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Cellulase of *Neurospora crassa*

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Mycelia and ungerminated conidia of *Neurospora crassa* were found to secrete extracellular endocellulase (EC 3.2.1.4). A simple induction system of potassium phosphate buffer (pH 6.0) plus inducer relied on the internal metabolic reserves of conidia or mycelia to provide energy and substrates for protein synthesis. Buffer concentration for optimum enzyme production was 100 mM, but at higher buffer concentrations enzyme production was inhibited. Cellobiose was clearly the best inducer, with an optimum effect from 0.05 to 1 mM. In deionized water, cellulase remained mostly associated with the cell, but a variety of salts stimulated the release of cellulase into the medium.

The study of cellulose decomposition is well documented in a number of organisms (9, 17, 19). This metabolic pathway is appropriately studied in *Neurospora crassa* because of the advantage of doing both biochemical and genetic cellulase studies in the same system (15). Earlier reports have described (i) the presence of cellulase and two β-glucosidases (4) and (ii) the location of β-glucosidase activity, both within the cells and on the surface of *Neurospora* (3). This report describes the production of an extracellular cellulase and conditions that foster the release of this enzyme into the external media.

MATERIALS AND METHODS

Chemicals. Cellobiose, *p*-nitrophenyl-β-D-glycopyranoside, and all other inducers and vitamins were from Calbiochem, Los Angeles, Calif.; copper sulfate, mercuric chloride, lead acetate, and silver nitrate were from Mallinckrodt Chemical Works, St. Louis, Mo.; carboxymethyl-cellulose 4MSP was from Hercules Power Co., Wilmington, Del.

Strains and media. Strains were maintained on glycerol-sucrose complete (GSC) medium in tubes (5). Conidia were produced in 2-liter wide-mouth flasks containing 300 ml of GSC medium. Vogel minimal medium was used throughout (32). Wild-type *Neurospora* strains were 74-OR8-1a and Emerson 692a, both from the Fungal Genetics Stock Center, Arcata, Calif. Strains 33(2-6), *gluc-2* (3), and 62(2-9) were selected in our laboratory for low aryl-β-glucosidase activity and excellent growth qualities. Exotic strains listed in Table 1 were a gift from David Perkins, Stanford University, and the Fungal Genetics Stock Center.

Induction in pregerminative conidia and mycelia. Conidia were harvested in sterile water, washed twice by centrifugation, and resuspended in 2 volumes of sterile distilled water. Mycelial fragments were removed by filtration through cheesecloth. The conidia were suspended in 0.1 N HCl for 5 min to inactivate external β-glucosidases that interfere with induction and were then washed again with 1 volume of distilled water. These cells were immediately inoculated into 0.1 M potassium phosphate buffer, pH 7.0, with a final optical density of 0.150 to 0.250 at 600 nm, light path of 1.0 cm. Induction medium routinely contained 1.0 mM cellobiose. Cultures were shaken in 125-ml Erlenmeyer flasks at 25°C. To determine the release of extracellular cellulase, samples were taken and centrifuged, and the supernatant was assayed. Remaining cells were frozen for assay at a later time.

Mycelia were induced in a manner similar to that used for conidia. To produce mycelia, conidia were inoculated into 50 ml of Vogel medium in 250-ml Erlenmeyer flasks with 2% sucrose, 1% vitamin stock solution, 100 mg of thiamine, 30 mg of riboflavin, 75 mg of pyridoxal, 200 mg of calcium pantothenate, 5 mg of *p*-aminobenzoic acid, 75 mg of nicotinamide, 200 mg of choline hydrochloride, 1 mg of folic acid, and 60 mg of inositol, and then shaken in a New Brunswick reciprocal shaker at 80 rpm at 25°C for 40 h. The growth medium was decanted, and the mycelium was washed into two batches of sterile, distilled water. Induction was started immediately by the addition of the pads to 40 ml of 0.1 M phosphate buffer, pH 7.0, and a final concentration of 1.0 mM cellobiose. Deionized water with inducer was substituted for buffered media in experiments designated to show retention and subsequent release of cellulase resulting from the addition of specific salts to the medium. The induction mixture was incubated at 25°C on a reciprocal shaker for 6 h. At harvest, the entire medium was poured through cheesecloth to separate mycelium. The clear filtrate was frozen for future assay. The mycelium was pressed on filter paper and frozen for future dry-weight determination.

