Cellulase Production by a Thermophilic \textit{Clostridium} Species

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Strain M7, a thermophilic, anaerobic, terminally sporing bacterium (0.6 by 4.0 μm) was isolated from manure. It degraded filter paper in 1 to 2 days at 60 C in a minimal cellulose medium but was stimulated by yeast extract. It fermented a wide variety of sugars but produced cellulase only in cellulose or carboxymethyl-cellulose media. Cellulase synthesis not only was probably repressed by 0.4% glucose and 0.3% cellobiose, but also cellulase activity appeared to be inhibited by these sugars at these concentrations. Both C₅ cellulase (degrades native cellulose) and C₆ cellulase (β-1,4-glucanase) activities in strain M7 cultures were assayed by measuring the liberation of reducing sugars with dinitrosalicillic acid. Both activities had optima at pH 6.5 and 67 C. One milliliter of a 48-h culture of strain M7 hydrolyzed 0.044-meq of glucose per min from cotton fibers. The cellulase(s) from strain M7 was extracellular, produced during exponential growth, but was not free in the growth medium until approximately 30% of the cellulose was hydrolyzed. Glucose and cellobiose were the major soluble products liberated from cellulose by the cellulase. ZnCl₂ precipitation appeared initially to be a good method for the concentration of cellulase activity, but subsequent purification was not successful. Isoelectric focusing indicated the presence of four C₆ cellulases (pI 4.5, 6.3, 6.8, and 8.7). The rapid production and high activity of cellulases from this organism strongly support the basic premise that increased hydrolysis of native cellulose is possible at elevated temperature.

The disposal of waste cellulose by anaerobic thermophilic bacteria has many potential advantages. In general, there is a positive correlation between temperature and rate up to the point where denaturation of enzymes becomes limiting. Anaerobic treatment of waste cellulose avoids the necessity for aeration, and in suitable fermentors at high substrate concentrations the process could approach thermal self-sufficiency. As a preliminary step in the investigation of large-scale degradation of cellulose by thermophilic anaerobes the properties of a typical representative of this class bacterium were examined.

MATERIALS AND METHODS

Chemicals. The media constituents and other chemicals were of reagent grade. Microcrystalline cellulose (Cellex-MX) and Bio Gel P-150 were purchased from Bio-Rad Laboratories, carboxymethylcellulose (CMC; 7H 3900/DP PS/0.65 to 0.85) was purchased from Hercules Incorporated, and diethylaminoethyl-Sephadex A-50 was obtained from Pharmacia. Absorbent cotton from Johnson and Johnson was used in C₅ cellulase assays.

Isolation. Strain M7 was representative of a large number of isolates from manure, hay, grass, wood chips, sewage sludge, and compost collected in the Vancouver area. Cellulolytic bacteria were enriched by incubation with filter paper, which was then inoculated into 1% cellulose medium. Dilutions were made into 1% cellulose semisolid tubes and finally into Hungate roll tubes (1). Cellulolytic colonies were further purified by repeated alternate transfer on 0.6% cellobiose and 0.5% cellulose agar plates. The isolates were preserved by storage at −20 C in cellulose medium plus 10% (vol/vol) glycerol and also by freeze drying.

Culture. The growth of strain M7 and other organisms in cellulose enrichment media was assayed by measuring increments in adenosine 5'-triphosphate extracted from the optically dense medium, which often contained organic material from the inoculum. In later experiments growth was determined by measuring increasing protein concentration.

The basal salts medium and anaerobic techniques were those of Blackburn (1). Yeast extract (Difco) at 0.5% was included in all growth media unless otherwise stated. Soluble carbon sources were autoclaved separately at a concentration of 10% before addition to the growth medium. Filter paper medium contained a piece of Whatman no. 1 paper (1 by 5 cm) in 5 ml of growth medium in rubber-stopped tubes (18 by 160 mm). Cellulose medium usually contained 1% microcrystalline cellulose.

Semisolid media, roll tubes, and plates contained 0.7, 1.5, and 2.5% agar, respectively. The reduced agar medium was cooled to 50 C before the plates were poured. The plates were placed in an anaerobic jar (BBL) with palladium pellet catalyst, and the jar
was evacuated and flushed three times with oxygen-free carbon dioxide and hydrogen in a ratio of 90:10. The resazurin in the medium was completely reduced within 20 min. The plates were inoculated under a stream of oxygen-free carbon dioxide and immediately replaced in the jar, reevacuated, flushed with carbon dioxide-hydrogen, and incubated.

