Cellulase Production by *Thermomonospora curvata* Isolated from Municipal Solid Waste Compost

FRED J. STUTZENBERGER

Department of Microbiology, Weber State College, Ogden, Utah 84403

Received for publication 26 April 1971

A cellulolytic, thermophilic actinomycete (previously isolated from municipal refuse compost samples) was identified as *Thermomonospora curvata*. A determination was made of the optimal conditions for cellulase production by *T. curvata* when grown at 55 C in a medium containing mineral salts, cellulose, and yeast extract. The pH and temperature optima (pH 6.0 and 65 C) for the cellulase produced by *T. curvata* were identical to those previously observed for the cellulase extracted from crude compost samples. Such similarities, together with the prevalence of *T. curvata* in compost samples and its ability to grow at composting temperatures, indicate that this actinomycete could possibly be considered as a major cellulose decomposer in the municipal refuse composting process.

During the last four decades, the per capita production of solid waste in the United States has doubled and is believed to be increasing at the rate of 4% per annum (15). The disposal of this municipal solid waste has become an important problem in the improvement of environmental quality. Composting (microbiological degradation of solid organic wastes) is currently under consideration as a method for the disposal of biodegradable municipal refuse. The Bureau of Solid Waste Management, U.S. Public Health Service, in cooperation with the Tennessee Valley Authority, has established an open windrow composting plant at Johnson City, Tenn., for the evaluation of this disposal method.

The paper content of the municipal solid waste often averages over 50% (1), and, therefore, cellulose degradation by the indigenous microbiota is of major importance in the composting process. In a recent report (21), the isolation of three species of cellulolytic flora from municipal solid waste compost samples was described. A thermophilic actinomycete was chosen for further study due to its prevalence in compost samples and its ability to grow rapidly at composting temperatures (50 to 60 C) on cellulose-agar media. The purposes of the present study were: (i) to identify this actinomycete, (ii) to determine optimal conditions for its cellulase (EC 3.2.1.4) production, and (iii) to compare its extracellular Cx cellulase with the crude Cx cellulase activity previously observed in compost extracts (21). Such a comparison would aid in evaluating this actinomycete as a contributor to cellulose degradation during the composting of municipal solid wastes by the open windrow process.

**MATERIALS AND METHODS**

**Culture identification.** We are indebted to Thomas Cross, University of Bradford, Bradford, England, for his identification of the thermophilic actinomycete used in these studies. The medium used for morphological observations was half-strength nutrient agar consisting of the following: beef extract, 0.5 g; yeast extract, 1.0 g; peptone, 2.5 g; NaCl, 2.5 g; agar, 15.0 g; distilled water, 1 liter (final pH 7.0 to 7.2). Cultures were incubated at 40, 49, and 55 C, and morphological development was observed at intervals as long as 5 days.

**Culture maintenance.** Cultures were maintained at 4 C on the microcrystalline cellulose-agar medium previously described (21).

**Medium for cellulase production.** The composition of the soluble portion of the medium routinely used for cellulase production was as follows: (NH4)2SO4, 1.0 g; MgCl2-6H2O, 0.1 g; 1.0 M potassium phosphate buffer (pH 8.0), 100 ml; 10% yeast extract (Difco) solution, 10.0 ml; distilled water to 1 liter. Samples (50 ml) of the mineral salts solution were autoclaved in 250-ml Erlenmeyer flasks with 50 mg each of the microcrystalline cellulose (Avicel; FMC Corp., American Viscose Div., Newark, Del.) and absorbent cotton (ground to pass 20-mesh screen). The yeast extract solution was sterilized by filtration through membrane filters (Millipore Corp.) and added aseptically after autoclaving. Cultures were routinely incubated for 10 days at 55 C in a rotary water bath shaker (model G-76; New Brunswick Scientific Co.) at 180 rev/min.

**Measurement of culture growth.** Growth of the

1 Present address: New Zealand Department of Agriculture, Ruakura Soil Research Station, Hamilton, N.Z.
actinomycete in shake cultures was estimated by measuring the increase of cell nitrogen. Nitrogen determinations were made by the method of Johnson (11), with twice-washed cells from 5-ml samples of culture.

