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Cellulolytic Activity of *Clostridium acetobutylicum*

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Clostridium acetobutylicum **NRRL B527 and ATCC 824 exhibited extracellular and cell-bound endoglucanase and cellobiase activities during growth in a chemically defined medium with cellobiose as the sole source of carbohydrate. For both strains, the endoglucanase was found to be mainly extracellular (70 to 90%) during growth in continuous or batch cultures with the pH maintained at 5.2, whereas the cellobiase was mainly cell associated (60 to 90%). During continuous cultivation of strain B527 with cellobiose as the limiting nutrient, maximum production of the endoglucanase and cellobiase occurred at pH values of 5.2 and 4.8, respectively. In the carbon-limited continuous cultures, strain 824 produced similar levels of endoglucanase, cellobiosidase, and cellobiase activities regardless of the carbon source used. However, in ammonium- or phosphate-limited cultures, with an excess of glucose, only 1/10 of the endoglucanase was produced, and neither cellobiosidase nor cellobiase activities were detectable. A crude extracellular enzyme preparation from strain B527 hydrolyzed carboxymethylcellulose and phosphoric acid-swollen cellulose readily and microcrystalline cellulose (Avicel) to a lesser extent. Glucose accounted for more than 90% of the reducing sugar produced by the hydrolysis of acid-swollen cellulose and Avicel. Strain B527 did not grow in medium with acid-swollen cellulose as the sole source of carbohydrate, although it grew readily on the products obtained by hydrolyzing the cellulose in vitro with a preparation of extracellular cellulase derived from the same organism.**

Cellulose is the most abundant biopolymer occurring in nature and in agricultural and many industrial wastes. The fermentation of cellulosic materials by anaerobes potentially combines the desirable objectives of (i) waste disposal and (ii) the production of fuels and chemicals independent of petrochemical feedstocks. The industrial production of acetone, *n*-butanol, and ethanol by fermentation of, for example, molasses by *Clostridium acetobutylicum* and related species has long been possible (26, 27). The solvent-producing strains of *Clostridium* sp. are capable of fermenting a wide variety of carbohydrates, including hexoses (6) and pentoses (9, 14, 19). The potential of these strains for the industrial production of acetone, *n*-butanol, and ethanol would be greatly enhanced if they were able to hydrolyze cellulose. Compere and Griffith (6), in their study of solvent production from cellulose by several strains of *Clostridium* sp., obtained results that were equivocal for two reasons: (i) the use of a complex basal medium probably permitted some solvent production in the absence of cellulose fermentation, and (ii) the nature and source of the cellulose used was not indicated. Allcock and Woods (1) have reported the production of an inducible endoglucanase [endoglucanase is endo-1,4-β-glucanase or 1,4-(1,3; 1,4)-β-D-glucan 4-glucanohydrolase (EC 3.2.1.4)] and a cellobiase [cellobiase is β-D-glucosidase or β-D-glucoside glucohydrolase (EC 3.2.1.21)] by *Clostridium acetobutylicum* P270. This industrial strain, however, was unable to grow on crystalline or amorphous cellulose as the sole carbon source.

In a recent report, we have described the production and some general properties of the endoglucanase and cellobiase activities of *Clostridium acetobutylicum* NRRL B527 (16). In this paper, further studies on the cellulolytic activity of strain B527 are reported, and the activity of another strain, *Clostridium acetobutylicum* ATCC 824, is described.

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MATERIALS AND METHODS

Bacteria. *Clostridium acetobutylicum* ATCC 824, ATCC 4259, and ATCC 10132 and *Clostridium butylicum* ATCC 14823, NRC 33005, NRC 33006, and NRC 33007 were kindly provided by Roger Latta, Division of Biological Sciences, National Research Council of Canada, Ottawa, Canada. *Clostridium acetobutylicum* NRRL B527 and *Clostridium butylicum* NRRL B592 and NRRL B593 were obtained from the Northern Regional Research Laboratory, Peoria, Ill. *Clostridium acetobutylicum* IFO 3853 and IFO 3854, *Clostridium butyricum* Prazmowski IFO 3315 and IFO 3858, and *Clostridium kainantoi* IFO 3353 were obtained from the Institute for Fermentation, Osaka, Japan. *Clostridium beijerinckii* ATCC 858, ATCC 11914, ATCC 14949, and ATCC 25752 were obtained from the American Type Culture Collection, Rockville, Md. *Clostridium butylicum* 374 and *Clostridium butyricum* 31 were from the culture collection of the Department of Microbiology, University of Guelph. For culture maintenance and preservation, cells were grown in Reinforced Clostridial Medium (Oxoid Ltd., Basingstoke, Hampshire, England) for 24 to 48 h and were either frozen in 20% (vol/vol) glycerol at −20°C (34) or freeze-dried in ampoules.

