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Optimization of Cellulase Production by *Aspergillus niger* NCIM 1207

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ABSTRACT

Aspergillus niger NCIM 1207 produces high levels of extracellular β-glucosidase and xylanase activities in submerged fermentation. Among the nitrogen sources, ammonium sulfate, ammonium dihydrogen orthophosphate, and corn-steep liquor were the best for the production of cellulolytic enzymes by *A. niger*. The optimum pH and temperature for cellulase production were 3.0–5.5 and 28°C, respectively. The cellulase complex of this strain was found to undergo catabolite repression in the presence of high concentrations of glucose. Glycerol at all concentrations caused catabolite repression of cellulase production. The addition of glucose (up to 1% concentration) enhanced the production of cellulolytic enzymes, but a higher concentration of glucose effected the pronounced repression of enzymes. Generally the growth on glucose- or glycerol-containing medium was accompanied by a sudden drop in the pH of the fermentation medium to 2.0.

Index Entries: *Aspergillus niger;* catabolite repression; cellulase inactivation.

INTRODUCTION

Cellulose is degraded by the synergistic action of three kinds of enzymes in the cellulase complex, namely, exo-1,4-β-D-glucanase, endo-1,4-β-D-glucanase and β-glucosidase. *Trichoderma reesei* is known to produce

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an active cellulase complex capable of degrading crystalline cellulose efficiently, but the low amounts of β-glucosidase limit the rate and extent of cellulose saccharification. Mutants of *T. reesei* producing high levels of endoglucanase, cellobiohydrolase (*1–4*), and β-glucosidase (*3,5*) have been isolated, and their culture conditions have been standardized (*6–8*). Similarly, culture conditions for production of cellulases by *Fusarium arenaceum* (*9*), Basidiomycetes sp. (*10*), and *Pellicularia filamentosa* (*11*) have also been optimized. Earlier we have reported the hyperproduction of β-glucosidase (*12*) and β-xylosidase (*13*) enzymes by *Aspergillus niger* NCIM 1207. The mutants of this strain exhibiting altered colony morphology and spore color were isolated, which produced comparatively high levels of β-glucosidase activity (*14*).

The synthesis of cellulases in microbes is repressed when glucose or other readily metabolizable compounds are added to the growth medium. In most of the organisms studied, induction and catabolite repression have been shown to function together (*15,16*). In general, catabolite repression seems to occur under various conditions in which catabolism exceeds the requirement of cells for biosynthetic purposes. Less information about this regulatory mechanism is available concerning eukaryotes. Though the specific role of proteases in the inactivation of cellulases has not been indicated in such fungi as *Trichoderma harzianum* (*17*) and *Trichoderma longibrachiatum* (*18*), only recently Bagga and coworkers (*19*) have shown that the increased levels or proteases in glucose- and glucerol-containing media caused the inactivation of cellulolytic enzymes in *Aspergillus nidulans*.

The present communication describes the optimum conditions for the production of cellulolytic enzymes by *Aspergillus niger* NCIM 1207. Some aspects of catabolite repression of these enzymes by glucose and glycerol have also been reported.

MATERIALS AND METHODS

Chemicals

Cellulose-123 powder was obtained from Carl Schleicher and Schull Co., Dassel, FRG. Solka Floc SW40 was purchased from Brown Co., Berlin, NH. *p*-Nitrophenyl-β-D-glucoside (pNPG) was obtained from Koch-Light Co., Coinbrook Buck, UK. Larch-wood xylan was from Fluka AG, Buchs, Switzerland. Dinitrosalicylic acid was from Reidel-de-Haen, Seelze-Hannover, FRG. All the other chemicals used were of Analar grade.

Microorganisms

Aspergillus niger NCIM 1207 was isolated in our laboratory. It was maintained on potato dextrose agar (PDA) slants and subcultured once in every 3 mo.