**Assay for cellulase activity.** Before the assay for free cellulase, culture fluids were clarified by centrifugation at 40,000 × g for 30 min at 2 C. Due to the multiplicity of components in the cellulase complex (5), activity was assayed by two methods generally similar to those recommended by Mandels and Weber (14). The activity of the C₁ component (the prehydrolytic or activating enzyme which is necessary for the hydrolysis of resistant substrates such as cotton fibers) was measured as follows. A 3.2-ml amount of the clarified culture fluid and 0.8 ml of sodium acetate buffer (1.0 M) were added to 90 mg of surgical grade absorbent cotton (Johnson and Johnson Co., New Brunswick). The final pH of this reaction mixture was 6.0 to 6.2. After incubation for 15 min at 65 C, the increase in soluble reducing sugars was measured by the method of Nelson (16), with glucose as the reducing sugar standard. Production of reducing sugar in the C₁ assay system was proportional to incubation time until a reducing sugar concentration equivalent to approximately 35 μg of glucose per ml was reached (about 20 min under optimal conditions). The C₂ cellulase component (the enzyme which hydrolyzes activated cotton and soluble cellulose derivatives) was assayed by the hydrolysis of carboxymethylcellulose (CMC) as previously described (21). The C₁ and C₂ activities are expressed in milliunits and units, respectively, according to the recommendations of the International Union of Biochemistry (10). The standard deviations of the C₁ and C₂ assays (each calculated from data obtained with four groups of four replicate samples) were ±3.8 and ±7.4%, respectively.

**RESULTS**

**Identification of the actinomycete.** Figure 1 is a photograph of the thermophilic actinomycete after growth on half-strength nutrient agar for 48 hr at 49 C. Note the long aerial hyphae with the single spores on simple or dichotomously branching sporophores. This actinomycete appeared to be very similar to *Thermomonospora curvata* previously described (8, 9) and has been classified as such by Cross.

**Optimal conditions for cellulase production.** Attempts to grow *T. curvata* in a mineral salts-cellulose medium without addition of yeast extract were unsuccessful. Production of high cell mass [about 1 mg (dry cell weight)/ml of medium] but insignificant cellulase production occurred when cultures were grown on a mineral salts medium containing cellobiose and yeast extract. Maximal cellulase production was attained with the mineral salts medium containing a yeast extract concentration of at least 0.1% and microcrystalline cellulose. The cellulose concentration was critical in its effect on cellulase production (Fig. 2). An increase in microcrystalline cellulose (Avicel) concentration from 0.05% (w/v) to 0.1% doubled the production of free cellulase (C₂), but an increase to 0.5% Avicel caused a 70% reduction in activity. Reduction in cellulase production was accompanied by an accumulation of reducing sugars in the medium; this accumulation routinely occurred during growth when the Avicel concentration exceeded 0.1%. The use of 50 mg of ground absorbent cotton in addition to the Avicel increased C₁ and C₂ production slightly (26 and 12%, respectively), but addition of more cotton caused no further stimulation. Therefore, 50 mg of Avicel and 50 mg of ground cotton were employed as the cellulose source in each 50-ml culture.

Results of studies to determine the optimal pH for cellulase production are shown in Fig. 3. Maximal cellulase production was attained when the initial pH of the medium was adjusted to 8.0 with phosphate buffer (final concentration, 0.1 M). After 10 days of culture incubation, a reduction in pH to 7.5 to 7.7 was routinely observed.

![Fig. 1. Gross morphology of Thermomonospora curvata. This culture was grown on half-strength nutrient agar (Oxoid) at 49 C for 48 hr. X 300.](image-url)
**CELLULOSE PRODUCTION BY T. CURVATA**

**Fig. 2.** Effect of microcrystalline cellulose (Avicel) concentration on cellulase (Cₙ) production by Thermomonospora curvata. The maximal cellulase activity observed in each of triplicate determinations was assigned a value of 100%.

**Fig. 3.** Effect of pH on cellulase (Cₙ) production by Thermomonospora curvata. Each point is the mean of two determinations.

**Fig. 4.** C₁ and Cₓ cellulase production by Thermomonospora curvata in the mineral salts medium containing optimal concentrations of yeast extract, cellulose, and hydrogen ions. Each point is the mean of two determinations.

