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Studies on Cellulase Production by *Clostridium thermocellum*

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Summary. *Clostridium thermocellum* ATCC 27405 (and its improved cellulase-producing mutant, AS-39) is an anaerobic thermophile that produces endo-β-glucanase and exo-β-glucanase when grown on cellobiose or cellulose as major carbon source (Shinmyo et al. 1979). The site of cellulase accumulation was at least 95% extracellular. Optimum conditions for endo-β-glucanase production in flasks included 1% (w/v) cellobiose, 0.2% (w/v) urea as a nitrogen source, 0.1 M morpholinopropane-sulfonic acid buffer, an initial pH of 7.4, and a yeast extract concentration of 0.6% (w/v). An improved medium (GS medium) was devised for future studies. Xylan was degraded by an extracellular enzyme (s) produced during cultivation on cellobiose, although *C. thermocellum* does not grow on xylan.

McBee (1950) extensively studied the anaerobic cellulolytic bacteria isolated up to 1950. He concluded that the organisms previously isolated as thermophilic anaerobic bacteria all belonged to *Clostridium thermocellum*. Later studies by Weimer and Zeikus (1977) and Ng et al. (1977) on other *C. thermocellum* strains essentially confirmed McBee's observations, except his claim that the organism was able to utilize xylose as carbon source.

A major stimulus for the fermentative utilization of this particular microorganism for the breakdown of cellulose is the fact that the main cellulose hydrolysis products (cellobiose and glucose) do not inhibit the production of cellulase by *C. thermocellum*; i.e., the enzyme appears to be constitutive (Shinmyo et al. 1979). Its thermophilic and anaerobic nature further contributes to making *C. thermocellum* a potentially rewarding

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subject for study and exploitation. Ultimately, optimization of its growth and enzyme production will result in more efficient cellulose hydrolysis. Efforts in this direction are described in this report.

Materials and Methods

Microorganisms. C. thermocellum ATCC 27405 was obtained from the American Type Culture Collection. A cellulase-overproducing mutant strain, AS-39 (Shinmyo et al., 1979), isolated after UV irradiation of ATCC 27405, was also used in this investigation.

Culture Medium. The composition of the original culture medium was slightly modified from the CM3 medium of Weimer and Zeikus (1977) as follows (g/l): KH_2PO_4, 1.5; K_2HPO_4, 2.9; $(NH_4)_2SO_4$, 1.3; $MgCl_2 \cdot 6H_2O$, 1.0; $CaCl_2 \cdot 2H_2O$, 0.15; yeast extract (Difco Laboratories, Detroit, MI), 2.0; cysteine hydrochloride, 1.0; $FeSO_4 \cdot 7H_2O$, 1.25 mg/l; and 1 ml of a 2% resazurin (Kodak, Rochester, NY) aqueous solution. The cellobiose (Calbiochem, La Jolla, CA) concentration was 1% unless otherwise specified. The pH was adjusted with NaOH to 7.0 before sterilization. For plating, 1.75% agar was added to the medium.

Cultivation. The microorganisms were cultivated in Hungate tubes (Bellco, Vineland, NJ), in anaerobic flasks as described by Daniels and Zeikus (1975), and in petri dishes placed in an anaerobic jar (BBL, Cockeysville, MD) using Gas-paks (BBL) for anaerobiosis. The cultivation temperature was 60°C.

When soluble carbon sources were tested using Hungate tubes (working volume 10 ml), the medium was autoclaved in three portions: a 10% carbon source solution, a 1% cysteine solution at pH 7.0, and a solution containing the rest of the components suspended in 80% of the final volume (pH 7.0). After sterilization, 1 ml of carbon source and 1 ml of cysteine solution were added to 8 ml of the rest of the medium. The tubes were preincubated for 10 to 20 min at 60°C until the indicator showed only small amounts of dissolved oxygen. When 4 ml of log phase inoculum per 100 ml of medium were used, bubbling with nitrogen was unnecessary if cysteine was added just before inoculation, as described above. In the case of insoluble carbon sources (e.g., xylan), the polymer was not autoclaved separately.