Media and growth of organisms. The chemically defined medium described by Andersch et al. (2) was used throughout these studies. Sodium dithionite was omitted from the medium because it was inhibitory to growth. Media were prepared under oxygen-free nitrogen. Growth of the organisms was initiated with a 10% (vol/vol) inoculum of cells in the late exponential phase of growth, and the atmosphere above the inoculated culture was briefly gassed with 100% CO_2. The incubation temperature was 34°C. Growth of the culture was monitored by measuring the optical density at 675 nm (OD_{675}) with a Spectronic-20 spectrophotometer with optically matched test tubes (15 by 125 mm). The readings were adjusted for deviations from the turbidity analog of

Beer's Law with the tables of Toennies and Gallant (36). The resulting values were referred to as adjusted optical density. In some experiments, OD_{675} was also measured with a Gilford Stasar II spectrophotometer. For maximum accuracy, samples with an OD_{675} of greater than 0.7 were diluted with known volumes of fresh medium to reduce the observed OD_{675} to values below 0.7.

Continuous cultivation studies were performed with a Multigen bench-top culturing apparatus equipped with a C-30 chemostat culture vessel (model F-1000; New Brunswick Scientific Co., Inc., Edison, N.J.). The pH was controlled by the addition of 2 M KOH with an automatic pH controller (type 45 AR; Chemtrix; Analytical Instrument Service Ltd.). All connections were made with black butyl rubber tubing. Anaerobiosis was maintained by sparging the culture continuously with oxygen-free nitrogen at a flow rate of 8 ml/min. Samples were collected on ice after passage of five culture volumes of medium. Culture purity was checked by Gram stain, by streaking on plates of reinforced clostridial agar in an anaerobic chamber, and by fermentation product analysis. When the chemostat was operating at steady state (at all pH values studied), clostridial or sporulating forms of the organism, observed by phase contrast microscopy, accounted for no more than 1% of the cells.

The relationship between growth and carbohydrate concentration in the chemostat was linear to 111 mM glucose (the highest concentration tested). The OD_{675} of the culture at 111 mM glucose was approximately 4. In the carbon-limited cultures, growth was limited to an OD_{675} of approximately 3.

Screening cultures for endoglucanase activity. Cultures on agar plates were screened for endoglucanase activity by the Congo red staining method of Teather and Wood (35).

Preparation of samples for enzyme assays. Cells were sedimented by centrifugation at 10,000 × *g* for 10 min at 4°C. The culture supernatant was collected and concentrated (usually 15-fold) by ultrafiltration at 0°C through an Amicon PM 10 membrane. The concentrated material was freed from low-molecular-weight metabolic products and medium components by dilution with 4 volumes of ice-cold 0.02 M sodium phosphate buffer, pH 6.0, followed by reconcentration by ultrafiltration. This process was repeated three times. Enzyme activities detected in this fraction were considered to be extracellular. The cells were washed once with 0.02 M sodium phosphate buffer, pH 6.0, and resuspended in the same buffer to give a cell density equivalent to a 15-fold concentration. The wash buffer was added to the supernatant culture fluid and concentrated as described above. Enzyme activity detected in the cell suspension was assumed to be cell bound.

Preparation of phosphoric acid-swollen cellulose. Acid-swollen cellulose was prepared from microcrystalline cellulose (Avicel, PH-105; FMC Corp., Philadelphia, Pa.) as described by Wood (39).

