Cellulolytic Activity of *Thermomonospora curvata*: Nutritional Requirements for Cellulase Production

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Received for publication 29 November 1971

The use of a minimal medium for cellulase (C₁ and C₄) production by *Thermomonospora curvata* increased extracellular C₁ activity (measured by rate of cotton fiber hydrolysis) 11-fold compared with the previously used yeast extract medium. Ground cotton fibers supported the highest cellulase production when compared to other soluble and insoluble carbohydrate sources. Maximal cellulase production occurred at 45°C, slightly less at 55°C, and was insignificant at 65°C (the highest temperature at which cellulase activity appeared stable). At a temperature of 55°C, an optimal pH of 8.0, and a cotton fiber concentration of 8 mg/ml, shake cultures of *T. curvata* degraded about 75% of the cellulose during the 10-day period.

Composting (microbiological degradation of solid waste materials) provides a large-scale method for recycling the biodegradable organic matter in municipal refuse. At the Public Health Service–Tennessee Valley Authority Joint Composting Project (Johnson City, Tenn.), sorted municipal refuse averaged about 50% cellulose at the initiation of the 7-week open-windrow composting process (15). The identification of a thermophilic actinomycete, *Thermomonospora curvata*, as a potentially important cellulose decomposer in the process has been reported (14). Many studies have been made on the nutritional requirements for cellulase (EC 3.2.1.4) production by mesophilic fungi such as *Trichoderma viride* (6, 9, 16) and *Myrothecium verrucaria* (12, 13, 17). Relatively little work of a similar nature has been published on cellulase production by thermophilic actinomycetes active in high-temperature (50 to 65°C) cellulose decomposition during composting. This report describes the nutritional requirements for growth, cellulase production, and cellulose degradation by *T. curvata* in a minimal medium.

**MATERIALS AND METHODS**

**Culture maintenance.** Cultures of *T. curvata* were maintained at 4°C on a 1% agar medium containing the mineral salts and vitamins listed below. The microcrystalline cellulose, Avicel, (PMC Corp., American Viscose Div., Newark, Del.) was added in concentrations of 0.5 to 0.7% together with 0.05% of type 12M31P carboxymethylcellulose (Hercules, Inc., Charlotte, N.C.) which improved suspension of the Avicel in the medium.

**Minimal medium for cellulase production.** The minimal medium for cellulase production consisted of: (NH₄)₂SO₄, 2.0 g; MgCl₂·6H₂O, 0.2 g; CaCl₂, 11 mg; thiamine and biotin, 1 mg each; 1.0 M potassium phosphate buffer (pH 8.0), 100 ml; and distilled H₂O to 1 liter. Several sources of carbohydrate were tested in this medium, including glucose and cellobiose (Sigma Chemical Co., St. Louis, Mo.), Avicel microcrystalline cellulose, Whatman type CF11 fibrous cellulose powder (W. & R. Balston Ltd., England), and ground, surgical-grade absorbent cotton (Johnson and Johnson Co., New Brunswick, N.J.). These materials were added in varying amounts (50 to 500 mg) to 50 ml of mineral salts in 250-ml Erlenmeyer flasks and sterilized by autoclaving. Stock solutions of the vitamins were autoclaved separately and added aseptically.

**Cellulase production.** Each flask was inoculated with about 122 μg (dry cell weight) of washed cells preserved in a frozen suspension. Cultures were routinely incubated at 55°C in a New Brunswick Scientific Co. G-76 rotary water-bath shaker (180 rev/min) for 10 days. Samples (5 ml) for the assay of cellulase activity, cell nitrogen, extracellular protein, and residual cellulose were taken at 1, 3, 6, and 10 days.

**Cellulase assays.** After clarification of culture fluids by centrifugation (40,000 × g for 30 min at 2°C), cellulase activity was assayed by two methods.
arsenomolybdate activities International respectively, according added. C1 fibers and soluble cellulose derivatives) was measured in reaction mixtures containing 3.5 ml of 4% carboxymethylcellulose (type 7L; Hercules, Inc.) which had a degree of substitution of 0.7. To this substrate 0.1 ml of 1.0 M sodium acetate buffer (pH 6.0) and 0.4 ml of 10-fold-diluted culture fluid were added. C1 and Cx assay systems were incubated for 10 min at 65°C. The liberation of soluble reducing sugars during the incubation was measured by the arsenomolybdate method of Nelson (8). C1 and Cx activities were recorded as milliunits and units, respectively, according to the recommendations of the International Union of Biochemistry (3).

Other methods. Extracellular protein was determined by the method of Lowry et al. (5).

Dry cell weight increase was estimated by determining cell nitrogen by the method of Johnson (4). T. curvata cells grown on 0.4% cellobiase averaged 11.5% nitrogen, and this value was used to estimate dry cell weight in flasks where the insoluble cellulolytic substrate made routine dry cell weight analysis impossible.