Enzyme Production

Shake-flask experiments were carried out in 500-mL Erlenmeyer flasks with 100 mL of fermentation medium containing 2% cellulose-123 powder, as described by Reese and Mandels (*20*). Fermentation medium was inoculated with spores (approx 10^5 spores/mL of fermentation medium) from 10-d-old culture grown on PDA and incubated at 28°C on a rotary shaker (150 rpm). Samples were removed at various time intervals and centrifuged on a tabletop centrifuge (3000 rpm) for 20 min. After centrifugation, the supernatant was analyzed for extracellular enzyme activities and soluble protein.

Enzyme Assays

Endoglucanase (CMCase; Endo-1,4-β-D-glucanase, EC 3.2.1.4) activity was assayed according to the method of Mandels et al. (*21*) with slight modification. The total assay mixture of 1 mL contained 0.5 mL of carboxymethylcellulose (CMC, 1% w/v) in citrate buffer (50 m*M*, pH 4.5) and 0.5 mL of suitably diluted enzyme. It was incubated at 50°C for 30 min and the reducing sugars released were measured as glucose equivalents. Xylanase (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8) activity was determined under conditions similar to those described above, except that 1% xylan was used instead of CMC. The reducing sugars in the samples were measured as xylose equivalents.

β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) activity was estimated according to the method described by Eberhart (*22*), using pNPG as substrate. The total 1 mL of reaction mixture consisted of 0.9 mL of pNPG (1 mg/mL) and 0.1 mL of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 65°C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm, after developing the color with 2 mL of 2% sodium carbonate.

Acidic protease was estimated at pH 3.0 by the method of Makonen and Porath (*23*), using hemoglobin as substrate. Hemoglobin (1%, w/v) was prepared in distilled water, and the pH of the solution was adjusted to 3.0 by the addition of 1*N* HCl. The assay mixture consisted of 0.9 mL of hemoglobin solution and 0.1 mL of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 35°C for 30 min. The reaction was terminated by the addition of 3 mL of trichloroacetic acid (5% w/v), and the proteins were allowed to precipitate for 30 min at room temperature. This was followed by filtration. The filtrate was analyzed for the hydrolysis product by measuring the absorbance at 280 nm.

Enzyme Units

Enzyme activity is expressed in international units (IU) as micromoles of glucose, xylose, *p*-nitrophenol, or tyrosine formed per minute per milliliter of culture filtrate.

Analytical Methods

The reducing sugars in the samples were determined by the dinitrosalicylic acid (DNS) method (24). Protein was estimated according to the method of Lowry et al. (25), with bovine serum albumin as standard.

Effect of Glucose/Glycerol Addition on Cellulase Production

To see the effect of glucose/glycerol on the production of cellulolytic enzymes, varying concentrations (0.1–5.0%) of each was added separately to the fermentation medium just before inoculation with spores. In addition to the other parameters, the production of acid protease in the culture filtrate was monitored in the experiments. In these experiments, the culture filtrates were dialyzed against 0.05*M* citrate buffer, pH 4.5, to remove unutilized glucose or glycerol that might interfere with the assay of cellulase enzymes.

RESULTS

The effect of nitrogen source on the extracellular cellulase production by *Aspergillus niger* NCIM 1207 was investigated in shake-flask cultures using Reese medium with different organic and inorganic nitrogen sources. The amount of supplemented nitrogen was calculated to result in 0.3 g of total nitrogen/liter of the fermentation medium. As Table 1 shows, the production of cellulases was higher when the organism was grown in a medium containing organic nitrogen sources other than urea. However, the levels of β-glucosidase were highest when corn-steep liquor was used as the sole nitrogen source. Growth of *A. niger* on such nitrogen sources as NH_4Cl, $NaNO_3$, $(NH_4)_2HPO_4$, and KNO_3 resulted in no change in the initial pH of the medium, whereas the pH of the medium at the end of fermentation reached 6.5 when urea was used as the sole source of nitrogen. Among the inorganic nitrogen sources, ammonium sulfate and ammonium dihydrogen orthophosphate were the best for the production of all three enzyme components. Table 1 also shows that, in general, activities of all the enzymes were low in the cases in which the pH of the medium at the end of fermentation remained above 5.0.