Enzyme assays. Endoglucanase activity was assayed by measuring the amount of reducing sugar released from 1.25% (wt/vol) carboxymethylcellulose (sodium salt; medium viscosity; degree of substitution, approximately 0.7; degree of polymerization, approximately 1,100; Sigma Chemical Co., St. Louis, Mo.) in 30 min at 39°C by the method of Nelson as modified by Somogyi (32). The assay was conducted in 0.1 M sodium acetate buffer, pH 5.2. The final assay volume was 0.2 ml. One unit of activity was defined as that amount of enzyme catalyzing the formation of 1 nmol of reducing sugar (D-glucose) per min.

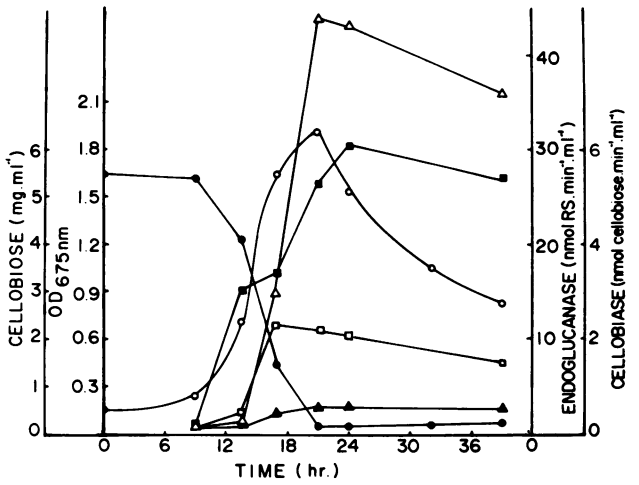


FIG. 1. Production of endoglucanase and cellobiase by *Clostridium acetobutylicum* B527 during growth in a batch culture with cellobiose as the source of carbohydrate and the pH controlled at 5.2. Symbols: ■, cell-bound cellobiase; □, extracellular cellobiase; ▲, cell-bound endoglucanase; △, extracellular endoglucanase; ●, residual carbohydrate expressed as cellobiose; ○, OD.

Cellobiase activity was determined by measuring the amount of glucose released from 29 mM (1% [wt/vol]) cellobiose after 60 min of incubation at 39°C in 0.1 M sodium acetate buffer, pH 5.2, in a final assay volume of 0.4 ml. The reaction was started by the addition of enzyme solution and stopped by heating the mixture in a boiling water bath for 10 min. The mixture was cooled for 5 min in a water bath, 0.1 ml of 5% Triton X-100 was added, and the free glucose was determined by using the glucose oxidase reagent (3). One unit of enzyme activity was defined as that amount of enzyme catalyzing the hydrolysis of 1 nmol of cellobiose per min.

For endoglucanase and cellobiase assays, the control treatment was performed by incubating the enzyme and substrate separately and mixing them together just before boiling to correct for possible endogenous formation of product.

Cellobiosidase activity was determined by measuring the amount of *p*-nitrophenol liberated from 5 mM *p*-nitrophenylcellobioside (Sigma) after 30 min incubation at 39°C in 0.1 M sodium acetate buffer, pH 5.2. One unit of enzyme activity was defined as that amount of enzyme that formed 1 nmol of *p*-nitrophenol per min. For a control, the enzyme was inactivated by heating in a boiling water bath for 20 min before incubation with substrate. To determine whether the major product of hydrolysis from *p*-nitrophenylcellobioside was cellobiose, samples of the reaction mixture were analyzed by using a high-pressure liquid chromatography system (Waters Associates). Sugars were separated on an Aminex HPX-87P column (300 by 7.8 mm; Bio-Rad Laboratories, Richmond, Calif.) fitted with cationic and anionic de-ashing microguard columns (Bio-Rad) with water (0.6 ml/min) as the eluant and a column temperature of 85°C. Eluted carbohydrate peaks were monitored with a refractive index detector (model 7510; Erma Optical Works, Tokyo, Japan).

