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Cellulolytic and Physiological Properties of *Clostridium thermocellum*

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Abstract. Three strains of *Clostridium thermocellum* obtained from various sources were found to have nearly identical deoxyribonucleic acid guanosine plus cytosine contents that ranged from 38.1 – 39.5 mole-%. All strains examined fermented only cellulose and cellulose derivatives, but not glucose, or xylose or other sugars. The principal cellulose fermentation products were ethanol, lactate, acetate, hydrogen and carbon dioxide. Growth of *C. thermocellum* on cellulose resulted in the production of extracellular cellulase that was non-oxygen labile, was thermally stable at 70° C for 45 min and adsorbed strongly on cellulose. Production of cellulase during fermentation correlated linearly with growth and cellulose degradation. Both the yield and specific activity of crude cellulase varied considerably with the specific growth substrates. Highest cellulase yield was obtained when grown on native cellulose, α-cellulose and low degree of polymerization cellulose but not carboxymethylcellulose or other carbohydrate sources. Cellulase activity was not detected when cells were grown on cellobiose. Crude extracellular protein preparations lacked proteolytic and cellobiase activity. The pH and temperature optima for endoglucanase activity were 5.2 and 65° C, respectively, while that of the exoglucanase activity were 5.4 and 64° C, respectively. The specific activity at 60° C for exoglucanase and endoglucanase of crude cellulase obtained from cells grown on cellulose (MN300) was 3.6 μmoles reducing sugar equivalents released per h (unit)/mg of protein and 1.5 μmole reducing sugar equivalent released per min (unit)/mg of protein, respectively. The yield of endoglucanase was 125 units per g of cellulose MN300 degraded and that of exoglucanase was 300 units per g of cellulose MN300 degraded. Glucose and cellobiose were the hydrolytic end products of crude cellulase action on cellulose, cellotetraose and cellotriose in vitro.

Key words: Thermophilic – Anaerobic cellulolytic bacteria – *Clostridium thermocellum* – Cellular growth properties – Cellulose degradation – Cellulase (exoglucanase and endoglucanase) production – Cellulase properties.

Cellulose is the most abundant natural biopolymer and comprises the majority of solid waste material in the United States. The utilization of cellulose as a chemical and energy source has been extensively reviewed (Wilke, 1975). However, studies on microbial degradation of cellulosic material have mainly been confined to mesophilic fungi and bacteria. Evidence suggests that cellulolysis is very active among thermophiles (Cooney and Ackerman, 1975; Cooney and Wise, 1975; Romanelli et al., 1975; Stutzenberger et al., 1970).

Thermophilic, anaerobic, cellulolytic bacteria have been isolated by McBee (1948, 1950), Enebo and Lundin (1951), and Lee and Blackburn (1975), although their cellulolytic properties remain ill-defined. Many of the strains are similar to *Clostridium thermocellum* of McBee (1950). Certain physiological features of *C. thermocellum* have been a source of conflicting reports. McBee (1948, 1950) was unable to grow *C. thermocellum* on glucose while Patni and Alexander (1971 a, b) obtained good growth on glucose and xylose, and also demonstrated the presence of glycolytic enzymes.

Recently, Lee and Blackburn (1975) described the cellulolytic properties of an isolate similar to *Clostridium thermocellulaseum* that proliferated on cellulose, glucose and numerous other mono- and disaccharides. We describe here, cellular growth and cellulolytic features of several strains of *C. thermocellum* that grow on cellulose (β1,4 glucan) and its derivatives but not on glucose or other sugars. The crude cellulase of *C. thermocellum* has activity comparable to that described for fungal cellulase (Bailey et al., 1975); however, the mode of action differs significantly from that of fungal systems.

MATERIALS AND METHODS

Organisms. Clostridium thermocellum strain LQ8 was provided by L. Y. Quinn, Iowa State University, Ames, Iowa. Strains N1 and H1 were respectively obtained by enrichment and isolation from Shatin, Hong Kong sewage digestor sludge and effluent of the University of Wisconsin Charmany Farms thermophilic dairy manure digestor. Enrichment cultures were initiated by inoculation of 1 ml of digestor contents into anaerobic culture tubes of CM3 medium. Pure cultures were obtained by repeated transfer in CM3 medium followed by agar dilution in the same medium that contained 4% agar (Difco, purified). Well isolated colonies were picked and transferred to liquid medium. Cultures were routinely incubated at 60° C without shaking.