Disks of Whatman no. 1 paper were soaked in 0.01% solutions of biotin, thiamine, Casamino Acids hydrolysate (Difco), or 10% glutamine and then placed on the surface of 0.5% cellulose basal agar plates spread with the organism. The plates were examined for zones of growth stimulation after incubation.

All cultures were grown anaerobically at 60 C.

Cellulose assay. The cellulose assay was based on the determination of reducing sugar liberated from cotton or CMC as the substrate. The estimation of reducing sugar was carried out using the dinitrosalicyclic acid reagent (4). One unit of cellulase was defined as the amount of enzyme which would release 1 μeq of glucose from cotton or CMC per min at 60 C.

The reaction mixture for the cellulase assay contained 1 ml of enzyme solution or culture supernatant and the substrates in 1 ml of 0.1 M sodium phosphate buffer, pH 6.5. C. cellulase (β-1,4-glucanase) was assayed using 10 mg of CMC, and C. cellulase (required for C6, to act on native cellulose) was assayed using 50 mg of cotton. After incubation at 60 C for 10 min for C6 and 60 min for C6, the reaction was stopped by the addition of 2 ml of dinitrosalicyclic acid reagent. The tubes were placed in a boiling-water bath for 50 min and then cooled to room temperature. The C6 cellulase assay mixtures were filtered. The optical densities of the C6 filtrates and the C6 assays were measured at 570 nm using a Bausch and Lomb Spectronic 20 colorimeter and converted to glucose equivalents. Cellulase units were read directly from a standard curve because optical density (or glucose equivalents) was directly proportional to only low concentrations of C6 cellulase. In the C6 assay the substrate was in limiting concentration.

Other assays. Adenosine 5'-triphosphate was assayed by the method of Stanley and Williams (22). Protein was extracted in 1% sodium dodecyl sulfate at 100 C for 15 min; it was assayed by the method of Lowry et al. (11). The total cellulose content in the culture was determined by the anthrone method (22) or gravimetrically (6). Bacterial cells adhering to the cellulose reacted to some extent with the anthrone reagent and were weighed as cellulose. The small yield of cells grown anaerobically lessened this as a problem.

Cellulase localization. Cultures of M7 in cellulose medium were centrifuged at 10,000 x g for 15 min, and the supernatant was assayed for extracellular cellulase. The pellet obtained from the above centrifugation was washed once with 0.85% NaCl and twice with 0.05 M sodium phosphate buffer, pH 7.0, and finally the pellet was treated at 4 C at 20,000 cycles for 90 s in 10-s bursts at maximum power in a Biosonik ultrasonic disintegrator (Bionik Scientific, Rochester, N.Y.). The sonicated mixture was centrifuged at 10,000 x g for 15 min to remove residual cellulose. The supernatant was assayed for cellulase activity.

Chromatography. Paper chromatography of the products of cellulose hydrolysis was performed on Whatman no. 1 filter paper using the solvent system n-butanol-pyridine-water (6:4:3) and the spray reagent alkaline AgNO3.

Concentration of cellulase activity. Ammonium sulfate was added at 4 C to 40 ml of culture supernatant containing 202 C6, mU/ml. The precipitate was dissolved in 5.0 ml of 0.05 M sodium phosphate buffer, pH 6.5, and dialyzed against the same buffer. The ammonium sulfate supernatant was also dialyzed, and both were assayed for C6 cellulase activity.

ZnCl2 precipitation was based on the method of Ensign and Wolfe (3). Two milliliters of a 10% ZnCl2 solution was added dropwise with vigorous stirring to 50 ml of a culture supernatant containing 205 C6, mU/ml. The precipitate was collected by centrifugation at 10,000 x g for 15 min and sonicated for 20 s at 20,000 cycles in 10 ml of 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.0, containing 10 mM ethylenediaminetetraacetate. The resulting suspension was centrifuged at 10,000 x g, and the precipitate was again sonicated in 10 ml of 0.1 M citrate buffer, pH 5.0, and centrifuged. C6 cellulase activity in the ethylenediaminetetraacetate and citrate extracts was assayed.