Cellulase assay. Throughout this work we have confined our attention to the cellulase activity that cleaves the cellulose molecule internally (5). The most definitive way to detect this activity is to measure the decline in viscosity of a soluble carboxymethyl-cellulose preparation caused by internal en-

TABLE 1. *Relative enzyme activities in standard and exotic Neurospora strains*[a]

Strain origin and stock no.[b]	Enzyme activity[c]		
	Cellobiase[d]	Aryl-β-glucosidase[e]	Cellulase[f]
Puerto Rico, 429	190	810	1,365
North Africa, 430	338	1,272	266
Java, 431	1,190	1,400	207
Fiji, 432	116	1,169	320
Philippines, 433	1,190	1,400	786
Liberia, 434	277	1,043	46
Fiji, 435	348	1,102	222
Singapore, 436	1,133	1,227	2
New Zealand, 643	1,751	1,029	693
Costa Rica, 851	255	1,570	1,945
Costa Rica, 852	361	1,279	575
Liberia, 961	177	928	592
Liberia, 967	217	868	273
U.S.A. 988	785	725	1,695
U.S.A. 262	1,820	1,680	2,672

[a] Pregerminative conidia were induced for 6 h under standard conditions with 0.1 M phosphate buffer and 1 mM cellobiose (see text). β-Glucosidase assays were done on whole cells with phenethyl alcohol to alter permeability.

[b] Fungal Genetics Stock Center number.

[c] Expressed as change in optical density per minute at a 410 mM concentration per 1 optical density unit of cells.

[d] Defined as thermolabile β-glucosidase at 60°C for 1 min.

[e] β-Glucosidase stable at 60°C for 1 min.

[f] Cellulose assay was of extracellular enzyme using standard procedure in text.

zymatic cleavage of the β-glucosidic bonds. Cellulase assay mixture contained 2.5 ml of 1% sodium carboxymethyl-cellulose in potassium phosphate buffer, 0.1 M, at pH 5.0, which was prepared routinely by mixing for 1 min in a Waring blender. Also added were 1.0 ml of enzyme solution at pH 7.0 and 1.5 ml of 0.1 M phosphate buffer at pH 6.0. The final reaction mixture had a total volume of 5.0 ml and a final pH of 6.0. The above components were mixed gently and incubated at 40°C for 20 min, and then the flow rate was measured in an Ostwald Viscosimeter tube. Calculation of units has been reported earlier (5).

RESULTS

A typical experiment that demonstrates the release of cellulase from *Neurospora* mycelia is shown in Fig. 1. The cells were grown for 40 h in Vogel medium (27). Equal portions of cells were washed with deionized water and suspended in 0.1 M phosphate buffer, pH 7.0, with 1.0 mM cellobiose as inducer. Cells were separated by filtration through Whatman no. 1 filter paper, and the filtrate was assayed for cellulase activity (see Materials and Methods). Cells incubated with phosphate buffer released higher levels of extracellular cellulase in the medium. This positive response in phosphate media was typical both of 40-h mycelia and of postgerminative conidia in other experiments. The experiment raised the question of the possible role of phosphate buffer in stimulating intracellular cellulase production, as opposed to facilitation of release of cellulase into the medium.

