	80.6

REFERENCES

1. Allen, A. and R. E. Andreotti. 1982. Cellulase production in continuous and fed-batch culture by *Trichoderma reesei* MCG80. *Biotechnol. Bioeng. Symp.* **12:** 451-459.
2. Ghose, T. K. and V. Sahai. 1979. Production of cellulase by *Trichoderma reesei* QM9414 in fed-batch and continuous flow culture with cell recycle. *Biotechnol. Bioeng.* **21:** 283-296.
3. Ghose, T. K. 1987. Measurements of cellulase activities. *Pure Appl. Chem.* **59:** 257-268.
4. Hendy, N. A., C. R. Wilke, and H. W. Blanch. 1984. Enhanced cellulase production in fed-batch culture of *Trichoderma reesei* C-30. *Enzyme Microb. Technol.* **6:** 73-77.
5. Hendy, N. A., C. R. Wilke, and H. W. Blanch. 1982. Enhanced cellulase production using Solka-Floc in a fed-batch fermentation. *Biotechnol. Lett.* **4:** 785-788.
6. Kirk, T. K. and T. W. Jeffries. 1996. Roles for microbial enzymes in pulp and paper processing, *ACS Symp. Ser. 655*, *ACS*, Washington DC, U.S.A.
7. Lejeune, R. and G. V. Baron. 1995. Effect of agitation on growth and enzyme production of *Trichoderma reesei* in batch fermentation. *Appl. Microbiol.* **43:** 249-258.
8. Mandels, M. 1981. Cellulase. *Annu. Reports Ferment. Process* **5:** 35-78.
9. Mandels, M. and R. E. Andreotti. 1978. Problems and challenges in the cellulose to cellulase to fermentation. *Process Biochem.* **12:** 6-13.
10. Mclean, D. D. and K. Abear. 1986. Fed-batch production of cellulase using *Trichoderma reesei* Rut C-30. *Can. J. Chem. Eng.* **64:** 588-597.
11. Mclean, D. D. and M. F. Podruzny. 1985. Further support for fed-batch production of cellulases. *Biotechnol. Lett.* **7:** 683-688.
12. Montenecourt, B. S. and D. E. Eveleigh. 1997. Preparation of mutants of *Trichoderma reesei* with enhanced cellulase production. *Appl. Environ. Microbiol.* **34:** 777-782.
13. Morikawa, Y., M. Kawamori, Y. Ado, Y. Shinsha, F. Oda, and S. Takasawa. 1985. Improvement of cellulase production in *Trichoderma reesei. Agric. Biol. Chem.* **49:** 1869-1871.
14. Mukhopadhyay, S. N. and R. K. Mailk. 1980. Increased production of cellulase of *Trichoderma* sp. by pH cycling and temperarture profiling. *Biotechnol. Bioeng.* **22:** 2237-2250.
15. Pourquie, J., M. Warzwoda, F. Chevron, M. Thery, D. Lonchamp, and J. P. Vandecasteele. 1988. Scale up of cellulose production and utilization, pp. 71-86. *In* J. P. Aubert, P. Beguin, and J. Milet. (eds.), *Biochemistry and Genetics of Cellulose Degradation*, Academic Press, London, U.K.
16. Rakshit, S. K. and V. Sahai. 1989. Cellulase production by partially catabolite resistant of *Trichoderma reesei. J. Gen. Appl. Microbiol.* **35:** 441-450.
17. Tangnu S. K., W. B. Harvey, and C. R. Wilke. 1981. Enhanced production of cellulase, hemicellulase, and β-glucosidase by *Trichoderma reesei* (Rut C-30). *Biotechnol. Bioeng.* **23:** 1837-1849.
18. Watson, T. G., I. Neligan, and L. Lessing. 1984. Cellulase production by *Trichoderma reesei* (Rut C-30) in fed-batch culture. *Biotechnol. Lett.* **6:** 667-672.
19. Wood, T. M. 1989. Mechanisms of cellulose degradation by enzymes from aerobic and anaerobic fungi, pp. 17-35, *In* M. P. Coughlan (ed.), *Enzyme Systems for Lignocellulose Degradation,* Elsevier Applied Science, London, U.K.
20. Wood, T. M. and K. M. Bhat. 1988. Method for measuring cellulase activities. *Methods in Enzymology* 160, Academic Press Inc., New York, U.S.A.
21. Yu, X. B., H. S. Yun, and Y. M. Koo. 1999. Cellulase production in fed-batch culture by *Trichoderma reesei* Rut C-30. *J. Microbiol. Biotechnol.* **9:** 44-49.
22. Yu, X. B., H. S. Yun, and Y. M. Koo. 1998. Production of cellulase by *Trichoderma reesei* Rut C-30 in a batch fermentor. *J. Microbiol. Biotechnol.* **8:** 575-580.