Isoelectric focusing. Thirty milliliters of dialyzed culture supernatant containing 194 mU of C6 cellulase activity and 0.35 mg of protein per ml was added to an LKB-8100 (LKB Produkter A.B., Stockholm, Sweden) electrofocusing column with LKB ampholine carrier ampholytes, giving a pH gradient from pH 3.0 to 10.0 after 1,000 V for 48 h. Ficol (Pharmacia) was used to give a density gradient.

RESULTS

General characteristics of strain M7. Zones of cellulose clearing were observed within 7 days when enrichments from manure were inoculated into cellulose semisol medium (Fig. 1a and b). Some cellulolytic zones were seen after 7 days, surrounding small colonies in high dilution roll tubes (Fig. 1c). Small, colorless, subsurface colonies with good zones of clearing were observed on agar plates after 5 days of incubation (Fig. 2). Disintegration of filter paper in liquid media occurred after 1 to 2 days of incubation and appeared to be associated with the production of a yellow pigment.

The cells stained gram negatively and were straight or slightly curved rods 0.6 by 4.0 μm. Terminal spores were produced on solid but rarely in liquid media. Motility was not observed. The temperature optimum for growth as measured by rate of increase in optical density at 660 nm in a glucose medium incubated in a linear thermal gradient was between 58 and 63 C. The optimum pH was 6.0 to 6.5. No growth occurred aerobically. The mean genera-
tion time ($t_d$) was 35 min in the cellobiose growth medium. Strain M7 grew on the mono-
saccharides L-(+)-arabinose, D-fructose, D-(+)-galactose, D-glucose, D-(+)-mannose, L-(+)-
rhamnose, D-(−)-ribose, and D-xylose; it fer-
mented the disaccharides cellobiose, lactose, maltose, and sucrose, but not melibiose; it fer-
mented the trisaccharides trehalose and raffi-
nose, but not melezitose; it fermented starch,
glycogen, dextrin, inositol, mannitol, sorbitol,
and salicin, but not inulin, dulcitol, and glycerol. Growth occurred in the absence of complex
nutrients but was stimulated by yeast extract,
Casamino Acids, glutamine, biotin, and thia-
mine.

**Rate of extracellular cellulase production.**
The locations of $C_1$ and $C_x$ cellulase activities
are shown in Table 1. No $C_1$ and almost no $C_x$
cellulase activity was associated with strain M7
cells. Almost no cellulase was detected in cell
washings, and the cell-free enzyme was stable
to sonication. $C_1$ and $C_x$ extracellular cellulase
activities were produced at similar rates, being
at a maximum after 3 days of incubation. The
time course of cellulase production is more
clearly seen in Fig. 3. Maximal activities of $C_1$
and $C_x$ were detected after 48 h of incubation
and decreased slightly after further incubation.
There was an initial lag of 28 h before cellulase
activity was detected and before cell protein
reached its doubling time of 120 min. There
was, however, a considerable decrease in cel-
lose during this period.

**Growth conditions and cellulase produc-
tion.** Strain M7 produced $C_x$ cellulase in the
cellobiose basal medium without other supple-
ments. The addition of yeast extract stimulated the growth and production of cellulase activity (Table 2). Maximum cellulase activity was produced in 0.5% yeast extract medium, although the production per cell was higher at 0.2 and 0.3% yeast extract. The addition of tryptose, proteose, peptone, or Casamino Acids gave similar results.

Maximum C₅ cellulase activity was observed in 1.0% cellulose medium after 72 h of incubation (Table 3). A lower concentration of cellulose (0.5%) was almost as good, and higher concentrations gave lower cellulase activities and low pH values.

Although strain M7 grew on a wide range of carbon sources after 3 days, it produced C₅ cellulase activity only on cellulose (89 mU/ml) and CMC (56 mU/ml). The addition of either cellobiose or glucose at 0.05% to the cellulose culture medium resulted in a partial inhibition of C₅ cellulase production; 0.3% cellobiose or 0.4% glucose gave complete inhibition. In these cultures there was also a decrease in cell yield relative to that in the cellulose medium alone.

The presence of Tween 80 in the cellulose medium at concentrations as low as 0.1% resulted in a 73% decrease in C₅ cellulase production and a 63% decrease in cell yield. Growth and cellulase production were inhibited by increasing concentrations of Tween 80 up to 0.6%, where no growth occurred.