The effect of phosphate buffer on production of internal cellulase, as well as extracellular cellulase, is shown in Fig. 2. Conidia were induced with 1.0 mM cellobiose at concentrations of phosphate buffer, pH 7.0, from 0 to 500 mM. After 6 h of incubation in the same medium, cells were extracted (see Materials and Methods) and the total extractable cellulase was compared with extracellular cellulase in the medium. Total cellulase in the culture could be calculated by adding these two values, since only a trace of the cellulase activity remained in the cell debris. Internal cellulase was highest at 100 mM buffer, with a decline in external and total activity at 500 mM buffer. This experiment shows that there is a buffer concentra-

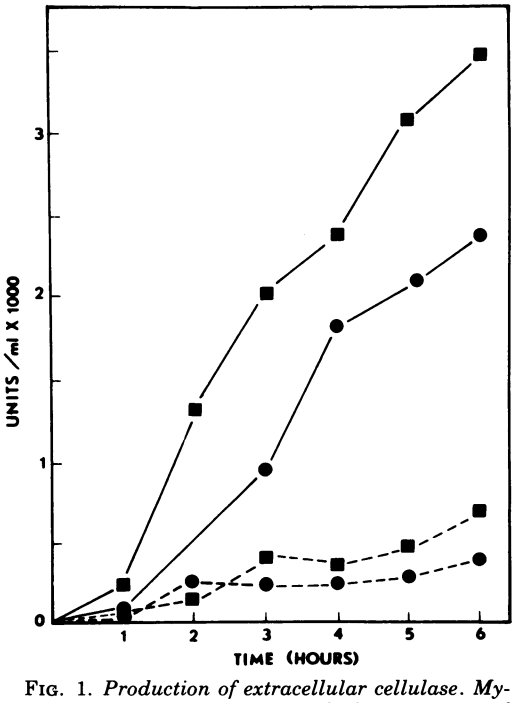

FIG. 1. *Production of extracellular cellulase. Mycelia were grown for 40 h, washed in water, and suspended in 0.1 M phosphate buffer, pH 7.0 (unbroken lines), or in water (broken lines), each containing 1 mM cellobiose. Symbols:* (■) *Strain 62(2-2);* (●) *strain 692a. Released enzyme was assayed as described in the text.*

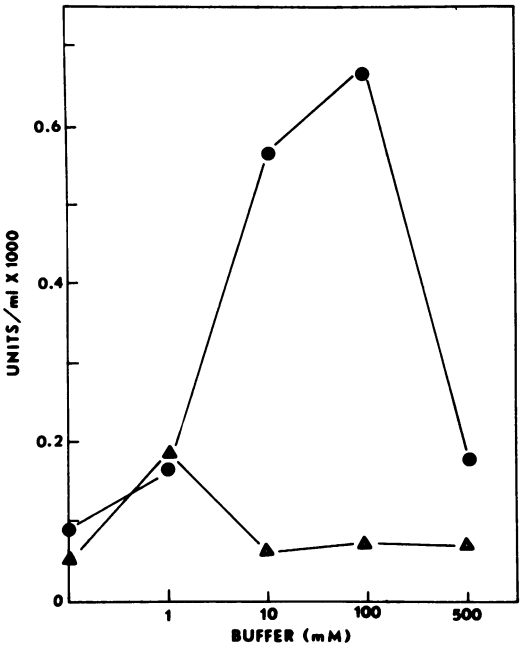

FIG. 2. *Cellulase production from postgerminative conidia related to buffer concentration. Potassium phosphate buffer was at pH 6.0 with strain 74-OR8-1a. Symbols:* (△) *Cellulase extracted from cells;* (●) *cellulase in medium.*

tion optimum for total cellulase production that differs from the optimum of cellulase retention in the cell.

Release of cellulase by buffer after induction in water medium is shown in Fig. 3. Mycelia were grown for 40 h in minimum medium with sucrose. Each 125-ml flask contained 50 ml of medium, and the mycelia contents were closely equivalent in wet weight. Mycelial pads were washed with distilled water and placed in flasks containing water and 1.0 mM cellobiose. As the induction proceeded, potassium phosphate buffer was added to selected flasks at 2- or 4-h intervals to bring the molarity to 0.1 M. Samples of medium were analyzed for cellulase activity. Cellulase was released more readily after addition of buffer, establishing levels in 30 min that were comparable to cellulase levels in cultures that had been induced with phosphate buffer from the beginning. This short span in the appearance of cellulase in the medium suggests that the potassium phosphate buffer acts directly to release preformed enzyme already at or near the cell surface. The mycelia exposed to cellobiose and phosphate buffer from the start of the experiment released cellulase continuously, thus suggesting that the process is unlike the shock release of periplasmic enzymes (7, 26). By contrast, relatively low concentration of ions are required for the continuing gradual release of cellulase from *Neurospora* cells.