Media. Cells were routinely grown on CM3 medium as described by Weimer and Zeikus (1977). CM medium was identical to CM3 medium except MN300 cellulose was replaced by other carbohydrate sources as indicated. The carbohydrate solutions were autoclaved and added separately.

Anaerobic Culture Methods. The anaerobic culture technique of Hungate (1969) as modified by Bryant (1972) was used throughout this investigation. Anaerobic culture tubes used for growth studies were 18 × 142 mm (Belcco), and 25 × 142 mm tubes (Bellco) were used for isolation of organisms. All test tubes contained 10 ml of medium. Culture tubes were gassed with N_2 and sealed with a neoprene stopper. Larger quantities of cells and culture supernatant were obtained from fermentor cultures that contained, 2 l, 8 l, or 12 l of medium.

Cellulosic Substrates. The following substrates were used: cellulose MN300 (Macherey, Nagel & Co., 5160 Düren, Germany), ashless powdered cellulose, Whatman No. 1 (W. & R. Balston, Ltd., London), α-cellulose fiber (Sigma), carboxymethylcellulose sodium salt or CMC-Na (Sigma), carboxylmethylcellulose 7HS (Hercules Incorporated, Delaware). Avicel microcrystalline cellulose (American Viscose Corporation, Marcus Hook, Pennsylvania), low degree of polymerization cellulose prepared as described below, 4-O-β-D-glucanopyranosyl-D-glucose (Cellobiose) from Sigma and ^{14}C-cellulose UL (ICN Pharmaceuticals, Inc., Cleveland, Ohio).

Measurement of Growth and Fermentation Products. Growth, residual cellulase and fermentation products were measured by the methods described by Weimer and Zeikus (1977).

DNA Base Composition Analysis. DNA was isolated from 6 g of frozen cells that were lysed by treatment in 100 ml of 0.05 M Tris-(hydroxylmethyl)-aminomethane and 0.02 M ethylene diaminetetraacetic acid buffer pH 8.0 that contained 0.1% sodium dodecyl sulfate and 150 mg of pronase (Sigma) for 75 min at 60° C. DNA was then purified by the method of Marmur (1961). Mole percent guanosine plus cytosine content (% G + C) was determined by the thermal denaturation method of DeLey (1970), using a Gilford-240 Spectrophotometer equipped with a Gilford 2527 Thermoprogrammer. *Escherichia coli* DNA (Sigma) served as a standard.

Protein Determination. Total protein was measured by the method of Lowry et al. (1951) using bovine serum albumin (Sigma) as standard.

Assay for Proteolytic Activity. Proteolytic activity was determined by incubating 1 ml of bovine serum albumin (10 mg/ml) with 1 ml of 10-fold concentrated extracellular protein (prepared as described below) at 60° C for 1 h. Release of amino acids was measured by the ninhydrin method of Moore and Stein (1954).

Determination of Cellodextrins and Glucose. Cellodextrins and glucose were assayed as follows:

a) Thin layer chromatography (TLC): Silica gel plates (20 × 20 cm from EM Reagents) with a 0.25 mm layer thickness were used. Chromatograms were developed at 25° C in isopropanol/ethyl acetate (9:7:4, v/v). For cellobiose and glucose, a formic acid/ethyl acetate/acetone (2:1:1, v/v) solvent was also used. After good resolution was achieved, the plates were air-dried and sprayed with aniline phthalate. Color was developed by heating at 110° C for 15 min. Unreacted sugars located by standards were extracted and quantified by the method of Millett et al. (1964). ^{14}C radioactivity was determined by mixing the samples (100 µl – 1 ml) with 9 ml Liquid Scintillator-Aquasol (New England Nuclear) and counted with a Packard Tri-Carb 3375 Liquid Scintillation Spectrometer.

b) Liquid chromatography: Samples of cellodextrins and glucose were applied to a Bio-Gel P-2, 200 – 400 mesh column (15 × 130 mm) and were eluted with glass distilled water at a flow rate of 16 ml/h. The sugars were monitored by differential refractometer and quantified by integration of the corresponding peaks.