The effect of initial pH on cellulase production by *A. niger* NCIM 1207 is shown in Fig. 1. In all cases (except for the initial pH values of 2.0 and 8.0) the pH values at the end of fermentation were between 3.0–3.5. It was also observed that growth at alkaline pH values caused a strong decrease in cellulase production. The maximum production of cellulases was observed at pH values between 3.0 and 5.5.

To study the effect of temperature on enzyme production, the organism was grown at four temperatures: 23, 28, 35, and 40°C. The production of

Table 1
Effect of Different Nitrogen Sources on the Production of Cellulolytic Enzymes by *Aspergillus niger* NCIM 1207

Nitrogen source*	Enzyme activities (IU/mL)				
	CMCase	Xylanase	β-Glucosidase	pH	Protein (μg/mL)
$(NH_4)_2SO_4$	0.83	15.4	5.0	3.5	621
NH_4Cl	0.27	8.4	0.37	5.5	ND
NH_4NO_3	0.25	8.1	0.67	5.3	ND
$(NH_4)_2HPO_4$	0.22	7.9	0.48	6.0	ND
$NH_4H_2PO_4$	0.88	18.5	5.6	3.2	783
$NaNO_3$	0.25	8.1	0.43	5.8	ND
KNO_3	0.23	7.7	0.20	5.3	ND
$Ca(NO_3)_2$	0.45	13.5	3.0	4.8	405
Casamino acids	0.90	8.6	3.4	3.8	648
Bacto peptone	0.90	20.0	2.75	3.6	962
Proteose peptone	1.20	24.0	4.0	3.2	1243
Yeast extract	1.02	11.5	4.1	3.3	1160
Corn steep liquor	1.05	18.6	6.0	2.9	1135
Bacto tryptone	0.92	11.6	2.6	3.8	1010
Urea	0.15	6.45	0.27	6.5	243

ND: Not determined.
*Supplemented nitrogen was 0.3 g of total nitrogen/liter of fermentation medium.

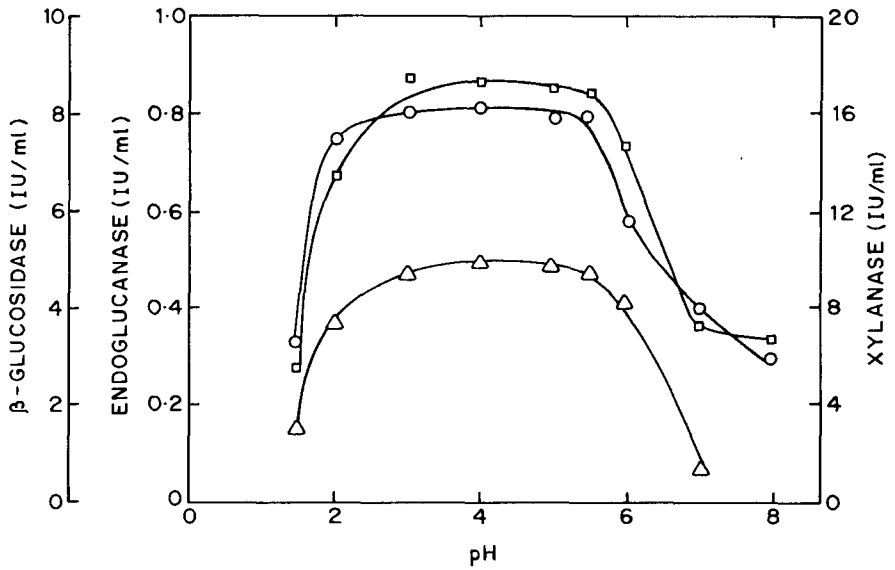

Fig. 1. Effect of pH on the production of cellulolytic enzymes by *A. niger* NCIM 1207: endoglucanase, ○——○; xylanase, □——□; β-glucosidase, △——△.

