0273-2289/92/3701—0011$02.00

Protection of *Aspergillus niger* Cellulases by Urea During Growth on Glucose or Glycerol Supplemented Media

D. V. GOKHALE,* S. G. PATIL, AND K. B. BASTAWDE

NCIM Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India

Received March 18, 1992; Accepted June 1, 1992

ABSTRACT

The cellulase enzymes of *Aspergillus niger* were found to undergo catabolite repression in the presence of glucose and glycerol accompanied by sudden drop in pH of the fermentation medium below 2.0. This sudden drop in pH caused inactivation of cellulolytic enzymes produced by *Aspergillus niger*. The supplementation of nitrogen sources, especially urea, protects *A. niger* cellulases from inactivation caused by a sudden drop in pH, since urea helped to maintain the pH of the fermentation medium between 3.5 and 4.5. The role of urea in the protection of cellulase was more prominent when it was used in combination with glycerol (5%).

Index Entries: Catabolite repression; *Aspergillus niger;* urea; cellulases; glucose and glycerol; pH inactivation.

INTRODUCTION

Catabolite repression of the synthesis of inducible enzymes by readily metabolizable compounds like glucose and glycerol has been observed in many microorganisms (*1–4*). In most of the organisms studied, induction and catabolite repression has been reported to function together (*5,6*). In prokaryotes, catabolite repression is considered to occur mostly at the transcriptional level (*7*). Limited information about catabolite repression

*Author to whom all correspondence and reprint requests should be addressed.

in eurkaryotes is available. The synthesis of acid protease and polygalacturonase is repressed by glucose at the transcriptional and translational levels respectively (*8–10*). Highly complicated and variable mechanisms are involved in controlling the synthesis of enzymes constituting the cellulase complex (*11*). The protease produced during catabolite repression caused the inactivation of cellulase enzymes in *Aspergillus nidulans* (*12*). It was also shown that a drastic drop in pH of the medium was responsible for inactivation of cellulase enzymes in the presence of glucose and glycerol (*13–18*).

In our previous work, we have shown that *A. niger* NCIM 1207 produced high levels of β-glucosidase (*19*) and β-xylosidase (*20*) activities. Mutants of this strain showing different levels of endoglucanase, xylanase, and β-glucosidase activities were isolated (*21*). Our recent report indicated that a sudden drop in the pH below 2.0 of the fermentation medium was responsible for the inactivation of cellulase enzymes when *A. niger* was grown in a medium containing glycerol and glucose (*22*).

In present studies, we report the effect of the added urea on the production of cellulolytic enzymes of *A. niger* when grown in the medium containing glucose or glycerol.

MATERIALS AND METHODS

Cellulose-123 powder was obtained from Carl Schleicher and Schull Co., Dassel, Germany. *p*-Nitrophenyl-β-D-glucoside (pNPG) was obtained from Koch-Light Co., Coinbrook, Buck, UK. Larch wood xylan was from Fluka AG, Buchs, Switzerland. Carboxymethylcellulose was purchased from Sigma Chemical Co., St. Louis, MO. *Aspergillus niger* NCIM 1207 was isolated in our laboratory, maintained on potato dextrose agar (PDA) slants, and subcultured every three months.

A. niger was cultivated in 500-mL Erlenmeyer flasks with 100 mL of fermentation medium containing 2% cellulose powder and 5% glucose or glycerol (*23*). The medium was inoculated with the spores (approx 10^5 spores/mL) from a 10-d-old culture grown on PDA slants and incubated at 28 °C on a rotary shaker (150 rpm). Samples were removed at various time intervals and centrifuged in a table top centrifuge (3000 rpm) for 20 min. The supernatant was analyzed for extracellular enzyme activities. To study the effect of the added urea on the cellulase production, *A. niger* was grown in glucose- or glycerol-containing medium with varying concentrations of urea. The culture filtrate, after centrifugation was dialyzed against 0.05*M* citrate buffer (pH 4.5), before assaying for enzyme activities.

Endoglucanase (CMCase; endo-1,4-β-D-glucanase, EC 3.2.1.4), xylanase (1,4-D-β-xylan xylanohydrolase, EC 3.2.1.8), and aryl-β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) activities were determined as

Table 1
Effect of Different Nitrogen Sources on Production of *Aspergillus niger* Cellulases During Growth in Medium Containing Glucose (5%)

Nitrogen sources, %	Enzyme activities, IU/mL*			
	Endoglucanase	Xylanase	β-glucosidase	pH
—	0.27	1.2	0.9	1.5
$(NH_4)_2HPO_4$				
0.1	0.45	21.3	1.0	2.7
0.5	0.52	11.3	0.8	2.9
1.0	0.91	19.3	3.3	4.3
$NaNO_3$				
0.1	0.34	10.6	1.4	4.0
0.5	0.97	27.7	3.3	3.4
1.0	0.43	8.7	0.5	3.8
Urea				
0.1	0.90	13.5	2.3	3.4
0.5	1.30	18.0	3.0	3.7
1.0	1.43	18.7	3.8	3.6

*The enzyme activities were determined after 12 d of fermentation.

described earlier (22). Enzyme activity is expressed in International Units (IU) as μmoles of glucose, xylose, and *p*-nitrophenol produced per min per mL of culture filtrate. The reducing sugars in the samples were determined by the dinitrosalicylic acid method (24).