Hydrolysis of carboxymethylcellulose, acid-swollen cellulose, and Avicel. The extent of hydrolysis of cellulose was determined by assaying the amount of reducing sugars

TABLE 1. Effect of culture pH on edoglucanase and cellobiase activities exhibited by *Clostridium acetobutylicum* B527[a]

pH	Activity (sp act) of[b]:			
	Endoglucanase		Cellobiase	
	Extracellular	Cell bound	Extracellular	Cell bound
4.2	0.9 (100)	0.4 (1)	0	0.1 (0.2)
4.6	14.7 (798)	3.8 (12)	0.2 (12)	2.0 (6)
4.8	53.0 (871)	10.9 (33)	4.0 (66)	14.2 (42)
5.2	93.9 (1,024)	13.1 (41)	8.6 (90)	4.6 (14)
5.5	40.9 (635)	6.3 (17)	5.4 (84)	7.3 (20)
6.0	18.7 (401)	3.0 (10)	1.2 (25)	2.7 (9)

[a] Activities were measured during growth in continuous culture with cellobiose (17.5 mM) as the limiting nutrient at a dilution rate of 0.1 h^{-1}.
[b] A unit of activity is defined as 1 nmol of reducing sugar or cellobiose hydrolyzed per min per ml of culture. All assays were conducted at pH 5.2 in 0.1 M sodium acetate buffer. Specific activity (shown in parentheses) is expressed as units of activity per milligram of protein.

formed by using the Nelson-Somogyi reagent (32). The amount of D-glucose formed was measured with the glucose oxidase reagent (3). The assay was performed in 0.1 M sodium acetate buffer, pH 5.2, at 39°C in the presence of 25 μg of chloramphenicol per ml, added to prevent microbial growth. At selected times, samples were removed and heated in a boiling water bath for 15 min. The insoluble cellulose was sedimented by centrifugation at 10,000 × *g* for 10 min. The cellulose-free supernatant fluid was assayed for reducing sugars and D-glucose. Enzyme which had been inactivated by heating in a boiling water bath for 20 min was incubated with the substrate and served as a control.

Preparation of hydrolysis products of cellulose for use as growth substrates. Acid-swollen cellulose was hydrolyzed with the concentrated extracellular enzyme prepared from *Clostridium acetobutylicum* B527 by ultrafiltration as described above. The reaction mixture contained 1% (wt/vol) acid-swollen cellulose, 0.39 mg of enzyme protein per ml (49.7 U of endoglucanase activity per ml), and 0.05 M sodium acetate buffer, pH 5.2. Chloramphenicol was omitted from this reaction. After 24 h at 39°C, the reaction was stopped by heating in a boiling water bath for 15 min. The cellulose-free supernatant fluid obtained by centrifugation was incorporated into the chemically defined medium, and the mixture was filter sterilized through a membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and used for growth studies.

Estimation of protein. Protein was estimated by the method of Lowry et al. (17) with bovine serum albumin as a standard.

Estimation of cellobiose. Residual carbohydrate was estimated by the phenol-sulfuric acid method with cellobiose as a standard (8).

RESULTS

Screening cultures for endoglucanase activity. Twenty-one strains of solvent-producing *Clostridium* spp. were tested for growth in a chemically defined medium containing 0.2% (wt/vol) phosphoric acid-swollen cellulose. None of these strains was able to use the cellulose as a source of carbohydrate. The strains were then screened for the ability to hydrolyze carboxymethylcellulose by the substrate overlay method described by Teather and Wood (35). Two strains of *Clostridium acetobutylicum*, NRRL B527 and ATCC 824, produced clearing zones indicating the production of endoglucanase activity. In the preliminary experiments, endoglucanase activity was detected in liquid batch cultures of strain B527 but not in the cultures of strain 824. Since the endoglucanase from B527 appeared to be more stable, it was studied first, and the results of these studies have been published (16).

Production of endoglucanase and cellobiase in a batch culture. *Clostridium acetobutylicum* B527 produced essentially cell-bound endoglucanase and cellobiase during growth in a batch culture (16). The enzyme activities, however, were unstable. The instability of the enzymes was thought to be caused by low pH. Hence, strain B527 was grown in a pH-controlled culture. Both endoglucanase and cellobiase activities increased with the growth of the organism and reached maxima at the stationary phase of growth (Fig. 1). The endoglucanase activity was largely extracellular. After 21 h of growth, about 95% of the total endoglucanase activity was extracellular, while only approximately 25% of the cellobiase activity was extracellular. The activities of the endoglucanase and cellobiase enzymes in this culture were 400- and 10-fold higher, respectively, than those previously reported for the culture without pH control (16).