Cellulase activity. The optical density (or glucose equivalents) was not directly proportional to C₅ cellulase concentration (Fig. 4). Similar plots were seen when optical densities were plotted against time of incubation, using constant enzyme concentration.

An increase in the CMC concentration in the standard C₅ assay resulted in a considerable

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**Table 1. Localization of cellulase activity**

| Incubation day | Extracellular cellulase activityᵃ (mU/ml) | Cellular cellulase activity (mU/ml) |
|---------------|------------------------------------------|----------------------------------|
|               | C₁  | C₅  | C₁  | C₅  |
| 1             | 4   | 10  | 0   | 0   |
| 2             | 17  | 161 | 0   | 0   |
| 3             | 31  | 272 | 0   | 13  |
| 4             | 28  | 269 | 0   | 0   |
| 5             | 28  | 267 | 0   | 0   |

ᵃ The culture was fractionated as described in Materials and Methods.

**Table 2. Effect of yeast extract on growth and C₅ cellulase production**

| Yeast extract (%) | C₅ activity (mU/ml) | Cell protein (mg/ml) | C₅ units/mg of cell protein |
|------------------|--------------------|----------------------|---------------------------|
| 0                | 34                 | 0.34                 | 0.100                     |
| 0.1              | 53                 | 0.38                 | 0.139                     |
| 0.2              | 81                 | 0.39                 | 0.208                     |
| 0.3              | 89                 | 0.47                 | 0.189                     |
| 0.4              | 109                | 0.81                 | 0.135                     |
| 0.5              | 124                | 0.92                 | 0.135                     |
| 0.6              | 68                 | 0.70                 | 0.097                     |
| 0.7              | 62                 | 0.64                 | 0.097                     |
| 0.8              | 50                 | 0.51                 | 0.098                     |

ᵃ Yeast extract was added to filter paper medium. Incubation was for 3 days. The results are the mean of three determinations.

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**Fig. 3. Rate of cellulase production. Strain M7 was grown in 500 ml of stirred cellulose medium. Samples were taken at 2-h intervals and assayed for cell protein (○), cellulose (●), C₁ activity (■), and C₅ activity (△).**
TABLE 3. Effect of cellulose concentration on growth and C₂ cellulase production

| Conc of cellulose (%) | h  | C₃ activity (mU/ml) | Cell protein (mg/ml) | pH of the supernatant |
|-----------------------|----|---------------------|----------------------|-----------------------|
| 0.5                   | 24 | 30                  | 0.2                  | 6.5                   |
| 1.0                   | 25 | 25                  | 1.8                  | 6.2                   |
| 2.0                   | 18 | 1.8                 | 6.2                  |
| 3.0                   | 17 | 0.4                 | 6.4                  |
| 0.5                   | 168| 2.2                 | 5.9                  |
| 1.0                   | 150| 2.8                 | 5.3                  |
| 2.0                   | 65 | 2.4                 | 5.2                  |
| 3.0                   | 80 | 1.9                 | 5.4                  |
| 0.5                   | 163| 1.6                 | 5.8                  |
| 1.0                   | 183| 2.9                 | 5.2                  |
| 2.0                   | 125| 2.9                 | 4.7                  |
| 3.0                   | 118| 3.6                 | 4.9                  |

* Cellulose was added to the basal medium without yeast extract.

Spots corresponding only to glucose and cellobiose were detected when cell-free cellulase acted on cellulose. In culture supernatants, however, three additional unidentified spots were detected on paper chromatograms. Glucose at 0.4% and cellobiose at 0.3% appeared to completely inhibit the hydrolysis of cellulose (Table 4). The high background in the assay due to sugars made it difficult to detect small concentrations.

Both C₁ and Cₓ cellulase activities were greatest at 67°C (Fig. 6). Cₓ activity decreased more rapidly than C₁ activity at temperatures in excess of 67°C and below 60°C. Very little C₁ or Cₓ activity was demonstrated below 45°C.

Both C₁ and Cₓ activities showed optima at pH 6.5 (Fig. 7). Cₓ activity had a broader pH tolerance than C₁ activity.

Concentration of cellulase activity. Ammonium sulfate at 90% saturation of the culture supernatant resulted in 20% recovery of Cₓ.