In flask experiments, nitorgen gas was bubbled into the medium at the start of a fermentation to eliminate residual dissolved oxygen and to maintain anaerobic conditions during sampling.

For auxonographic tests in plates, sterile medium without carbohydrate and supplemented with agar was seeded with *C. thermocellum* just after the addition of the cysteine solution; it was then poured into the petri dishes. After gel formation, a sterile carbon source was added with a spatula to one portion of the plate, and the plates were incubated in anaerobic jars. For experiments involving confluent growth on plates, the carbon source was incorportated into the agar before gel formation.

Stock Cultures. C. thermocellum was maintained in liquid medium at 4°C in Hungate tubes. To prepare the stock culture, a cell population was grown for 24 h on cellobiose. Cultures stored as long as 2 months could be used successfully.

Growth Estimations. Growth of *C. thermocellum* was estimated by absorbance measurements in a Klett-Summerson (New York, NY) colorimeter (red filter) when cellobiose was used as carbon source. Three hundred Klett units correspond to 1 mg/ml dry cell weight.

Assay of Enzyme Activities. Endo-β-glucanase (β-1, 4-glucan-glucanohydrolase, E.C. 3.2.1.4) was measured in two ways; details of both methods are given by Shinmyo et al. (1979). The method most often employed was the chromogenic assay (Huang and Tang 1976) using trinitrophenyl-carboxymethylcellulose (TNP-CMC); the liberation of soluble yellow color was measured at 344 nm. Culture supernatants were easily assayed by this method since sugars do not interfere. Occasionally, we used carboxymethylcellulose (CMC) as substrate and measured liberation of reducing sugar by the dinitrosalicylic acid method (Miller 1959). Sugar interferes in this assay and thus only ethanol-precipitated enzyme was used in the CMCase assay. One unit of TNP-CMCase activity liberates 0.01 absorbance unit per min, whereas one unit of CMCase liberates 1.0 μmole of reducing sugar (measured as glucose) per min. Xylanase activity was estimated on 2% xylan (Nutritional Biochemical Corp., Cleveland, OH) suspensions under similar conditions for the CMCase determination. For sugar determination after the enzymatic reaction, the polymer was eliminated by filtration (Millipore membrane HAW-PO2500, 0.45 μm pore diameter; Millipore Corp., Bedford, MA).

Ethanol Precipitation of Broths for Enzyme Determinations. Culture broth was centrifuged at 7500 x g for 10 min to remove the cells and, if present, polymeric carbon source. The supernatant fluid was cooled in an ice bath, and cold ethanol was poured into it very slowly; the mixture was continuously mixed by manual shaking. The final ethanol concentration was 70% (v/v). The mixture was incubated overnight without shaking at 4°C. Precipitate was separated by centrifugation at 7500 x g for 12 min. at 4°C. The supernatant fluid was removed and the pellet resuspended in 0.05 M citrate buffer, pH 4.5. These preparations were practically free of sugar and both CMCase and xylanase activities could be performed without interference.

Results

Extracellular Nature of Endo-β-glucanase. Although the cellulase of *C. thermocellum* is considered to be an extracellular enzyme (Weimer and Zeikus 1977), the relative amount inside or bound to cells has not been investigated. Gordon et al. (1978) reported that an amount equal to 35% of the CMCase found extracellularly could be liberated from particulate material of a cellulose-grown culture by 2% Triton. However, this amount represents the sum of cellular cellulase and extracellular enzyme bound to cellulose particles. To determine whether a significant amount of cellulase also exists inside or bound to cells, *C. thermocellum* AS-39 was grown in liquid medium containing cellobiose as major carbon source, then washed and subjected to one of the following three threatments: (a) 30 min grinding with quartz sand in 0.02 M citrate buffer (pH 4.5); (b) 2-h incubation at 45°C in 0.42% (w/v) lysozyme in 0.01 M Tris-HCl buffer (pH 7.2); or (c) one min sonication. In no case did the cellular activity amount to over 5% of the extracellular endo-β-glucanase.