Determination of residual cellulose in cultures of T. curvata was made in the following manner: insoluble cellulose from 5-ml samples was sedimeted by centrifugation, washed once with distilled water, re-sedimented, and extracted by the method of Crampton and Maynard (2) to remove lignins, hemicellulose, and xylosans. The sample was then treated with 10 ml of 67% (v/v) H2SO4, incubated for 1 hr at room temperature, and diluted 1:100. The sugar content of this dilution was measured by the anthrone method of Scott and Melvin (11). The cellulose standard used in these tests was Avicel microcrystalline cellulose.

RESULTS

Growth requirements. Both thiamine and biotin were essential for growth of T. curvata. The addition of choline chloride (1 \( \mu \)g/ml) was found to be slightly stimulatory (average of 11% increase) to cellulase production and was routinely employed in later experiments.

Effect of carbohydrate source on cellulase production. The type and concentration of carbohydrate was found to be critical for maximal cellulase production in the minimal medium. Although the presence of cellobiase allowed rapid growth (1.4 mg of dry cell weight per ml at 24 hr) and liberation of extracellular protein (0.5 mg/ml), relatively little Cx activity and no C1 activity could be detected in the culture fluids. Similar results were obtained in the presence of glucose. Several insoluble carbohydrate sources were then tested.

Absorbent cotton (ground to pass a 20-mesh screen) proved to be the best substrate for cellulase production, although the concentration was critical (Fig. 1). Maximal production of both C1 and Cx cellulase (110 milliunits of C1 and 5.9 units of Cx) was achieved when the ground-cotton concentration was 7 to 8 mg/ml, whereas extracellular protein increased in proportion to the concentration of ground cotton in the medium. At the optimal ground-cotton concentration, the maximal reducing sugar accumulation in the medium was about 50 \( \mu \)g/ml (as compared to the glucose standard). An increase in cotton concentration to 10 mg/ml tripled the reducing sugar accumulation. A comparison is made in Table 1 to illustrate the relative effectiveness of ground cotton and other substances in cellulase production.

Effect of aeration, temperature, and pH on cellulase production. Cultures of T. curvata produced an average of 2.7 times as much C1, 4.2 times as much Cx, and 5.9 times as much extracellular protein when shaken at 180 rev/min as compared to stationary cultures at the same temperature (55°C). The extremes of temperature which allowed detectable growth of T. curvata were 36°C and 62°C. The maximal rate of growth occurred in the range of 50 to 55°C, with peak dry cell weights averaging about 1.2 mg/ml. The rate of growth at 45°C
was slower (about 40% less growth at 3 days of incubation), but maximal dry cell yields at 10 days were slightly higher (about 1.4 mg/ml). The temperature for maximal cellulase production was 45 C (Fig. 2). Increasing the incubation temperature from 45 to 55 C resulted in a 15% decrease in cellulase production. Incubation at 60 C eliminated almost all cellulase production and growth.

The stability of cellulase in the culture fluids was a factor to consider in the determination of temperature effects on cellulase production. In a medium containing insoluble cellulose as the sole carbon source, the growth of a culture might be limited at higher temperatures by the instability of the cellulase liberated into the medium. To determine whether the cellulase of culture fluids was being thermally denatured at temperatures above 55 C, stability in the range of 60 to 75 C was determined. The results are given in Fig. 3 and 4. C, activity (as measured by cotton fiber hydrolysis) appeared to be relatively stable at 60 C, slowly inactivated at 65 C, and inactivated rapidly at 70 (half-life of about 20 min) and 75 C (half-life of about 2.3 min). C, activity (as measured by carboxymethylcellulose hydrolysis) appeared to be somewhat more stable. No inactivation occurred during 1 hr of exposure at 65 C; halflives at 70 and 75 C were 36 min and 5 min, respectively. Inactivation curves for both C, and C, appeared to be biphasic.

The effect of the initial pH of the medium on cellulase production is shown in Fig. 5. No cellulase production occurred at pH 6.0. An increase in the initial pH from 7 to 8 doubled cellulase production, with the optimum occurring at pH 8.0. During the 10-day incubation period, the pH of the culture fluids dropped from the initial 8.0 to the range of 7.2 to 7.6.

The municipal, solid waste, composting environment from which T. curvata was isolated (the open-window composting process at the Public Health Service-Tennessee Valley Authority Joint Composting Project) reached temperatures in the range of 55 to 65 C and pH values in the range of 7.0 to 8.5 (15). To test the rate of cellulose degradation under such conditions, flasks containing the minimal

Table 1. Effect of various carbohydrate sources on cellulase production by T. curvata*

| Carbohydrate source   | $C_1$ production (relative %) | $C_x$ production (relative %) |
|-----------------------|-------------------------------|-------------------------------|
| Cellulose             | 0                             | 30                            |
| Fibrous cellulose powder (Whatman) | 45                             | 74                            |
| Microcrystalline cellulose (Avicel) | 69                             | 69                            |
| Ground cotton         | 100                           | 100                           |

*Data represent cellulase production at the optimal concentration of each carbohydrate. These concentrations (milligrams per milliliter of medium) were: 1 to 2 for cellulose, 3 for Whatman cellulose powder, 4 for Avicel, and 8 for ground cotton.