Effect of pH on the production of endoglucanase and cellobiase in chemostat cultures. The effect of culture pH on endoglucanase and cellobiase production by strain B527 was studied in a chemostat with cellobiose as the limiting nutrient. Maximum endoglucanase activity was detected at pH 5.2 (Table 1). The endoglucanase was largely extracellular

TABLE 2. Endoglucanase, cellobiosidase, and cellobiase activities exhibited by *Clostridium acetobutylicum* ATCC 824 grown with various limited carbon sources[a]

Carbon source (concn)	Activity (sp act) of[b]:					
	Endoglucanase		Cellobiosidase		Cellobiase	
	Extracellular	Cell-bound	Extracellular	Cell-bound	Extracellular	Cell-bound
Glucose (83.3 mM)	104.6 (503)	18.6 (40)	16.5 (79)	3.2 (7)	2.9 (19)	10.8 (23)
Xylose (100.0 mM)	121.7 (485)	26.2 (43)	25.0 (100)	6.4 (11)	4.1 (16)	16.8 (28)
Cellobiose (29.3 mM)	148.1 (1,105)	16.7 (39)	8.7 (65)	0.7 (2)	1.9 (14)	4.0 (10)
Mannose (83.3 mM)	410.9 (1,553)	56.1 (117)	19.2 (73)	1.4 (3)	3.1 (12)	8.5 (18)

[a] Activities were measured during growth in a chemostat at pH 5.2 and a dilution rate of 0.05 h^{-1}.
[b] Units are as defined in footnote *b* of Table 1.

TABLE 3. Endoglucanase, cellobiosidase, and cellobiase activities produced by *Clostridium acetobutylicum* ATCC 824 with either phosphate or ammonium as the limiting nutrient[a]

Carbon source (concn)	Limiting nutrient (concn)	Activity (sp act) of[b]:					
		Endoglucanase		Cellobiosidase		Cellobiase	
		Extracellular	Cell-bound	Extracellular	Cell-bound	Extracellular	Cell-bound
Glucose (166.7 mM)	Phosphorus (0.03 mM)	12.2 (350)	1.2 (3)	0.3 (7)	0	0	0
Glucose (222.2 mM)	Ammonium (3.3 mM)	9.5 (245)	3.9 (5)	0.2 (8)	0.2 (0.2)	0	0
Xylose (200.0 mM)	Ammonium (3.3 mM)	36.7 (1,077)	9.1 (21)	1.7 (49)	0.6 (1)	0.4 (12)	1.7 (4)

[a] Activities were measured during growth in a chemostat at pH 5.2 and a dilution rate of 0.1 h^{-1}.
[b] Units are as defined in footnote *b* of Table 1.

(70 to 80%) at all pH values studied. Maximum cellobiase activity was produced at pH 4.8. A larger proportion (60 to 90%) of the cellobiase was cell bound at all pH values used, except at pH 5.2, at which value 65% of the total activity was extracellular.

Effect of the carbon source on the production of endoglucanase, cellobiosidase, and cellobiase. *Clostridium acetobutylicum* ATCC 824 was grown in a chemostat at pH 5.2 with carbon limitation. The endoglucanase, cellobiosidase, and cellobiase activities in these cultures were measured. Cultures grown on glucose, xylose, and cellobiose exhibited similar levels of endoglucanase activity (Table 2). The mannose-grown culture, however, displayed an enhanced (fourfold) level of endoglucanase activity. A major proportion (80 to 90%) of the activity was found in the culture supernatant. The levels of cellobiosidase activity detected in cultures grown on glucose, xylose, or mannose were similar but were only one-half to one-third of the value observed in the cellobiose-grown culture. About 80 to 93% of the cellobiosidase activity was extracellular. Cellobiose accounted for 85% of the hexose released during *p*-nitrophenylcellobioside hydrolysis by the extracellular enzyme of cells grown on xylose. The highest level of cellobiase was detected in the xylose-grown culture; only approximately 25% of this activity was detected in the cellobiose-grown culture. The distribution of cellobiase of strain 824 was similar to that in strain B527, in which 79 to 80% of the total activity was cell associated.