Table 2
Effect of Initial Temperature on Enzyme Production by *A. niger* NCIM 1207

Temperature	Enzyme activities (IU/mL)		
(°C)	CMCase	Xylanase	β-glucosidase
23	0.30	12.0	1.0
28	0.75	17.9	4.0
35	0.34	10.0	0.75
40	0.15	2.6	0.16

Table 3
Effect of Glucose or Glycerol on the Production of CMCase, Xylanase, and β-Glucosidase Enzymes by *Aspergillus niger* NCIM 1207

Carbon source	Enzyme activities (IU/mL)					Protein
	CMCase	Xylanase	β-glucosidase	Protease	pH	(μg/mL)
Control	0.83	15.4	5.0	0.012	3.5	621
C+Glucose (%)						
0.1	1.20	20.8	4.9	0.013	3.4	1100
0.5	1.35	16.8	6.5	0.015	2.9	1230
1.0	1.41	18.3	7.6	0.017	2.8	1400
3.0	0.67	10.9	4.0	0.016	2.3	1460
5.0	0.27	1.2	0.9	0.013	1.5	1900
C+Glycerol (%)						
0.1	1.20	16.5	3.4	0.012	3.2	750
0.5	0.60	10.8	2.2	0.011	3.1	760
1.0	0.49	7.3	1.7	0.013	2.8	990
3.0	0.36	5.8	1.8	0.015	2.5	1360
5.0	0.17	5.1	1.4	0.014	2.0	1700

C - Control

cellulases was monitored up to 10 d of incubation, and the values of enzyme activities are given in Table 2. The optimum temperature for the production of cellulases was 28°C, whereas growth of the organism at higher temperatures resulted in a decrease in enzyme production.

To study the effects of glucose and glycerol on the cellulase production, the organism was grown in a medium containing different concentrations of glucose or glycerol along with cellulose-123 powder. Data indicating the enzyme activites and the extracellular protein are given in Table 3. The observations indicate that no repression of enzyme production was observed up to 1.0% glucose, but higher concentrations of glucose did effect pronounced repression. There was a 1.5-fold increase in extracellular β-glucosidase at 1.0% glucose concentration. The extracellular protein

increased concomitantly with the increase in glucose concentrations. Unlike glucose, glycerol at all concentrations repressed the production of all three enzymes. The decrease in cellulase levels could be correlated to the amount of glycerol added. In general, glucose or glycerol at higher concentrations (5.0%) caused maximum repression of cellulases.

Figure 2 (A and B) shows the profiles of cellulase production, changes in pH, and extracellular protein on addition of glucose (1,3, and 5%) to the fermentation medium. At 1% glucose concentration, the endoglucanase and xylanase activities reached maximum after 7 d of incubation, after which only xylanase activity started declining. However, β-glucosidase activity continued to rise during further cultivation. The endoglucanase and β-glucosidase appeared after 4 d of incubation in the case of 3% glucose concentration, but these activities never reached the values of the control, in which no glucose was added. Maximum repression of all the cellulolytic enzymes was caused by 5% glucose. The addition of glucose always resulted in a sudden drop in the pH of the medium. Similar results were obtained when medium was supplemented with glycerol. It is notable that pH never dropped below 2.0 when 1% glucose was used, but the drop in pH was followed by a immediate rise to pH 2.7. It was observed that the production of acid protease was not significantly increased at all time intervals during growth on glucose (Fig. 3). We did not estimate the activities of neutral or alkaline proteases, since the pH of fermentation broth remained below 3.0 at all intervals of time.

DISCUSSION

The nitrogen sources in the fermentation medium for the production of cellulolytic enzymes vary from organism to organism. Ammonium sulfate, ammonium dihydrogen orthophosphate, and corn-steep liquor were the best nitrogen sources for the production of cellulases by *A. niger* NCIM 1207. Brown et al. (*26*) have reported that urea or corn-steep liquor was the best nitrogen source for the production of cellulases by *Penicillium pinophillum*. They have also indicated that β-glucosidase production was poor when inorganic nitrogen sources were used as sole nitrogen sources. We observed that *A niger*, when grown on urea as the sole nitrogen source, gave poor yields of all three enzyme activities. The inorganic nitrogen sources other than ammonium sulfate and ammonium dihydrogen orthophosphate were not suitable for the production of cellulases by *A. niger* NCIM 1207. Grajek (*27*) has shown that ammonium chloride and potassium nitrate were the best nitrogen sources for β-glucosidase production by *Sporotrichum pulverulentum*. The optimum pH and temperature for production of cellulases by our strain were found to be 3.0–5.5 and 28°C, respectively. These are the values at which most cellulolytic fermentations are generally run for maximum enzyme production.