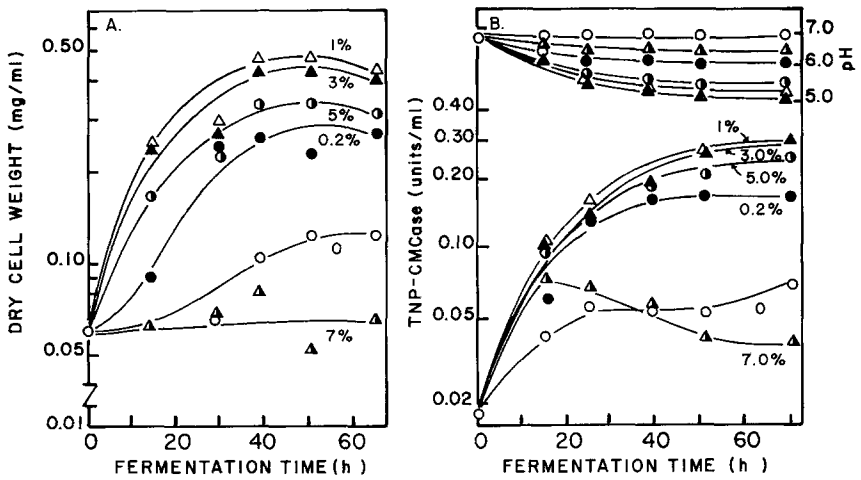

Fig. 1 A and B. Effect of cellobiose concentration on (A) growth and (B) pH and endo-β-glucanase production by *C. thermocellum* ATCC 27405. All flasks contained 0.2% yeast extract

Effect of Cellobiose on Growth and Cellulase Production. Studies were carried out with varying cellobiose concentrations to determine the optimum level of this favored carbon source. The results in Fig. 1 show a dependence of growth and enzyme production on cellobiose concentration. Concentrations as low as 0.2% cellobiose support growth and endo-β-glucanase production; maximum growth and enzyme production were both reached at 1% cellobiose. Concentrations higher than 1% resulted in decreased growth and enzyme production.

Studies on Xylan. Gordon et al. (1978) reported that *C. thermocellum* produces glucose, cellobiose, and xylose from Solka-floc. This would indicate that the hemicellulose portion of Solka-floc can be broken down by *C. thermocellum*. It was thus of interest to determine whether *C. thermocellum* could grow on xylan. When xylan was tested as major carbon source, there was no evidence of growth or enzyme production. The observation that xylanase activity developed when the cells were grown in the presence of cellobiose was of major interest (Table 1).

When agar medium containing xylan as major carbon source was streaked with a heavy cellobiose-grown inoculum, xylan disappeared, clearing the plates. In auxonographic tests, disappearance of xylan was also detected, although growth could not be observed. These data indicated that xylan does not serve as a source of carbon for growth but can be hydrolyzed when cells are grown on cellobiose.

Effect of Nitrogen Sources on Growth and Cellulase Production. A spectrum of nitrogen sources was tested for support of growth and cellulase formation in comparison to ammonium sulfate. Although the basal medium contained 2 g/l yeast extract to supply growth factors and 1 g/l cysteine for anaerobiosis, growth in the presence of these nitrogenous materials in the absence of other nitrogen sources was poor (Table 2). From these results, ammonium nitrate, potassium nitrate, urea, valine, and Casamino acids

Table 1. Production of carboxymethylcellulase (CMCase) and xylanase by *C. thermocellum* ATCC 27405 growing on cellobiose

Fermentation time (h)	Carbon source[a]	Dry cell weight (mg/ml)	CMCase (units/ml)	Xylanase (units/ml)
0	None	0.024	0.008	0.011
12		0.057	0.024	0.021
41		0.036	0.029	0.018
64		0.036	0.025	0.018
0	Cellobiose	0.027	0.038	0.052
12		0.465	0.199	0.135
41		0.435	0.204	0.143
64		0.420	0.184	0.124