**Fig. 5.** Effect of pH on cellulase (Cₓ) activity of cell-free culture fluids assayed after growth of Thermomonospora curvata for 6 days. Reaction mixtures were buffered with 0.1 M sodium acetate or 0.05 M potassium phosphate. Each point is the average of three determinations.

Figure 4 illustrates the course of C₁ and Cₓ production by *T. curvata* in the mineral salts medium having optimal yeast extract, cellulose, and hydrogen ion concentrations. Maximal C₁ and Cₓ activity occurred after approximately 6 days of growth at 55 C and diminished during further incubation. C₁ activity (liberation of reducing sugars from absorbent cotton) was low compared to the Cₓ activity (liberation of reducing sugars from CMC); the C₁/Cₓ ratio was approximately 1:450 after incubation of the cultures for 6 days. Cell nitrogen in flasks providing optimal conditions for cellulase production increased from 3 µg/ml at the time of inoculation to a range of 35 to 56 µg/ml at 6 days. This wide variation is due in part to the heterogeneous clumping growth of the *T. curvata*, the tendency
of the clumps to cling to the sides of the flasks, and the interference by the insoluble cellulose.

**Comparison of C₅ activities.** Cellulase production by *T. curvata* in the medium just described allowed a preliminary comparison to the C₅ activity observed in compost extracts (21). The C₅ activity in compost extracts had been studied in regard to pH, temperature, and substrate concentration optima, and therefore similar determinations were made on the C₅ enzyme produced by *T. curvata*. Figure 5 is a plot of C₅ activity versus pH when reaction mixtures of *T. curvata* culture fluids and CMC were buffered with either acetate (final concentration, 0.1 M) or phosphate (final concentration, 0.05 M). Maximal activity occurred at pH 6.0 in the presence of either acetate or phosphate buffers. Figure 6 illustrates the effect of temperature on the activity of C₅ enzyme produced by *T. curvata*. The increase in activity when the temperature was increased from 45 to 60 °C appeared to be quite linear. Maximal activity occurred at 65 °C; an increase to 75 °C reduced the activity to approximately one-third of the maximum.

The effect of substrate (CMC) concentration on C₅ activity was determined and the Kₘ for the system was calculated. Following the recommendations of Dowd and Riggs (3) for estimating Michaelis-Menten kinetic constants, a linear transformation of S/V versus S (7) was used to estimate the Kₘ (Fig. 7). The Kₘ for the reaction system was calculated to be 1.90% CMC based on a plot fitted to the data by the least-squares method.

**DISCUSSION**

Thermophilic actinomycetes have been found in abundance in high-temperature, well-aerated composts, and some species are active in cellulose degradation; at 60 to 65 °C, species of *Thermomonospora* and *Thermopolyspora* predominate (22). Therefore, it should be expected that a cellulolytic thermophile such as *T. curvata* would establish significant populations in a well-aerated compost of high cellulose content, such as the municipal solid waste material processed by the open windrow method at the Public Health Service Tennessee Valley Authority (PHS-TVA) Composting Project. The importance of its role in cellulose decomposition during the composting process has yet to be defined, due in part to the heterogeneity of the composting material and the complex microbiological ecosystem which is active during its degradation. One purpose of the present study was to evaluate the potential of *T. curvata* as a major cellulose decomposer during the composting process.

Although attempts to cultivate *T. curvata* in an unsupplemented mineral salts-cellulose medium were unsuccessful, addition of 0.1% yeast extract allowed growth and cellulase production. Such results are similar to those observed in a study (20) on cellulose degradation by *Gliomastix convoluta*: maximal degradation of cotton cloth was obtained when a yeast extract concentration of 0.1% was employed in a mineral salts medium. Studies on the nutritional requirements of *T. curvata* are currently under way to define the necessity of yeast extract during growth and cellulase production.
The availability of carbohydrate during growth (either as cellulose or as cellobiose) was a major influence on cellulase production. The presence of soluble reducing sugars in the medium inhibited cellulase production. This phenomenon is not unusual in studies on microbial cellulase production, particularly those involving cellulytic fungi of the genus Trichoderma (12, 13). Apparently, soluble sugars such as cellobiose (whether added to the medium or produced as hydrolysis products of cellulose by enzymatic attack) act as effective repressors on cellulase production when present in greater than trace amounts. Therefore, the optimal concentration of cellulose in the medium would be that which would allow growth and cellulase production without allowing accumulation of soluble sugar to act as a repressor. The use of 0.1% microcrystalline cellulose (Avicel) and 0.1% ground cotton appeared to meet that requirement in the present studies.