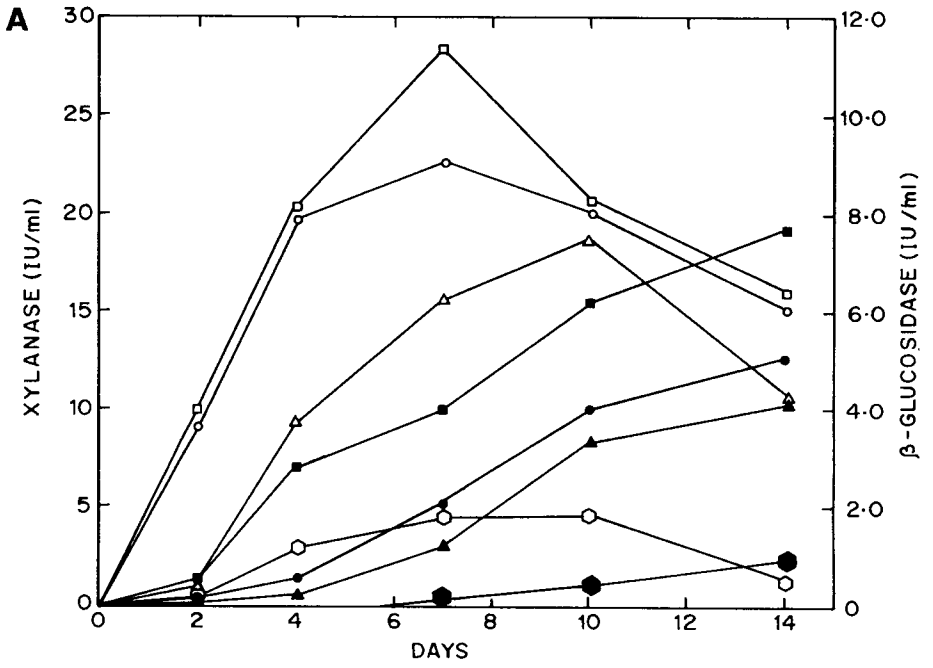

Fig. 2 (A). Effect of addition of glucose on the production of xylanase and β-glucosidase by *A. niger* NCIM 1207: open symbols, xylanase; closed symbols, β-glucosidase. Control, ○——○; 1% glucose, □——□; 3% glucose, △——△; 5% glucose, ⬡ — ⬡.

It is well known that, in general, glucose and glycerol have been shown to cause catabolite repression of cellulase production in fungi (*18,28–31*). The regulation of cellulase biosynthesis is brought about by feedback control in the presence of such readily metabolizable compounds as glucose and glycerol. Catabolite repression causes either cessation or decrease of enzyme production, but the reasons for decrease in enzyme activity are not clearly understood. The inactivation of cellulolytic enzymes by readily metabolizable sugars was attributed to the drastic drop in pH of the medium (*17,30,32–36*). The growth of *A. niger* NCIM 1207 in a medium containing higher concentrations of glucose or glycerol caused the pH to suddenly drop below 2.0. This sudden drop in pH could be responsible for inactivation of cellulases. In yeasts, proteolytic enzymes synthesized in the state of catabolite repression are known to degrade certain enzymes, resulting in their inactivation (*37,38*). Sandhu and Sidhu (*30*) have reported that proteases produced during catabolite repression cause inactivation of cellulolytic enzymes in *Aspergillus nidulans*. However, increased levels of proteases in *Trichoderma* species produced during catabolite repression were incapable of inactivating the cellulase enzymes in vitro (*17,18*). The possibility of inactivation of cellulases by proteases in *A. niger* has been