A variety of salts stimulated the release of cellulase. In experiments with mycelia grown for 40 h and then exposed to salt and inducer for 6 h, 90% of the cellulase was released in the presence of 0.1 M sodium or potassium phosphate. Similar results were obtained using potassium chloride, magnesium chloride, and sodium citrate (data not shown).

Induction and release of cellulase as a function of pH of the medium is shown in Fig. 4. Cells were induced for 6 h with 1 mM cellobiose in a range of pH from 5.5 to 8.5, using 0.1 M potassium phosphate buffer. Cellulase was released maximally into the medium at pH 7.0. Cellulase accumulated within the cells at pH 7.5 even though total production of cellulase was less than at pH 7.0. This observation has led us to use pH 7.0 as a standard condition in the routine production of extracellular cellulase.

To study the effectiveness of different potential inducers, pregerminative conidia of strains 74-OR8-1a and 33(2-6)A were induced for 6 h under standard conditions in 1.0 M solutions of the following compounds: cellobiose, *N*-acetyl-

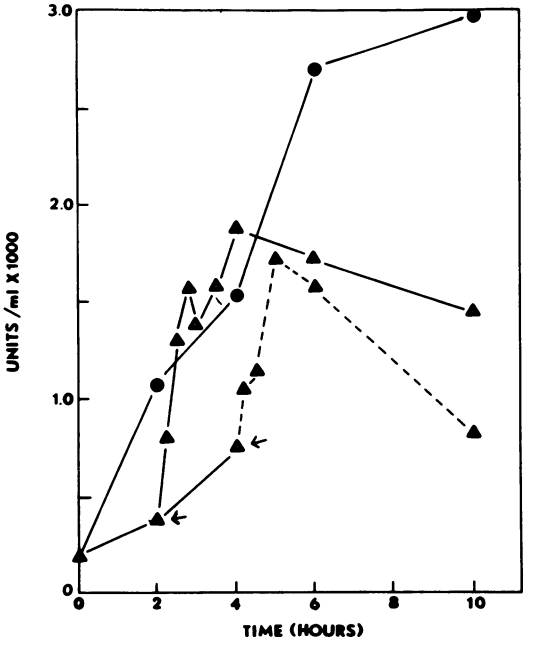

FIG. 3. *Release of cellulase from 40-h mycelia. Symbols:* (●) *Cells initially induced in buffer;* (△) *cells induced in water with buffer added at 2 or 4 h. Strain used was 62(2-2)a.*

glucosamine, amygdalin, arbutin, galactose, gentiobiose, glucono-Δ-lactose, glucose, xylose, laminaribiose, trehalose, maltose, and mannose. Only cellobiose showed a significant induction; maltose showed 10% of cellobiose, whereas the other compounds did not induce significant levels of cellulose (data not shown).

The relationship between cellobiose concentration and extracellular cellulase production was determined for four *Neurospora* strains (Fig. 5). Pregerminative conidia were exposed to cellobiose concentrations from 0 to 5.0 mM for 6 h under standard conditions. The optimum cellobiose concentration for wild-type strain 74-OR8-1a was 0.1 mM, whereas concentrations above this were less effective. Strains 33(2-6), *gluc-2*, and EM692a, wild type, were induced maximally at 1.0 mM cellobiose. Strain CM62(2-2), wild type, responded best to higher concentrations. In strain 74-OR8-1a, the decline in enzyme production with increasing concentrations of cellobiose concentration may have been due to production of glucose from cellobiose by this strain. Despite the prior treatment of cells with acid to remove surface aryl-β-glucosidase activity, it is possible that intracellular enzymes may release glucose within the cell before cellobiose has had an inducer effect. Glucose inhibits the induction not only of cellulase,