RESULTS

The effect of different nitrogen sources on production of cellulase enzymes by *A. niger* NCIM 1207 is summarized in Table 1. The culture was grown in fermentation medium containing glucose (5%) and varying concentrations of $(NH_4)_2HPO_4$, $NaNO_3$, or urea. Although all three nitrogen sources produced activities higher than those obtained without any additional nitrogen sources, urea was the best nitrogen source for cellulase production by *A. niger* NCIM 1207. In the case of $(NH_4)_2HPO_4$ and urea, the increase in activities could be correlated with the increase in concentration of nitrogen sources. The supplementation of these nitrogen sources helped to maintain the fermentation medium pH between 3.0 and 4.0. To investigate whether the addition of urea to glycerol-containing medium enhanced cellulase activities, the culture was grown in a medium containing glycerol (5 or 2%) and varying amounts of urea. It is clear from the

Table 2
Effect of Urea on the Production
of *Aspergillus niger* Cellulases Grown on Glycerol (2% or 5%)

Urea, %	Glycerol, 2% Enzyme activities, IU/mL				Glycerol, 5% Enzyme activities, IU/mL			
	pH	CMCase	Xylanase	β-glucosidase	pH	CMCase	Xylanase	β-glucosidase
—	2.6	0.35	6.3	2.1	1.8	0.17	5.1	1.4
0.1	4.7	0.65	12.5	4.4	2.0	0.25	9.0	1.6
0.5	4.8	0.75	11.5	11.6	4.6	0.35	12.5	10.2
1.0	6.2	0.50	10.4	5.9	4.6	0.55	14.0	13.0

results that activities of all the enzymes were increased, and the increment in enzyme activities was concomittant with the increase in urea concentration in the case of 5% glycerol (Table 2). The combination of glycerol (5 or 2%) and urea (1%) yielded β-glucosidase activity four times greater than that obtained in a medium containing glucose (5%) and urea (0.5%).

The course of enzyme production by *A. niger* during growth on the medium containing glucose (5%) and urea (0.5%) is shown in Fig. 1. All the enzyme activties had appeared after the 4th d of incubation. Both endoglucanase and xylanase activities reached their maximum level by the 6th d. The pH of the medium dropped to 2.5 during first 2 d of incubation and then remained above 3.5 during further incubation. Figure 2 shows the profiles of enzyme activities and changes in pH during the growth of *A. niger* on medium containing glycerol (5%) and urea (1%). In this case no lag phase was observed in the production of endoglucanase and xylanase enzymes. The pH profile was similar to those observed during growth in medium containing glucose and urea. The pH of the medium fluctuated between 3.5 and 4.5 throughout the period of fermentation.

DISCUSSION

Soluble carbon sources, like glucose and glycerol, have shown to cause catabolite repression of cellulase production in fungi (*16,25–27*). Catabolite repression causes either cessation or a decrease in enzyme production, but does not explain why the activity of the enzyme decreases. The most likely casual factor responsible for the decrease in enzyme activities is enzyme inactivation either by a drop in pH or possibly by proteases. Growth on glucose or glycerol is invariably accompanied by a drastic drop in pH of the medium that is responsible for the inactivation of extracellular cellulases (*15,17*). Reports on *T. harzianum, T. longibrachiatum* (*15, 16*), and *A. nidulans* (*12*) concluded that proteases produced during the state of catabolite repression causes inactivation of cellulases.