Determination of Crystallinity Index (CI). CI of cellulose were determined by the Wood Chemistry Analytical Lab. of the U.S.D.A. Forest Products Laboratory, Madison, Wisconsin by x-ray diffraction.

Preparation of Cellodextrins. Cellotetraose and cellotriose were prepared according to the method of Feather and Harris (1967). LP-cellulose, a cellulose with a low degree of polymerization of approximately 37 was prepared by the procedure of Kaustinen et al. (1969). The reaction time was 6 h at 55° C with perchloric acid as catalyst.

Analysis for Cellulolytic Activity. Crude extracellular protein was prepared by centrifuging strain N1 culture solution at 12000 × g for 30 min. The supernatant was concentrated 10-fold by ultrafiltration with a PM-10 membrane (Amicon) if necessary.

β-1,4-Glucan cellobiohydrolase or exo-glucanase (EC 3.2.1.74) activity was determined by incubating 1 ml of the crude extracellular protein with 2 mg of Avicel cellulose in 1 ml of 0.05 M ammonium acetate buffer, pH 5.0, at 60° C for 2 h. The reaction mixture was then filtered through a 0.45 µm membrane filter (Millipore). The amount of reducing sugar present in the filtrate was determined by the method of Nelson (1944) and Somogyi (1952) with glucose as a standard. One unit of exo-glucanase is expressed as 1 µmole of reducing sugar equivalents released per hour.

β-1,4-Glucan glucanohydrolase or endo-glucanase (EC 3.2.1.4) activity was determined colorimetrically by assaying reducing sugar equivalents released from carboxyethylcellulose (Sigma). Crude extracellular protein (10 – 200 µl) was incubated with 1 ml of 2% CMC solution at 60° C for 30 min. The total reaction volume was brought to 2 ml by the addition of 0.05 M ammonium acetate buffer pH 5.0. The reducing sugars were assayed by the method of Miller et al. (1960) with glucose as a standard. One unit of endo-glucanase is defined as 1 µmole of reducing sugar equivalents released per min. The term "cellulase" used here is referred to as both exo- and endo-glucanase activity.

β-Glucosidase or cellobiase (EC 3.2.1.21) activity was determined by using both cellobiose and p-nitrophenyl-β-D-glucoside (Aldrich) as substrates. Crude extracellular protein was dialyzed in a collodion bag (Schleicher & Schuell, Inc.) at 4° C for 24 h against 50 ml of 0.05 M ammonium acetate buffer, pH 5.0. The dialyzed protein (1 ml) was incubated with 4 mg of cellobiose in 2 ml of 0.2 M sodium citrate buffer pH 6.0 for 2 h. Glucose released was assayed by TLC as described above. Activity toward p-nitrophenyl-β-D-glucoside was determined by the method of Norkrans (1957) as modified by Berghem and Petterson (1974). β-Glucosidase (Sigma) was used as a control.

Hydrolysis of Cellulose and Cellodextrins. Avicel (10 mg) was suspended in 5 ml of 0.05 M ammonium acetate buffer, pH 5.0, and

incubated with 1 ml of 10-fold concentrated crude cellulase preparation (4.3 mg protein/ml) for 24 h at 60° C. The solution was filtered through a 0.45 μm membrane filter. Hydrolysis of ^{14}C-cellulose UL was similar as that described for Avicel except that 10 μl of ^{14}C cellulose (1.0×10^6 dpm/ml) was added in addition to 10 mg of cold Avicel cellulose.

Degradation of cellodextrins were carried by incubating 1 ml of cellodextrin solution (2 mg/ml) in 0.05 M ammonium acetate buffer pH 5.0 with 1 ml of crude extracellular protein (0.52 mg protein/ml) at 60° C for 4 h. All reaction mixtures were concentrated by vacuum evaporation at 4° C prior to analysis of hydrolytic products.