![Figure 4](image_url)

**FIG. 4.** Effect of enzyme concentration on cellulase assay. Varying quantities of culture supernatant, containing 0.45 mg of protein per ml, were assayed as described in Materials and Methods. The Cₓ cellulase activity (○) was assayed after 10 min and the C₁ cellulase activity (●) was assayed after 60 min.

![Figure 5](image_url)

**FIG. 5.** Effect of substrate concentration on the rate of CMC hydrolysis. A culture supernatant containing a 0.45 mg of protein per ml was assayed in digests containing different amounts of CMC (Cₓ, C₁ activity).

**TABLE 4.** Effect of glucose and cellobiose on cellulase activity

| % Sugar in assay | % Glucose inhibition | % Cellobiose inhibition |
|-----------------|---------------------|------------------------|
| 0.05            | 0                   | 5                      |
| 0.10            | 0                   | 4                      |
| 0.20            | 0                   | 49                     |
| 0.30            | 38                  | 100                    |
| 0.40            | 100                 | 100                    |

* C₁ cellulase activity was assayed in the presence of glucose or cellobiose. One milliliter of culture supernatant containing 0.45 mg of protein and 8.0 U of C₁ cellulase activity was used. The assays were incubated for 24 h.
The precipitation of C₃ cellulase activity by ZnCl₂ (Table 5) was partially successful, giving only 14 and 20% recoveries with some purification in the tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetae and sodium citrate supernatants, respectively.

Cellulase (130 U of C₁) from 100 ml of culture supernatants could be bound at 4°C to 50 g of crystalline cellulose in 0.05 M sodium phosphate buffer, pH 7.0. Only 15% could be eluted in 100 ml of distilled water and a further 10% by 100 ml of 1 M NaCl in 0.05 M sodium phosphate, pH 7.0.

Partial purification. Attempts to purify C₁ and C₃ cellulase activities from ZnCl₂ precipitates were unsuccessful. The cellulase appeared to bind to dextran as well as to cellulose columns. In the only experiment in which Bio Gel P-150 was used, there was a total loss of activity.

Isoelectric focusing (Fig. 8) was more successful in resolving C₁ cellulase activity into four fractions, with a total recovery in all fractions of 128%. The C₁ cellulase activity whose isoelectric point was at pH 6.8 contained 40% of the starting cellulase activity, with a protein content so low that it could not be measured accurately.

DISCUSSION
Strain M7 was typical of a large number of anaerobic thermophilic cellulolytic bacteria which were isolated from a wide variety of sources. Other isolates differed in the range of sugars fermented, and many did not utilize glucose, but most produced some yellow pigmentation. The cell morphology, terminal spores, and wide fermentative capacity of M7 made it similar to Clostridium thermocelluloseum (2). Strain M7 digested 1% (wt/vol) filter paper completely in 2 days, in contrast to 7 to 14 days noted for other anaerobic thermophiles by McBe (12) and Enebo (2).

Strain M7 grew well on agar plates and was

| Fraction            | Total activity (C₁ units) | Total protein (mg) | Sp act (U/mg) | Purification (fold) | Yield of activity (%) |
|---------------------|---------------------------|--------------------|---------------|---------------------|-----------------------|
| C₁ supernatant      | 10.28                     | 21.0               | 0.49          | 1                   | 100                   |
| ZnCl₂ supernatant   | 2.30                      | 3.75               | 0.62          | 1.3                 | 22                    |
| Tris-EDTA extract²  | 1.46                      | 1.63               | 0.90          | 1.8                 | 14                    |
| Citrate extract      | 2.10                      | 1.25               | 1.68          | 3.4                 | 20                    |

*Tris, Tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetae.
not as sensitive to O₂ as are many rumen anaerobes (10). Considerable care was taken to ensure that the strain was uncontaminated, since cellulose hydrolysis often appears to be more efficiently performed by mixed populations (2, 8). To ensure that non-cellulolytic contaminants were eliminated, the cultures were plated alternately on cellobiose and cellulose media. No contaminants were observed, and the strains lost none of their cellulolytic ability.

The C₁ and C₅ cellulase activities appeared to be completely extracellular and were not associated with the cells. This would explain the large zones of cellulose clearing produced around colonies. Cellulose disappeared before cell-free cellulase could be detected. It is probable that cellulase, when it was first produced, was bound to the cellulose. The binding of cellulase to cellulose was also observed in the purification studies. The binding of cellulase to substrate has been observed by Hungate (10) and Norkans (18).