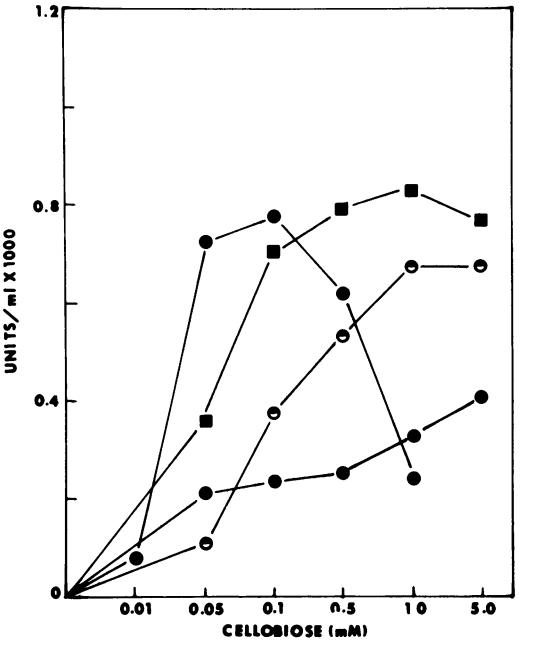

FIG. 5. *Extracellular cellulase production as a function of cellobiose concentration. Buffer was 0.1 M phosphate. Pregerminative conidia from strains:* (●) *74-OR8-1a;* (■) *33(2-6);* (⊕) *CM62(2-2);* (◓) *692a.*

but also of aryl-β-glucosidase and cellobiase as well (4). The mode of glucose action is probably via catabolite repression (11, 18).

Prevalent models for cellulose decomposition (1) involve the action of both endocellulase and exocellulase (β-glucosidase) to produce free glucose. Since there are two β-glucosidases in *Neurospora*, we wished to know whether either of these enzymes was reduced at levels of activity coordinate with the cellulase production in these strains. Such a coordination would distinguish one or both of the β-glucosidases as a constant element of the cellulose decomposition pathway. To test this point, a variety of standard and exotic strains were induced and assayed for cellobiase aryl-β-glucosidase and extracellular cellulase activities. There was little evidence for correlation of activity of either of the two β-glucosidases with the cellulase activity within individual strains (Table 1). Cellulase activity varied widely among exotic strains. Cellobiase activity was next most variable in activity, whereas aryl-β-glucosidase was the most constant from strain to strain. All of the standard laboratory wild-type strains that we tested fell roughly within the same range of cellulase activity and only varied by two- or threefold, whereas the extreme range in exotics was many hundred-fold under simple conditions of induction.

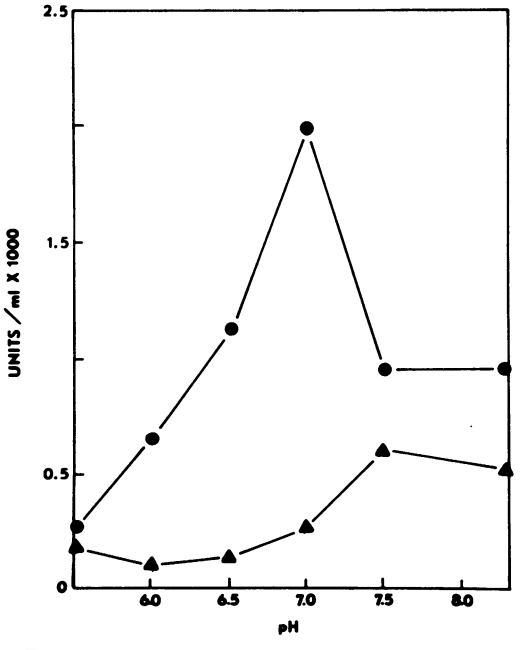

FIG. 4. *Effect of pH on cellulase production and release. Symbols:* (●) *Cellulase in media;* (△) *cellulase bound in cells. Pregerminative conidia of strain 74-OR8-1a were in 0.1 M buffer and assayed under standard induction conditions.*

Exotic strain Singapore 436 was examined in greater detail because of its very low cellulase activity in young cultures. Although this strain produced very low levels of cellulase activity under standard minimal induction conditions, the addition of vitamin supplements increased the levels of this enzyme activity. This suggests that future comparisons of enzyme activities of exotic strains with wild type should also be made with complex nutritional media as well as minimal media because of the varied nutritional capabilities of the exotic and standard wild-type strains.