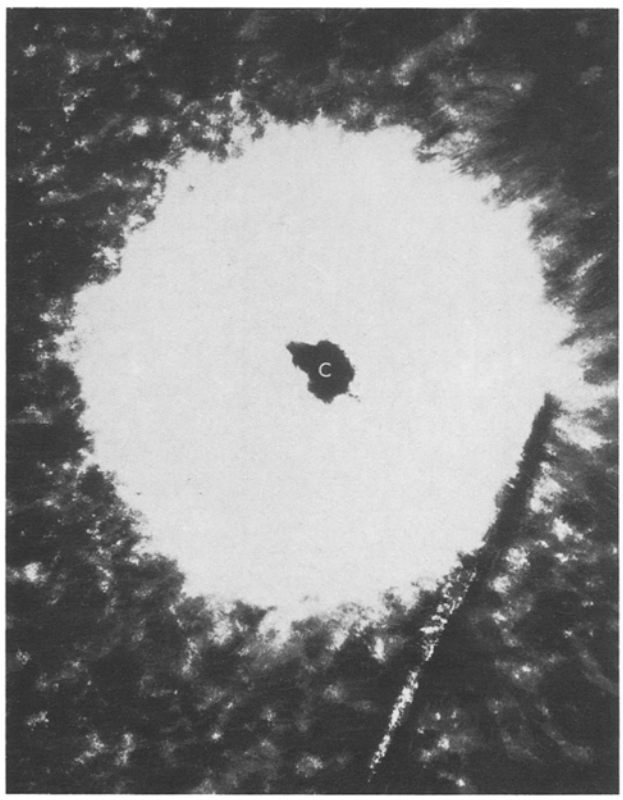
Adsorption of Cellulase to Cellulose. The affinity of cellulase for cellulose was determined by incubating 40 ml of crude cellulase (0.50 mg protein/ml) with varying amounts of Whatman No. 1 cellulose for 1 h at 4° C. These suspensions were then centrifuged twice at 10000 × g for 15 min to remove the residual cellulose. The supernatant absorbance was monitored at 280 nm and cellulase activity measured.

Determinations of Cellulase Physico-Chemical Properties. The effect of oxygen on enzyme activity was determined by incubating the crude extracellular protein under various concentration of oxygen at 4° C for 24 h. The exoglucanase and endoglucanase activities were then measured. The pH optima of the crude cellulase was determined by measuring the endoglucanase and exoglucanase activities at various pH values. Endoglucanase was assayed in 0.1 M citrate buffer for the pH range of 3.0 – 5.0 and in 0.1 M phosphate buffer for the pH range of 5.0 – 7.0. Universal buffer solutions (Britton and Robinson type) from pH 2.6 – 10.0 were used for assessment of exoglucanase (Dawson et al., 1972). All pH values were measured at 60° C. The terminal stability of endoglucanase and exoglucanase was determined by preincubation of the crude cellulase for 15, 30, and 45 min at the specified temperature prior to measurement of the cellulolytic activity.

RESULTS

General Cellular and Growth Characteristics

Strain LQ8, N1 and H1 displayed nearly identical cellular and growth characteristics. Deep colonies of all strains in CM3 agar roll tubes were tannish-yellow, roughly round, and filamentous. All three strains produced an active extracellular cellulase which was evident by complete solubilization of cellulose around colonies (Fig. 1 A). When grown on cellulose, all strains produced a yellow pigment that was not evident during growth on cellobiose. Shaking of cultures decreased growth on cellulose but had no effect when cells were grown on cellobiose. Microscopic examination suggested that the organism associated tightly with cellulose fibers (Fig. 1 B).