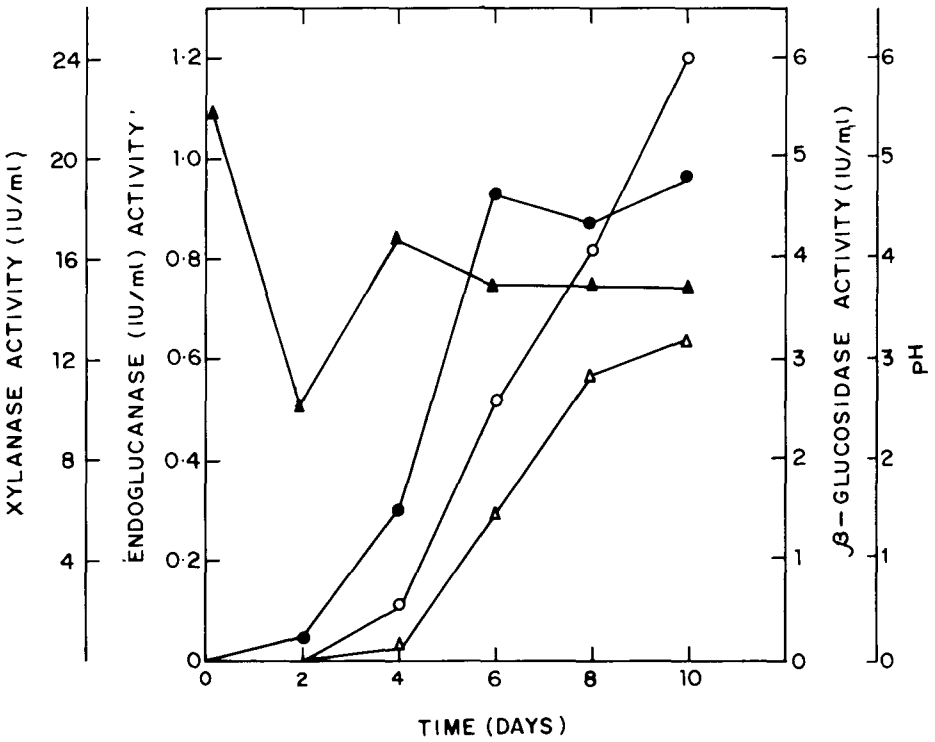

Fig. 1. Profile of extracellular enzymes and pH of *Aspergillus niger* during growth on medium containing glucose (5%) and urea (0.5%). Endoglucanase activity = ○; β-glucosidase activity = △; Xylanase activity = ●; pH = ▲.

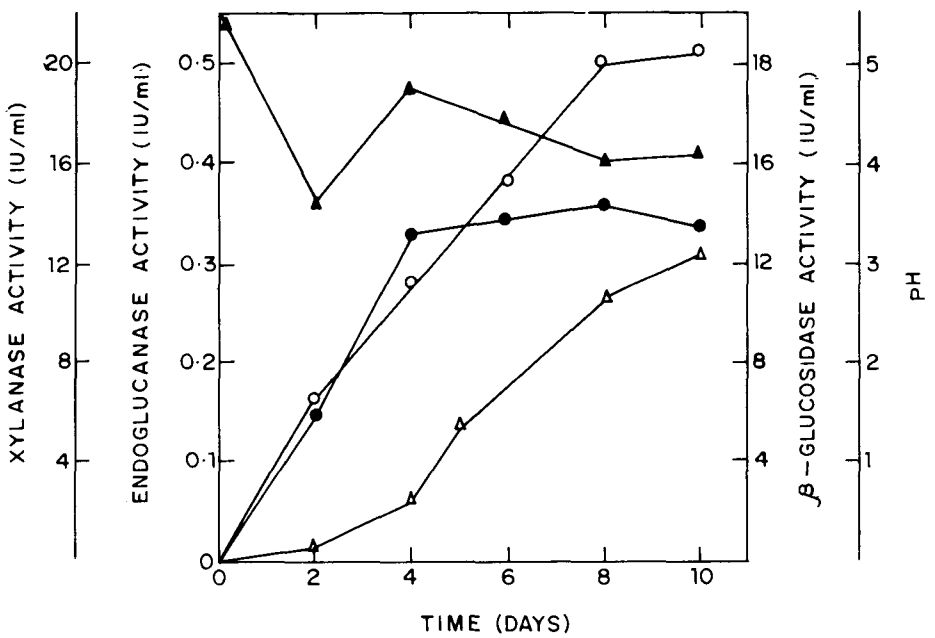

Fig. 2. Extracellular enzyme production and changes in pH of the culture filtrate during growth of *Aspergillus niger* in a medium containing glycerol (5%) and urea (1%). Endoglucanase activity = ○; β-glucosidase activity = △; Xylanase activity = ●; pH = ▲.

Earlier, we have demonstrated that growth of *A. niger* in a medium containing high concentrations of glucose or glycerol caused the pH of the fermentation medium to suddenly drop below 2.0. This sudden drop in pH could be responsible for the inactivation of cellulolytic enzymes produced by *A. niger* (*22*). The possibility of inactivation of cellulases by proteases in our strain was ruled out since it did not produce any significant levels of proteases during growth on glucose or glycerol. The mixture of urea and ammonium sulphate was used to stabilize the pH of the medium (*28*). The nitrogen sources, like $(NH_4)_2SO_4$, $NaNO_3$, and urea, raised the pH of the fermentation medium above 5.5 during growth of *A. niger* (*22*). We therefore used these nitrogen sources to stabilize the pH of the medium during growth on glucose- or glycerol-supplemented medium. The use of these nitrogen sources stimulated better activities of enzymes than media containing only glucose (Table 1). Urea appeared to be the best nitrogen source for the production of aryl-β-glucosidase activity when the organism was grown in glycerol-containing medium. The profile of pH changes during the growth of *A. niger* (Fig. 2) suggested that no inactivation of enzymes occurred, since the pH of the medium fluctuated between 3.0 and 4.0. This supports our earlier observation that the sudden drop in pH value below 2.0 was responsible for inactivation of cellulases produced during the growth of *A. niger* NCIM 1207 on a medium containing glycerol or higher concentration of glucose.