An initial pH value of 8.0 was optimal for cellulase production by T. curvata; optimal growth of most actinomycetes occurs in the pH range of 7 to 8 (22). This pH range is attained by the composting solid waste at the PHS-TVA Composting Project during the fourth to fifth week of the process and seldom exceeds pH 8.5 (21). This slightly alkaline pH together with the elevated temperatures would favor the growth of actinomycetes over that of the fungi.

When T. curvata was cultured at 55 C under optimal conditions of cellulose, yeast extract, and hydrogen ion concentrations, maximal accumulation of both C_1 and C_2 (4.5 units and 10 milliunits per ml, respectively) enzymes occurred at approximately 6 days. Activity of the cell-free culture fluids was much greater against the soluble, substituted cellulose (CMC) than against the insoluble cotton fibers. As pointed out by Mandels and Weber (14), hundreds of microorganisms have been studied as to their ability to produce cell-free enzymes which rapidly hydrolyze cellulose; yet the enzyme-containing filtrates generally act in a relatively slow manner on a highly resistant material such as cotton fibers. Since cotton fibers constitute one of the forms most resistant to enzymatic attack, it is predictable that culture fluids would act upon the insoluble fibers much more slowly than on a soluble cellulose such as CMC which is very susceptible to enzymatic hydrolysis.

The optimal pH and temperature (6.0 and 65 C) for C_2 activity in the cell-free culture fluids of T. curvata are identical to those observed for C_2 activity in extracts of various compost samples studied at the PHS-TVA Composting Project (21). Although pH 6.0 is somewhat higher than the optimal pH range (pH 4.5 to 5.2) for cellulases of fungi such as Trichoderma viride (17) and Myrothecium verrucaria (6), it correlates well with the pH optimum (pH 5.9) observed for cellulase of an actinomycete of the genus Streptomyces (19). The temperature optimum of 65 C for the C_2 enzyme of T. curvata is higher than that of most fungal β-glucanases (50 C); but a few, such as the β-1,6-glucanase of Sporotrichum pruinatum (18), have temperature optima at 60 to 70 C.

These two points of similarity (identical pH and temperature optima) between C_2 activities of compost extracts and T. curvata culture fluids suggest the possibility that this actinomycete produces most of the cellulase extracted from compost samples. However, numerous research groups have shown that many cellulases are similar in these two respects, and that most such β-glucanases have optimal activity at pH values of 4 to 6 and at temperatures of 40 to 60 C (2). Comparison of C_1 activities from both compost and culture fluids would perhaps be helpful (and more meaningful in application to cellulose degradation in composting), but unfortunately the C_1 activity of crude compost extracts is insignificant, probably due to the irreversible binding of the enzyme on insoluble cellulosic materials (18).

Comparison of the K_m values under identical conditions for C_2 activities from both sources appears to be of limited value. Relatively little information is available regarding K_m values for CMC hydrolysis by cellulase. Values of 0.5 and 1.6 mg/ml have been published in studies on the cellulases of M. verrucaria (6) and T. viride (18), respectively, but the effect of substrate concentration on reaction rate is complicated by the adsorption of the enzyme on the substrate (whether it be CMC or solid cellulose) in such a way that it is rendered inactive when the ratio of enzyme to substrate is relatively low (18). In the present study, a K_m value of 19.0 mg/ml (1.9%) was calculated for the C_x enzyme hydrolyzing a CMC with a degree of substitution (DS) of approximately 1.2. This high K_m value may be due to the high DS of the CMC, for it has been shown that the K_m for CMC increases with increase in DS (4). However, the K_m calculated for C_x activity of compost extracts studied under conditions identical to those in the present study was 8.3 mg/ml (21). The evaluation of this difference would probably require purification of C_x enzymes from both sources.