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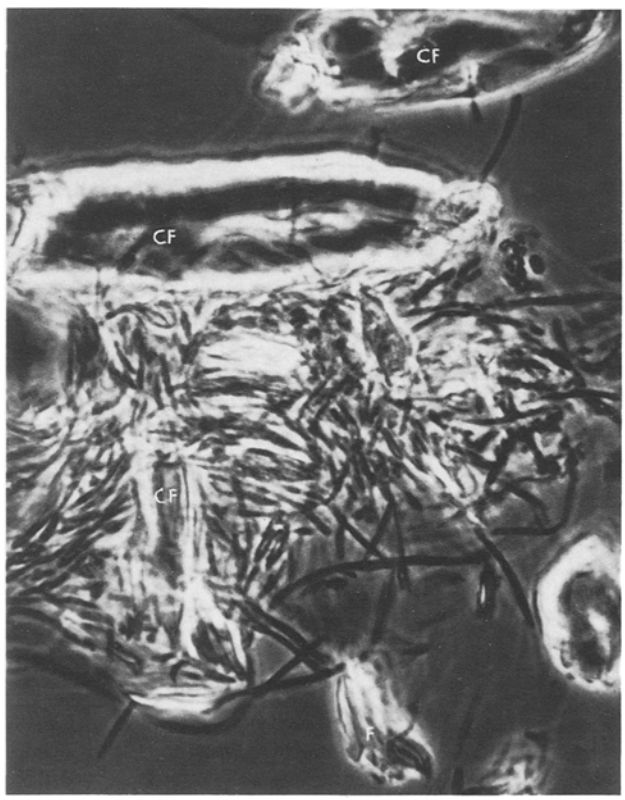

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Fig. 1 A and B. Association of *Clostridium thermocellum* and cellulose during growth. (A) Phase-photomicrograph (final magnification 81 ×) showing solubilization of crystalline cellulose (*clear circular area*) around a mature colony (*C*) of *C. thermocellum* strain LQ8 grown on cellulose agar. (B) Phase photomicrograph (final magnification 5184 ×) illustrating the binding of *C. thermocellum* (*long rods*) to crystalline cellulase fibers (*CF*) during growth in liquid medium

All three strains were obligately anaerobic, formed terminal oval spores and were Gram-negative rods of 0.5 – 0.6 μm by 4 – 5 μm. The primary fermentation products of strains N1 and H1 grown on cellulose were H_2, CO_2, ethanol, acetic and lactic acid as previously reported for *Clostridium thermocellum* strain LQ8 by Weimer and Zeikus (1977). No cellodextrins or glucose were detected during growth of the three strains on cellulose. The DNA base composition (% G + C) were 38.1, 39.5, and 38.8 for strains N1, LQ8, and H1, respectively.

C. thermocellum was examined for the ability to ferment various sugars by monitoring H_2 and CO_2 formation and growth after inoculation of culture tubes. All strains had identical metabolic response and fermented cellulose and its derivatives but not glucose, xylose, sucrose or trehalose. The optimum temperature for growth was 60 – 64° C. The rate of growth of *C. thermocellum* was compared on cellulose and cellobiose (Table 1). Essentially the same amount of growth was obtained from equal amounts of cellobiose and cellulose. The degradability of three different commercial cellulose by *C. thermocellum* is shown in Table 2. Despite significant differences in crystallinity index, the three cellulosic substrates were fermented to approximately the same extent. Growth and complete degradation of cellulose also occurred with Avicel microcrystalline cellulose as substrate.

Production of Cellulase during Growth

The correlation between rate of cellulase production, rate of cellulose degradation and growth rate was determined. Five 10 ml samples were sequentially withdrawn at 16 h intervals from a 2 l *C. thermocellum* strain LQ8 culture grown at 60° C. The samples were assayed for endoglucanase, exoglucanase and proteolytic activity. Optical density at 525 nm, total protein and total residual cellulose were subsequently determined. Production of cellulase increased progressively during exponential growth and correlated linearly with the rate of cellulose degradation, as shown in Figure 2. The rate of production of endo- and exoglucanase reached its maximum at approximately 56 h after inoculation. At this time, cellulose degradation was most rapid and cells were in the exponential phase of growth. The specific activities of endo- and exoglucanase remained relatively constant throughout growth. No proteolytic activity was detected in the culture solution at any time.