Strain M7 did not require organic nitrogen additives for growth on cellulose medium, unlike some aerobic, thermophilic bacteria (23). Both growth and cellulase production were stimulated by the addition of yeast extract up to 0.5%, although the highest differential rate of production was at 0.2% yeast extract. High concentrations of cellulose gave high yields of bacteria but apparently lower quantities of cellulase. The latter observation may have been due to adsorption of cellulase to undegraded cellulose. Other cellulolytic microorganisms have been observed to be inhibited by 1.0% cellulose (10, 12, 23), a concentration which was optimal for strain M7. Cellulose and/or CMC was required in the growth medium for cellulase production, although good growth was obtained in other carbohydrates. The role of cellulose as an inducer to fungal cellulases has been demonstrated by many workers, but in addition some oligosaccharides such as cellobiose (15), sophorose (13, 17), and lactose (14) allow for some cellulase production. It has been suggested (9) that cellulase is not induced on easily metabolized carbon sources.

Cellulase was not detected in cellulose media to which relatively high concentrations of cellobiose or glucose were added. It is assumed that this was a repression of cellulase synthesis rather than an inhibition of cellulase function, although similar concentrations of glucose (0.3%) and cellobiose (0.2%) apparently inhibit cellulase action. Gupta and Heale (5) found that the C₅ cellulase production by Verticillium alboatrum in CMC media was inhibited by a large number of sugars. They observed that slow growth rates were related to increased cellulase production.

The addition of Tween 80 (0.1%) inhibited the growth of strain M7 and decreased the yield of cellulase. Strain M7 differs from Trichoderma viride in this respect, as Tween 80 (0.2%) increased the cellulase production of this fungus in a cellobiose medium (20) or in a cellulose medium (19).

The C₁ and C₅ cellulase activities were produced at the same rate, and the ratios remained at approximately 1:7. This could indicate that one enzyme was responsible for both activities.

This speculation is corroborated by the similar temperature and pH profiles of the C₁ and C₅ activities, but is not corroborated by the multiple peaks of C₅ cellulase activity observed in isoelectric focusing.

![Isoelectric focusing](image)

**Fig. 8.** Isoelectric focusing. Two-milliliter fractions were collected, and C₅ cellulase activity (○), the optical density at 280 nm (●), and the pH (solid line) were measured.
CMC 7H was not in excess in the assay mixtures, and maximum velocities of enzyme action were, therefore, not obtained. The C₄ enzyme activities were thus considerably less than the possible maximum. Unfortunately, the fact that the substrate was limiting was not discovered until halfway through the investigation, at which time it was too late to change the assay conditions. CMC 7H was selected as having a relatively low degree of substitution and being more like native cellulose. H gave, however, very viscous solutions and could not be conveniently used at high concentrations. The concentration employed (0.5%) is that usually used in cellulase assays (16). The comparisons of relative amounts of C₃ activities in the experiments in this investigation are, however, quite valid and quantitative since they were taken from a very reproducible calibration curve. It is not possible to compare the C₃ cellulase activity with that of other workers, since our C₃ units were less than maximum and different workers use different CMCs. The rates of hydrolysis of native cellulose in nanomoles per minute per milliliter were 44 for strain M7, 63 for T. viride strain 6a (Table 3; reference 17), and 10 for Thermomonospora curvata (23). Strain M7 thus is one of the more active cellulase producers and is probably the most rapid producer, 2 days compared to at least 6 days for the other organisms quoted. This is consistent with the 1- to 2-day hydrolysis of 1% cellulose in growth media.

Strain M7 cellulase was most active at pH 6.5, unlike many fungal cellulases which have low pH optima (7, 16). Strain M7 cellulase had a high optimum temperature at 67°C, unlike most fungal cellulases but similar to Thermomonospora curvata (23). It resembled the C₄ enzyme of Enebo in its pH optimum (L. Enebo, Ph.D. thesis, Royal Institute of Technology, Stockholm, Sweden, 1964).

Zinc chloride precipitation at first appeared to offer a convenient method for the concentration and partial purification of the cellulase, but in many experiments very low recoveries and poor purification was obtained. C₄ cellulase activity was separated into four fractions by isoelectric focusing. One of the main fractions was considerably purified. Unfortunately, the C₁ activity of these fractions was not assayed, and it is thus not possible to determine whether C₁ and C₄ activities were separated.

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