In conclusion, it should be stated that the prevalence of T. curvata in compost samples, taken at the PHS-TVA Composting Project, its ability to grow at composting temperatures
(50 to 60 C), and its production of cellulase which has identical pK and temperature optima as that of compost extracts strongly indicate that this actinomycete may occupy a major role in cellulose degradation during the composting of municipal solid wastes. Studies are now in progress to determine the nutritional requirements of T. curvata in a chemically defined medium and to compare its ability to degrade various natural and synthetic cellulose materials. It is hoped that such studies will form the basis for successful manipulation of controlled composting conditions to increase the speed and effectiveness of cellulose degradation in the process.

ACKNOWLEDGMENTS

I thank both Hubert Lechevalier and Thomas Cross for their expert assistance in the identification of Thermomonospora curvata.

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.

LITERATURE CITED

1. American Public Works Association, Committee on Refuse Disposal. 1961. Municipal refuse disposal, p. 45. Interstate Printers and Publishers, Inc., Danville, Ill.
2. Bull, A. T. 1967. The enzymatic degradation of beta-glucans. A.T. Int. Biodet. Bull. 3:3-12.
3. Dowd, J. E. and D. S. Riggs. 1965. A comparison of estimates of Michaelis-Menten kinetic constants from various linear transformations. J. Biol. Chem. 240:863-869.
4. Eriksson, K., and B. H. Holmmark. 1969. Kinetic studies on the action of cellulase upon sodium carboxymethyl cellulose. Arch. Biochem. Biophys. 133:233-237.
5. Gilligan, W., and E. T. Reese. 1954. Evidence for multiple components in microbial cellulases. Can. J. Microbiol. 1:90-105.
6. Halliwell, G. 1961. The action of cellulolytic enzymes from Myrothecium verrucaria. Biochem. J. 79:185-192.
7. Hanes, C. S. 1932. Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis of amylase of germinated barley. Biochem. J. 26:1406-1421.
8. Henssen, A. 1957. Beitrag zur Morphologie und Systematik der thermophilen Actinomyceten. Arch. Mikrobiol. 26:373-414.
9. Henssen, A., and E. Schnepf. 1967. Zur Kenntnis thermophiler Actinomyceten. Arch. Mikrobiol. 57:214-231.
10. International Union of Biochemistry. 1965. Enzyme nomenclature, p. 39. Elsevier Publishing Co., Amsterdam.
11. Johnson, M. J. 1941. Isolation and properties of a pure yeast polypeptidase. J. Biol. Chem. 137:575-586.
12. Mandels, M., and E. T. Reese. 1957. Induction of cellulase in Trichoderma viride as influenced by carbon sources and metals. J. Bacteriol. 73:269-278.
13. Mandels, M., and E. T. Reese. 1960. Induction of cellulase in fungi by cellulobiose. J. Bacteriol. 79:816-826.
14. Mandels, M., and J. Weber. 1969. The production of cellulases. Advan. Chem. Ser. 95:391-414.
15. National Academy of Sciences—National Research Council. Committee on Pollution. 1966. Waste management and control, p. 13-14. National Academy of Sciences Printing and Publishing Office, Washington, D.C.
16. Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 158:375-380.
17. Okada, G., K. Nisizawa, and H. Suzuki. 1968. Cellulase components from Trichoderma viride. J. Biochem. 64:591-607.
18. Reese, E. T., and M. Mandels. 1963. Enzymatic hydrolysis of beta-glucons, p. 210. In E. T. Reese (ed.), Advances in enzymic hydrolysis of cellulose and related materials. Pergamon Press, London.
19. Sietsema, J. H., D. E. Eveleigh, and R. H. Haskins. 1968. The purification of cellulase and exo-laminarase and their role in the formation of Pythium sp. "protoplasm." Antonie van Leeuwenhoek J. Microbiol. Serol. 34:331-340.
20. Siu, R. G. H., and J. W. Sinden. 1951. Effects of pH, temperature, and mineral nutrition on cellulolytic fungi. Amer. J. Bot. 38:284-290.
21. Stutzenberger, F. J., A. J. Kaufmann, and R. D. Lossin. 1970. Cellulolytic activity in municipal solid waste composting. Can. J. Microbiol. 16:553-560.
22. Waksman, S. A. 1967. The Actinomycetes: a summary of current knowledge, p. 17-18. Ronald Press Co., New York.