Production of Cellulase from Different Cellulosic Substrates

In order to determine the most suitable cellulosic substrates for the production of cellulase, *C. thermocellum* strain N1 was grown in CM medium that contained 0.7% of the cellulosic substrates. The inoculum was grown on cellobiose. Fifty ml samples were withdrawn from each culture at early stationary

Table 1. Growth of *Clostridium thermocellum* on cellulose and cellobiose[a]

Strain	Substrate	OD (525 nm)		Doubling time (h)
		Initial	Final[b]	
LQ8	Cellulose	0.10	1.17	7.2
	Cellobiose	0.08	1.21	2.4
N1	Cellulose	0.10	1.20	6.8
	Cellobiose	0.10	1.15	2.1
H1	Cellulose	0.08	1.30	6.7
	Cellobiose	0.10	1.32	2.1

[a] Concentrations of cellulose and cellobiose were 0.7 and 0.68%, respectively. Experiments were carried out in anaerobic culture tubes at 60° C without shaking

[b] Readings were taken after 60 and 30 h for cellulose and cellobiose, respectively

Table 2. Effect of crystallinity index (CI) on cellulose degradation[a]

Cellulosic substrate	CI (%)	Gas production (μmoles) in gas phase	
		H_2	CO_2
MN300	58	82.0	103.5
α-Cellulose	67	72.4	87.8
Whatman No. 1	79	82.2	107.5

[a] Degradability of different cellulose preparations was measured by the amount of gas produced by *C. thermocellum* strain LQ8 after 156 h incubation at 60° C. Similar results were obtained for strains N1 and H1

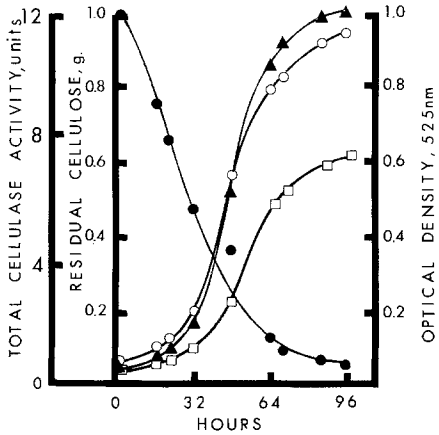
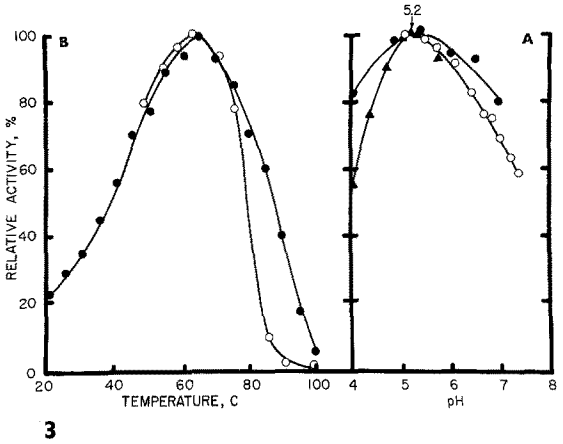


Fig. 2. Production of cellulase during growth. Sequential samples were withdrawn from a culture grown on MN300 cellulose and were assayed for exoglucanase activity (○——○), endoglucanase activity (□——□), OD_{525} (▲——▲), and residual cellulose (●——●)

Table 3. Production of cellulase from different substrates[a]

Substrates	Final cultures optical density (525 nm)	Endoglucanase		Exoglucanase	
		specific activity (units/mg)	yield (units)	specific activity (units/mg)	yield (units)
LP-cellulose	1.31	1.54	32.9	3.67	79.3
Whatman No. 1	1.1	0.96	26.9	2.14	59.5
MN300	1.0	1.52	25.1	3.62	59.7
α-Cellulose	0.9	1.21	15.7	2.66	34.6
CMS-7HS	0.3	1.60	13.1	3.36	27.6
Cellobiose	1.2	0	0	0	0

[a] Samples of culture supernatant from *C. thermocellum* strain N1 grown on different substrates at 60° C were assayed for the different parameters shown. Initial OD_{525} was 0.08