The effect of carbon source on the levels of enzyme produced by strain 824 was also studied in cultures with phosphate or ammonium limitation. The results showed marked reductions in the levels of enzyme produced in these cultures (Table 3) and in cultures grown under carbon limitation (Table 2). In the cultures with glucose as the carbon source, the endoglucanase activity detected in phosphate- or ammonium-limited cultures was only 1/10 of that in the glucose-limited cultures. Very little cellobiosidase activity and no cellobiase activity was detected. In the ammonium-limited, xylose-grown cultures, the endoglucanase, cellobiosidase, and cellobiase activities were only 1/3, 1/14, and 1/10, respectively, of those detected in the xylose-limited culture. The levels of these enzymes in the ammonium-limited cultures were higher in the presence of excess xylose than in the presence of excess glucose (Table 3).

General properties of the endoglucanase and cellobiase. The

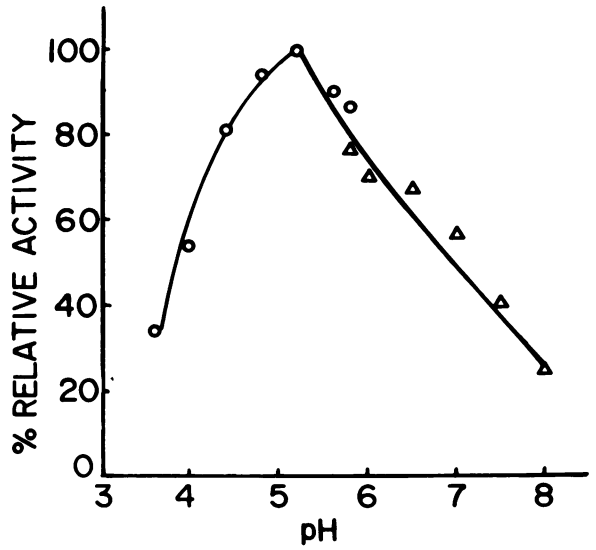


FIG. 2. Effect of pH on extracellular endoglucanase activity of *Clostridium acetobutylicum* ATCC 824. Buffers used were the 0.1 M sodium salts of acetate (○) and phosphate (△).

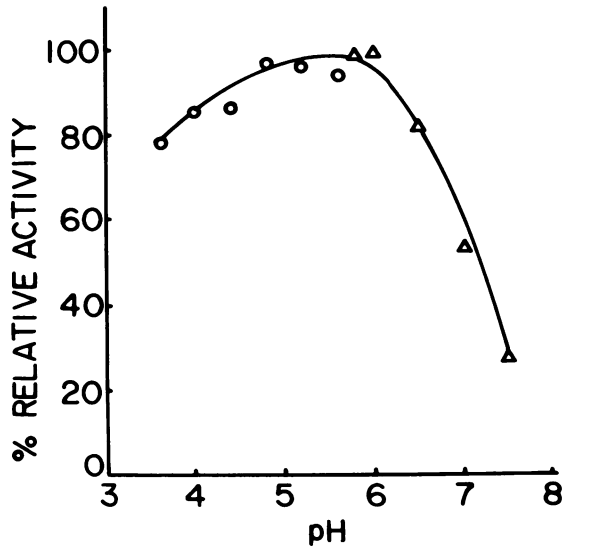


FIG. 3. Effect of pH on cell-bound cellobiase activity of *Clostridium acetobutylicum* ATCC 824. Buffers used were 0.1 M sodium salts of acetate (○) and phosphate (△).