ACKNOWLEDGMENT

We express our gratitude to Sandhya Sudge and Madhavi Deshpande for their valuable technical help during the course of this work.

REFERENCES

1. Egilsson, V., Gudnason, V., Jonasdottir, A., Ingvarsson, S., and Andresdottir, V. (1986), *J. Gen. Microbiol.* **132,** 3309–3313.
2. Hsu, E. and Vaughn, R. (1969), *J. Bacteriol.* **98,** 172–181.
3. Kilian, S., Prior, B., and Latigan, P. (1983), *Eur. J. Appl. Microbiol. Biotechnol.* **18,** 369–373.
4. Maldonado, M. C., Navarro, A. R., and Callieri, D. A. (1986), *Biotechnol. Lett.* **8,** 501–504.
5. Coughlan, M. P. (1985), *Biotechnol. Gen. Eng. Rev.* **3,** 39–109.
6. Gong, G. S. and Tsao, G. T. (1979), *Ann. Rep. Ferment. Proc.* **2,** 111–140.
7. Judewicz, N. and Kornblihtt, A. R. (1983), *Bioquimica General De Torres,* Ateneo, E. I., ed., Carminatti, Cardini, pp. 843–844.
8. Shinmyo, A., Mitsushima, K., and Terui, G. (1972), *J. Ferment. Technol.* **50,** 647–654.

9. Shinmyo, A., Davis, I. K., Nomoto, F., Tahara, T., and Enatsu, T. (1978), *Eur. J. Appl. Microbiol. Biotechnol.* **5,** 59–68.
10. Tahara, T., Doi, S., Shinmyo, A., and Terui, G. (1972), *J. Ferment. Technol.* **50,** 655–661.
11. Reese, E. T. and Mandels, M. (1971), *High Polymers* **5,** 1079–1094.
12. Bagga, P. S., Sandhu, D. K., and Sharma, S. (1989), *Proc. Biochem.* **24,** 41–45.
13. Herr, D. (1979), *Biotechnol. Bioeng.* **21,** 1361–1371.
14. Horton, J. C. and Keen, N. T. (1966), *Can. J. Microbiol.* **12,** 209–220.
15. Karla, M. K., Sidhu, M. S., and Sandhu, D. K. (1984), *Appl. Microbiol. Biotechnol.* **20,** 427–429.
16. Karla, M. K., Sidhu, M. S., and Sandhu, D. K. (1986), *J. Appl. Bacteriol.* **61,** 71–80.
17. Sandhu, D. K. and Sidhu, M. S. (1985), *J. Basic Microbiol.* **25,** 591–598.
18. Sidhu, M. S. and Sandhu, D. K. (1984), *Can. J. Microbiol.* **30,** 1377–1382.
19. Gokhale, D. V., Puntambekar, U. S., Vyas, A. K., Patil, S. G., and Deobagkar, D. N. (1984), *Biotechnol. Lett.* **6,** 719–722.
20. Gokhale, D. V., Puntambekar, U. S., and Deobagkar, D. N. (1986), *Biotechnol. Lett.* **8,** 37–38.
21. Gokhale, D. V., Puntambekar, U. S., Deobagkar, D. N., and Peberdy, J. F. (1988), *Enzyme Microb. Technol.* **10,** 442–445.
22. Gokhale, D. V., Patil, S. G., and Bastawde, K. B. (1991), *Appl. Biochem. Biotechnol.* **30,** 99–109.
23. Reese, E. T. and Mandels, M. (1963), *Meth. Carbohydr. Chem.* **3,** 139–143.
24. Fischer, E. H. and Stein, E. A. (1961), *Biochem. Prep.* **8,** 32–34.
25. Borgia, P. and Shepherd, P. S. (1977), *J. Bacteriol.* **130,** 812–817.
26. Manning, K. and Wood, D. A. (1983), *J. Gen. Microbiol.* **129,** 1835–1847.
27. Umezurike, G. M. (1981), *Biochem. J.* **199,** 203–208.
28. Grajeck, W. (1987), *Enzyme Microb. Technol.* **9,** 744–748.