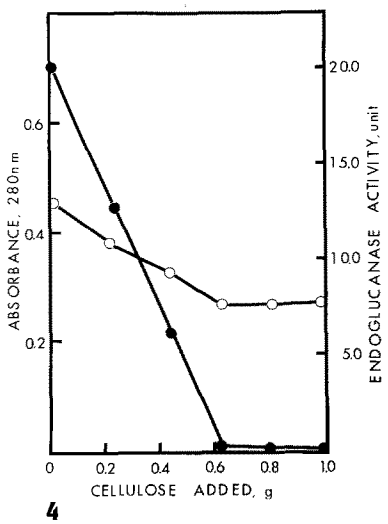


Fig. 3 A and B. Effect of pH and temperature on cellulase activity. (A) Exoglucanase activity in Universal buffer (●——●), and endoglucanase activity in citrate buffer (▲——▲) and phosphate buffer (○——○) were assayed and pH values determined at 60° C. 100% values of exoglucanase activity and endoglucanase activity correspond to 1.9 and 0.05 units, respectively. (B) The values for 100% activity of exoglucanase (○——○) and endoglucanase (●——●) were 2.1 and 0.06 units, respectively

Fig. 4. Affinity of endoglucanase for cellulose. Samples of crude cellulase (20 mg protein/40 ml) were reacted with varying amounts of Whatman No. 1 cellulose for 2 h at 4° C. Absorbance at 280 nm (○——○) and endoglucanase activity (●——●) were measured from the supernatant of the reaction mixture

phase. The supernatant was assayed for total and specific endoglucanase and exoglucanase activities. The results of the total yields and specific activities obtained from different substrates are given in Table 3. The yield of endoglucanase and exoglucanase was highest when grown on LP-cellulose. Specific activities of the enzymes obtained from growth on α-cellulose and Whatman No. 1 cellulose were relatively lower than those from other cellulosic substrates. No cellulase activity was detected when grown on cellobiose. Larger quantities of cellulase were produced by growing *C. thermocellum* strain N1 in 14 l fermentor cultures with CM3 medium. The fermentation was maintained at 60° C for 4 days without agitation. A mean yield of 125 units of endoglucanase and 300 units of exoglucanase activity was obtained per g of cellulose MN300 degraded.

General Properties of the Crude Cellulase

Both endo- and exoglucanase activities were independent of oxygen tension within a range of 0–100% of the gas phase at ambient pressure. As shown in Figure 3 A, the pH optima for endo- and exoglucanase were 5.2 and 5.4, respectively. The exoglucanase displayed a broader pH optimum than did the endoglucanase.

Figure 3 B illustrates that the temperature optima for endo- and exoglucanase activity were 65 and 62° C, respectively. Both enzymes remained thermally stable at 70° C for 45 min, but were completely denatured at 90° C after 10 min. The results were similar for preincubation times of 15 and 30 min. The endoglucanase displayed a strong affinity for its substrate (Fig. 4). Incubation of the crude cellulase with 600 mg of

Table 4. Effect of substrates on hydrolytic products of *C. thermocellum* cellulase

Substrate	Product ratio (w/w)		
	Cellotriose	Cellobiose	Glucose
Cellulose MN300	–	1	2
^{14}C-cellulose	–	1	2
Cellotetraose	1	4	4
Cellotriose	–	1	1
Cellobiose	–	+	–

– = not detected; + = detected but not quantified

cellulose resulted in approximately a 60% decrease in absorbance at 280 nm and a total disappearance of endoglucanase activity. Exoglucanase displayed a similar affinity for cellulose. The adsorbed protein was not removed by repeated washing with 0.05 M ammonium acetate buffer, pH 5.0, but 75% and 92% of the cellulolytic activity recovered by elution with 0.1 M Tris-HCl pH 9.0 and 40% glycerol in 0.1 M ammonium acetate buffer, pH 5.0 respectively.

The exo- and endoglucanase from the crude culture solution had specific activities of 3.6 and 1.5 units/mg, protein, respectively. No activity toward cellobiose or p-nitrophenyl β-D-glucoside was detected.

End Hydrolytic Products of Crude Cellulase

In order to gain an insight on the mode of cellulase action, various celluloses and cellodextrins were subjected to hydrolysis by the crude cellulase. The resultant hydrolytic products were analyzed (Table 4). Glucose was not released from attack on cellobiose. Equal amounts of glucose and cellobiose were detected when cellotriose and cellotetraose were used as substrates. Prolonged enzymatic hydrolysis of cellulose released twice the amount of glucose as cellobiose.