[a] All flasks also contained 0.2% yeast extract

Table 2. Effect of nitrogen sources on growth and endo-β-glucanase production[a]

Nitrogen source (0.27g/l nitrogen)	Dry cell weight (mg/ml)	Trinitrophenyl-carboxymethyl-cellulase	
		(Units/ml)	(Units/mg cells)
Ammonium sulfate (control)	0.337	0.184	0.546
Ammonium chloride	0.337	0.207	0.614
Ammonium nitrate	0.367	0.220	0.599
Sodium nitrate	0.217	0.155	0.714
Potassium nitrate	0.217	0.180	0.829
Urea	0.225	0.185	0.822
Glutamate	0.225	0.150	0.667
Aspartate	0.255	0.132	0.518
Lysine	0.217	0.164	0.756
Arginine	0.225	0.152	0.676
Leucine	0.217	0.139	0.641
Isoleucine	0.300	0.157	0.523
Valine	0.247	0.184	0.745
Casamino acids	0.300	0.194	0.613

[a] Duration of experiment was 22.5 h. All flasks contained 0.2% yeast extract

were selected for further study on the basis of high enzyme production on a specific and/or volumetric basis. These five sources were next compared to ammonium sulfate over a nitrogen concentration range of 0.02 to 0.5 g/l. Some of the results are shown in Fig. 2; the data on valine, KNO_3, and Casamino acids are omitted since these nitrogen sources were clearly less impressive than ammonium sulfate, ammonium nitrate, and urea at these higher concentrations. As shown in Fig. 2, ammonium nitrate and ammonium sulfate supported the highest level of growth, but urea utilization provided the

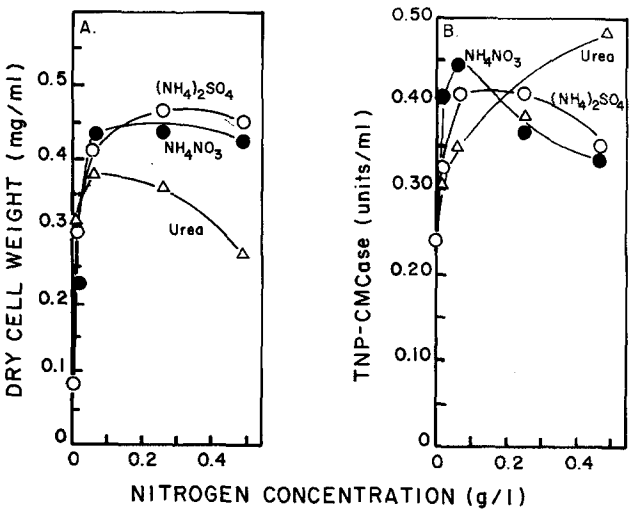

Fig. 2 A and B. Effect of nitrogen sources on (A) growth and (B) endo-β-glucanase production by *C. thermocellum* AS-39. The major carbon source was cellobiose. All flasks contained 0.2% yeast extract. The duration of the experiment was 24 h

highest titer of endo-β-glucanase. We also examined urea as a nitrogen source at even higher concentrations. In this experiment, the normal concentration of yeast extract (0.2%), as well as a higher level (0.6%), were used. Although urea was toxic to *C. thermocellum* AS-39 at levels greater than 0.5 g/l when the medium contained 0.2% yeast extract, this was not true at the higher yeast extract concentration; here, 1 g/l urea nitrogen could be tolerated and, in fact, led to a high cellulase titer.

Effect of pH on Growth and Cellulase Formation. Despite the presence of phosphate in the medium, pH during fermentation dropped to values between 5.0 and 6.0. The addition of morpholinopropane-sulfonic acid (MOPS) buffer retarded the decrease in pH, resulting in improved growth and cellulase formation. A concentration of 0.1 M MOPS buffer was optimal for both growth and enzyme formation.