DISCUSSION

The model that is generally proposed for the decomposition of cellulose by microbes (9, 17) agrees with our observation of an extracellular cellulase and two β-glucosidases in *Neurospora* (5, 8, 10). In fungi, three types of enzymes are believed to be directly involved in cellulose decomposition. The first, a C_1 enzyme, swells cellulose. The second type of cellulase, reported here, breaks cellulose into intermediate-size chains. Finally, β-glucosidase releases glucose sequentially from the ends of these shortened chains. Naturally occurring cellulose chains are probably too large to penetrate the cell wall spaces in *Neurospora* (13, 23, 24). Therefore, external cellulase activity is required for the preparation of cellulose as a carbon source.

Aryl-β-glucosidase of *Neurospora* is in a position to further degrade the initial products of cellulase activity from its location within the cell wall of *Neurospora* (3). Cellobiase, which is found only within the cell, can then break down the smaller β-glucosidic polymers that survive exposure to aryl-β-glucosidase of the cell wall.

Cellobiose is an effective inducer of cellulase in fungi (6, 12, 15) and also acts as an inducer for cellobiase and aryl-β-glucosidase in *Neurospora* (5). Neither glucose nor other monosaccharides tested perform an induction function, whereas maltose is an inducer 10% as effective as cellobiose.

Cellulase is not effectively released from mycelia if salts are absent from the induction medium. We propose two likely mechanisms to explain the retention of cellulase by mycelia induced in water alone. The first possibility involves a requirement for certain ions by a reaction step in the secretion of cellulase. Since vesicles in the hyphal tip of *Neurospora*, and other fungi, are involved in secretion of extracellular enzymes (1), this step could require the presence of ions for activation.

A second possibility for cellulose retention is that the enzyme is released in the presence of water alone, but is then bound to the cell wall by reaction analogous to ion exchange (22, 29). In this system, added salts would facilitate release of absorbed protein into the media (25). At present, we do not have evidence to decide between these two possibilities. It does not, however, seem that an alternate possibility of shock release (7, 26) can explain the gradual release of cellulase seen in continuous exposure of cells to 1 mM phosphate buffer.

Potassium or sodium phosphate buffer stimulates cellulase production up to 100 mM but inhibits enzyme production at higher concentrations. It is possible that these ions can interact with a component at the cell membrane such as cellobiose permease and in this way block the entry of the inducer, thus blocking enzyme production. This would lead us to predict that other saccharide permeases of *Neurospora* may be inhibited by high phosphate buffer concentrations (16, 20, 21). The related observation of a pH optimum for cellulase induction may reflect the pH optimum of a cellobiose permease. The primary effect of optimum pH could be regulation of entry of the inducer. A secondary effect might involve the release of the enzyme into the external media, since experiments show that release of cellulase from the cell is slightly retarded at pH values above 7.0.

Earlier work (15) showed that cellulase and cellobiase activity were produced coordinately in the *cell-1* mutant of *Neurospora*. By contrast, the present data show little correlation between levels of cellulase or cellobiase activities in the exotic strains that we have induced in simple media. We have also observed a lack of coordination between the levels of activity of either cellobiase or cellulase with aryl-β-glucosidase activity in exotic strains.

We have not found an effective enrichment technique to obtain mutants of *Neurospora* with altered cellulase activity, but we are pursuing the genetic basis of variation in cellulase activity in exotic strains. The heterogeneity of cellulase activity in these strains suggests involvements of more than one gene locus. We hope to ultimately determine the genetic contribution by appropriate crosses between exotic strains and standard laboratory strains.

ACKNOWLEDGMENTS

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