DISCUSSION

The three strains of *C. thermocellum* examined here display marked similarities in morphology, DNA base composition and physiology, particularly their inability to utilize glucose and other hexose sugars. These properties suggest that all strains are the same species, namely *Clostridium thermocellum* (Viljoen et al., 1962). The DNA base composition of other strains of *Clostridium thermocellum* or other thermophilic, cellulolytic clostridia have not been reported. Other thermophilic anaerobic, cellulolytic clostridia that resemble *C. thermocellum* in morphology but are able to ferment hexoses (Lee and Blackburn, 1975) may be different species or mixed cultures. Unsuspected contamination which results in the ability to ferment glucose is frequently encountered during the maintenance of pure cultures of *C. thermocellum* (McBee, 1950).

Growth of *C. thermocellum* on a soluble substrate such as cellobiose was more rapid than growth on an insoluble substrate, i.e. cellulose, indicating that the solubilization of cellulose is a rate limiting step for growth. Equivalent growth with cellulose or cellobiose indicates that cellulase production does not require large amounts of cellular ATP. Production of cellulase was at its maximum during the exponential phase of growth. Maximal endo- and exoglucanase activities were present at early stationary phase and proteolytic activity was absent from the culture during growth. Thus, the cellulase is truly extracellular and is not released by autolysis of the cells. The cellulase is also very stable. The specific activities of both endoglucanase and exoglucanase remained constant throughout the course of fermentation.

Cellulase is not a constitutive enzyme because no cellulase activity could be detected during growth on cellobiose. It has not yet been determined if cellobiose represses cellulase. Cellulase production might be induced by short chain cellulose molecules. Cellulose with low degree of polymerization gave the best yield of cellulase compared to native cellulose. Cellulose M300 was chosen as the substrate for enzyme production because of the limited availability of LP-cellulose. Although carboxymethyl cellulose induced cellulase, the yield was low because it did not support active growth. In this respect, CMC resembles a gratituous inducer for cellulase.

The exoglucanase yield is relatively low in *C. thermocellum*. It is possible that a cellulose solubilizing factor associated with the membrane or cell wall is involved since degradation of cellulose was interrupted by agitation of the culture, and the cellulase had an absolute affinity for cellulose. The current assay for exoglucanase activity only measures the number of short chain cellulose molecules released and gives no indication of their sizes and distribution; thus, it is not possible to account for the total exoglucanase activity expected. On the other hand, the endoglucanase is very active. Endoglucanase activity assayed in vitro can account for the complete degradation of cellulose in vivo, provided that complete decrystallization of cellulose into long chain β-glucan molecules occurred. Judging from the non-specificity of the types and ratios of cellodextrins released upon hydrolysis of various substrates in vitro, the endoglucanase acts randomly over the linear cellulose molecule. Glucose which appears as a hydrolytic product is probably a secondary breakdown product from cellodextrins released initially. Unlike cellulase systems in other organisms (Reese, 1956), *C. thermo-*

cellum does not have an extracellular cellobiase. This suggests that cellodextrins are readily transported into cells before being further degraded by the cellulase. This proposal is supported by the demonstration of intracellular cellodextrin phosphorylase in *C. thermocellum* strain 651 by Alexander (1972a, b).

Anaerobic digestion of solid waste has been shown to proceed more readily at thermophilic than at mesophilic temperatures (Cooney and Ackerman, 1975; Cooney and Wise, 1975; Pfeffer, 1974). *C. thermocellum* was the most predominant cellulolytic organism in the thermophilic dairy manure digestor examined here. In pure culture, *C. thermocellum* ferments cellulose vigorously, but the rate of fermentation is more rapid in mixed culture (Enebo and Lundin, 1951; McBee, 1950) indicating that interactions among several microbial species probably control the rate of cellulolysis in complex systems. Recently, Weimer and Zeikus (1977) have demonstrated a growth rate dependent metabolic interaction between *C. thermocellum* and *Methanobacterium thermoautotrophicum* co-cultured on cellulose.

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