In the above experiment, the initial pH had been adjusted to 7.0 before autoclaving. To determine the best starting pH, a series of flasks containing 0.1 M MOPS buffer was adjusted to pH values ranging from 5.5 to 7.8. Figure 3 shows that growth took place when the initial pH was between 6.5 and 7.8; optimum growth occurred at an initial pH of 7.0. Extracellular endo-β-glucanase production occurred optimally at an initial pH of 7.4. In this case, the pre-autoclaving pH was 7.5 and the pH at 44 h was 6.8.

Improved Medium. As a result of the studies described above, a new medium (GS medium) was developed. The compositions of this medium and the original medium are shown in Table 3. Performance of the parent culture (ATCC 27405) and mutant AS-39 is shown in Fig. 4. It can be seen that both parent and mutant produce about 60% more endo-β-glucanase in the new medium.

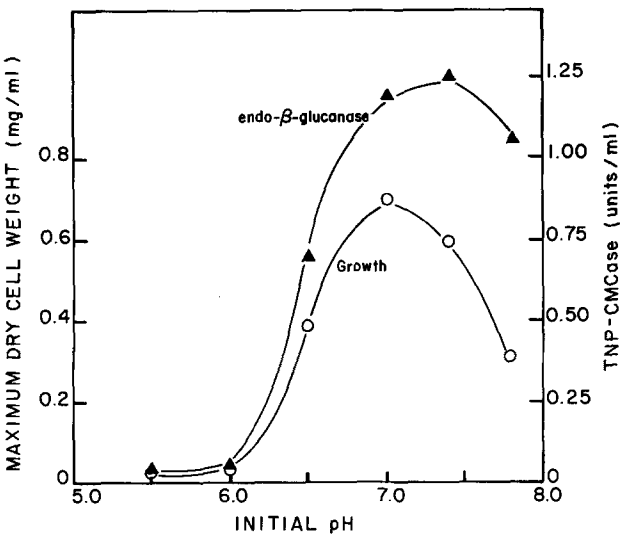

Fig. 3. Effect of inital pH on growth and endo-β-glucanase production by *C. thermocellum* AS-39. Medium contained cellobiose as major carbon source and 0.1 M morpholinopropanesulfonic acid (MOPS) buffer. Samples were taken for analysis at 21, 27, and 44 h. Maximum values are plotted. Initial pH values were determined after autoclaving; pre-autoclave pH values were the same except for the highest pH points, which were 7.5 and 8.0, instead of 7.4 and 7.8, respectively

Table 3. Composition of media

Ingredients	Initial medium (modified CM3)	New medium (GS)
KH_2PO_4	1.5 g	1.5 g
K_2HPO_4	2.9 g	2.9 g
$(NH_4)_2SO_4$	1.3 g	–
Urea	–	2.14 g
$MgCl_2 \cdot 6H_2O$	1.0 g	1.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g	0.15 g
$FeSO_4 \cdot 6H_2O$	1.25 mg	1.25 mg
Cysteine-HCl	1.0 g	1.0 g
Yeast extract	2.0 g	6.0 g
Resazurin	2.0 mg	2.0 mg
Cellobiose	10.0 g	10.0 g
Morpholinopropane-sulfonic acid	–	20.9 g
Final volume	1 l	1 l
pH	7.0	7.5[a]