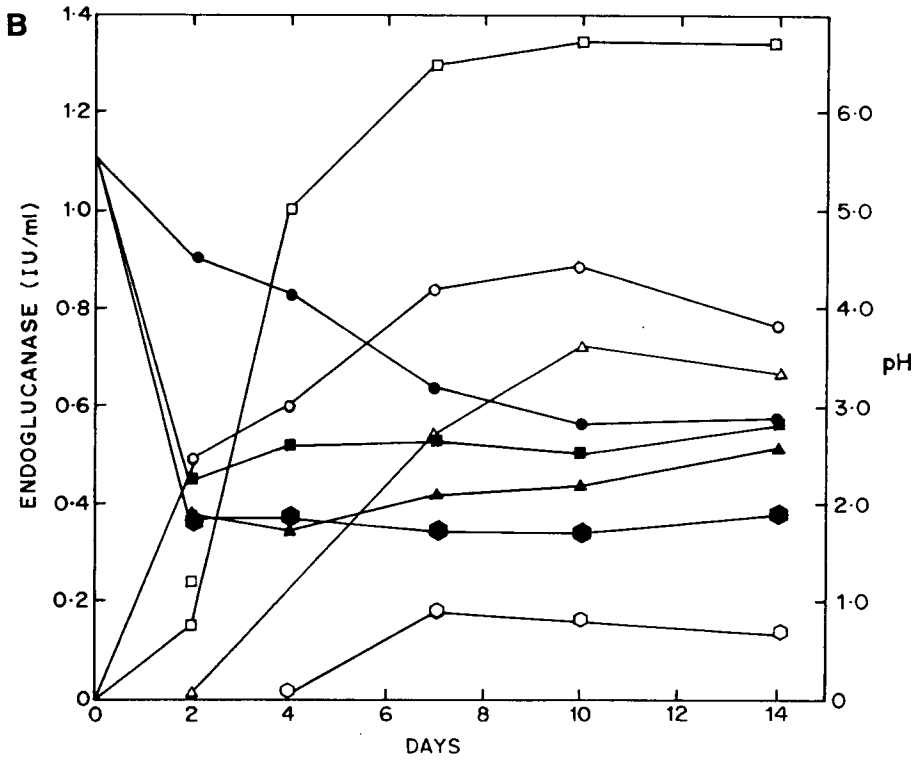

Fig. 2 (B). Endoglucanase and changes in pH of the culture filtrate of *A. niger* NCIM 1207 after addition of glucose: open symbols, endoglucanase; closed symbols, pH. Control, ○——○; 1% glucose, □——□; 3% glucose, △——△; 5% glucose, ⬡——⬡.

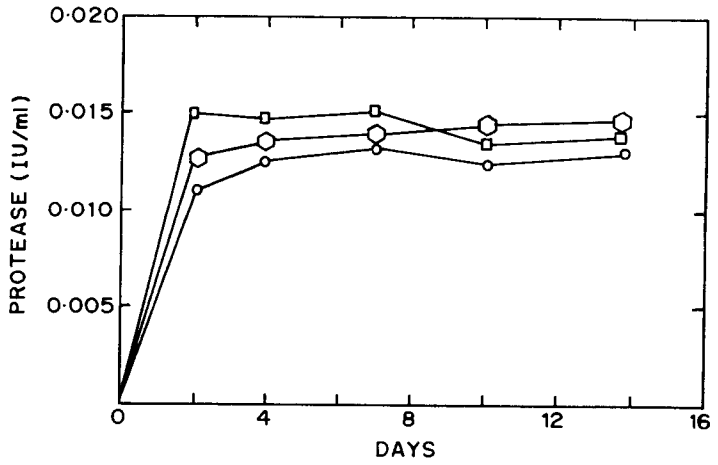

Fig. 3. Extracellular acid protease profiles during growth of *A. niger* NCIM 1207 on glucose-supplemented medium: control, ○——○; 1% glucose, □——□; 5% glucose, ⬡——⬡.

ruled out, since this strain did not produce any significant levels of proteases during growth on glucose or glycerol. From these results, it was concluded that pH value suddenly dropping below 2.0 may be responsible for inactivation of cellulase enzymes when *A. niger* is grown in a medium containing glycerol and higher concentrations of glucose.

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