Fig. 2. Influence of temperature on cellulase ($C_1$ and $C_x$) production by T. curvata. Each point is the mean of two determinations.

Fig. 3. Semilogarithmic plot of $C_1$ cellulase inactivation in cell-free culture fluids at various temperatures. Each point is the mean of two determinations.
medium, with 8 mg of cotton per ml, were shaken for 10 days at pH 8.0 and at 55 C. Figure 6 illustrates culture growth and rate of cellulose degradation. After a 1-day lag period, dry cell weight increased to about 1 mg/ml at 3 days and about 1.2 mg/ml by 10 days. During that time, the cellulose content of the flasks decreased from 8 mg/ml to about 2 mg/ml. The ground cotton used in these studies averaged over 99% cellulose and, therefore, cotton dry weight was considered equivalent to cellulose dry weight. Most of the soluble sugar released by the enzymatic hydrolysis of this cellulose was utilized by the cells; however, a small amount of reducing sugar (average of 53 µg of glucose per ml) was detectable in the extracellular fluid at the termination of the 10-day incubation period.

C₁ and C₅ cellulase activity, together with extracellular protein, were also measured (Fig. 7). After the initial 1-day lag period, C₁ and C₅ activity of the extracellular culture fluid increased rapidly to maxima at 6 days (about 110 milliunits/ml and 5 units/ml for C₁ and C₅, respectively). Incubation beyond 6 days resulted in decreases in both C₁ and C₅ activity, although extracellular protein continued to accumulate.

**DISCUSSION**

Besides the obvious advantages of a chemically defined medium versus a complex one (ease of enzyme purification, better resolution of effects by inducers and inhibitors, and decreased interference with chemical assays) the medium described here increased C₁ cellulase production 11-fold in comparison with the

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**Fig. 4.** Semilogarithmic plot of C₅ cellulase inactivation in cell-free culture fluids at various temperatures. Each point is the mean of two determinations.

**Fig. 5.** Influence of pH on cellulase (C₁ and C₅) production by T. curvata. Each point is the mean of two determinations.

**Fig. 6.** Increase in dry cell weight and decrease in insoluble cellulose in cultures of T. curvata grown at pH 8 at 55 C with ground cotton (8 mg/ml) as the sole source of carbohydrate. Each point shows the average and range of four determinations.
yeast extract medium previously reported (14). The cause of this increase might be the elimination of soluble sugars from the medium.

The failure of *T. curvata* to produce maximal amounts of cellulase when growing on a soluble sugar such as glucose or cellobiose as the source of carbohydrate is not unusual; similar observations have been made in studies on cellulase production by *T. viride* (6), *M. verrucaria* (13), and *Pseudomonas fluorescens* var. *cellulosa* (18). Cellobiose, the predominant product when the cellulose polymer is hydrolyzed by cellulase, appears to act as an inducer in low concentrations, but as a repressor of cellulase production in concentrations normally employed in growth media (7). Earlier reports (1, 9, 16) and the present study indicate that cotton fibers, the most resistant of the substrates normally employed in cellulase assay systems and the best indicator of C, cellulase activity (7), are an excellent carbohydrate source for cellulase production. Its worth in this regard probably lies in its relative resistance to rapid enzymatic hydrolysis; in an actively growing cellulolytic culture, it would act as a reservoir to allow gradual release of soluble sugars in quantities small enough to act as an inducer rather than as a repressor of cellulase production. That the concentration of absorbent cotton is critical in cellulase production by *T. curvata* also seems to indicate that the optimal substrate must supply ample substrate for growth, yet not reach the threshold concentration where inducer becomes repressor. In this study, cultures growing on the optimal concentration of ground, absorbent cotton (7 to 8 mg/ml) never accumulated more than about 55 µg of reducing sugar in the medium (compared to a glucose standard). An increase in absorbent cotton concentration to 10 mg/ml tripled the accumulation of reducing sugar and decreased cellulase production. The inducer-repressor capacity of cellobiose, the ability of sophorose (a beta 1-2 glucoside) to act as a potent inducer of cellulase production (7), and the significance of the crystal structure of cellulose in the production of cellulase (9) comprise an interesting area of metabolite control which has received relatively little attention on the molecular level.

The optimal pH and temperature range for cellulase production by *T. curvata* was pH 7.5 to 8.0 and 45 to 55 C, respectively. This pH and temperature range is reached by composting municipal solid waste during the 5th to 7th week of the open-windrow process (15). Under the conditions employed in the laboratory, cultures were able to degrade about 75% of the cotton cellulose within 10 days. Although cellulose degradation proceeds at a slower rate in the actual composting process, control of pH and temperature during the earlier stages of composting might be profitable in the search for faster and more complete cellulase degradation.

ACKNOWLEDGMENTS

I acknowledge the advice of David Updegraff, concerning the procedure for cellulose analysis, and the technical assistance of James Richardson.

This investigation was supported by Public Health Service grant no. EC-00420-01 from the Bureau of Solid Waste Management and by the Ronald V. Jensen Center for Environmental Studies, Weber State College.

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