[a] After autoclaving, the pH was 7.4

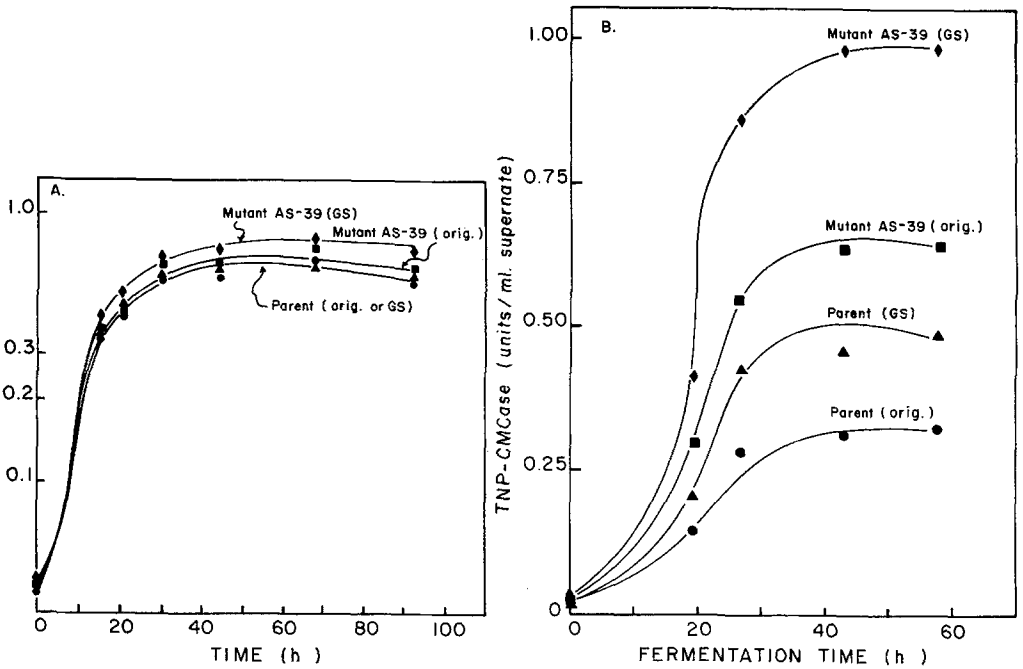

Fig. 4 A and B. Growth and endo-β-glucanase production of *C. thermocellum* ATCC 27405 (parent) and AS-39 (mutant) in the original and new (GS) media: (A) growth; (B) trinitrophenyl-carboxymethylcellulase (TNP-CMCase)

Discussion

The extracellular nature of endo-β-glucanase of *C. thermocellum* was noted in this work: no more than 5% of the activity could be liberated by various methods of cell disruption. This finding indicates that the 35% figure obtained in a cellulose fermentation by Gordon et al. (1978) is mainly due to cellulase adsorbed to insoluble cellulose rather than to cellular or intracellular enzyme.

The optimum level of 1% cellobiose for growth and endo-β-glucanase production observed in the present study only applies to medium composition at the start of the fermentation. No doubt the toxicity of higher cellobiose concentrations could be eliminated by feeding cellobiose during the fermentation.

The observation that *C. thermocellum* can produce xylose (in addition to glucose and cellobiose) from Solka-floc (Gordon et al 1978) indicates an ability to break down the xylan component of hemicellulose. Our observation of the lack of growth on xylan in liquid or solid media confirms earlier results of others (D.I.C. Wang, C.L. Cooney, and R.F. Gomez, unpublished data). These investigators noted the development of xylanase activity during growth on cellulose; in the present work, we found this hydrolytic activity during growth on cellobiose. The fact that *C. thermocellum* can produce xylanase is significant, since most natural cellulose sources contain hemicellulose. At present, we do not know whether the xylanase represents a separate enzyme or is an

activity of the cellulase enzyme(s) of *C. thermocellum*. At any rate, the inability of *C. thermocellum* to grow on xylan is evidently caused by its inability to utilize xylose. Unlike McBee (1948, 1950, 1954), we have failed to obtain growth of ATCC 27405 on xylose (Shinmyo et al. 1979). Since the American Type Culture Collection states that ATCC 27405 is the same as strain 157 of McBee (1948), it is possible that the organism has lost the capacity to grow on xylose or that the xylose preparation of McBee was contaminated with cellobiose. Ng et al. (1977) found three strains of *C. thermocellum* (LQ8, Nl, and Hl) to be incapable of growth on xylose.

Studies on nitrogen source utilization resulted in an increase in endo-β-glucanase production. This increase was obtained by adding 2.1 g/l urea and 6.0 g/l yeast extract instead of 1.3 g/l $(NH_4)_2SO_4$ and 2.0 g/l yeast extract.

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