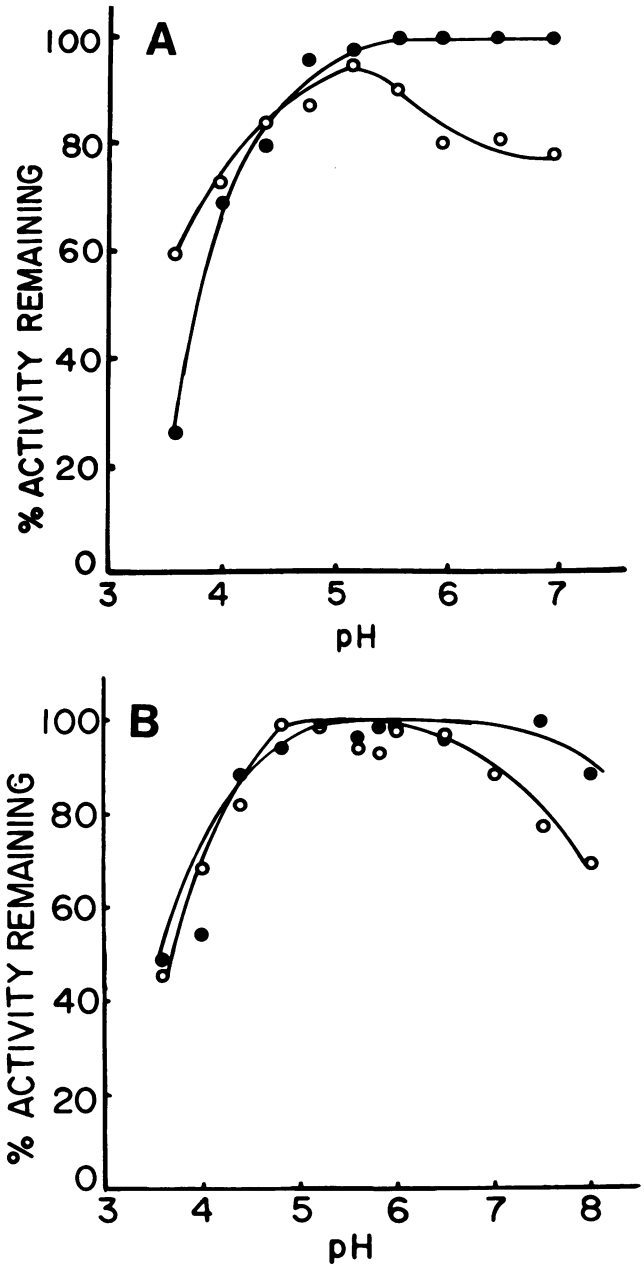


FIG. 4. The pH stability curves for the endoglucanase (●) and cellobiase (○) of *Clostridium acetobutylicum* B527 (A) and ATCC 824 (B). Enzyme solutions were exposed to 25 mM sodium acetate buffer (pH 3.6 to 5.6) or sodium phosphate buffer (pH 6.0 to 8.0) for 1 h, and activity remaining was assayed in 250 mM sodium acetate buffer, pH 5.2.

extracellular endoglucanase and cell-bound cellobiase of strain B527 exhibited optimal activity at pH 5.2 and 5.6, respectively (16). The endoglucanase of strain 824 had a similar pH optimum (Fig. 2). However, its cellobiase had a broader pH activity spectrum (4.8 to 6.0) (Fig. 3) than that of strain B527. The endoglucanases of strains B527 and 824 were stable at pH levels above 5.2 but were unstable on the acidic side of this pH value (Fig. 4A and B). The cellobiase of strain B527 was most stable at pH 5.2 (Fig. 4A); however, that of strain 824 was stable over the range from pH 4.8 to 6.5 (Fig. 4B).

Substrate inhibition was exhibited by the cellobiase of strain 824 (Fig. 5). This was anticipated in the light of a similar phenomenon previously reported for the cellobiase of strain B527 (16).

Hydrolysis of soluble and insoluble cellulose. Carboxymethyl cellulose was hydrolyzed by the enzyme preparation of strain B527 (Fig. 6A). In 4 h of incubation, 8% of the carboxymethylcellulose was hydrolyzed, and glucose constituted 28% of the total reducing sugar produced.

About 30% of the phosphoric acid-swollen cellulose was hydrolyzed in 24 h of incubation (Fig. 6B), with glucose accounting for more than 90% of the reducing sugar produced. In an experiment in which all the reagents were saturated with oxygen-free nitrogen and the reaction mixtures were incubated in an atmosphere of nitrogen, no difference was observed between this anaerobic and the aerobic incubations (Fig. 6B) in terms of the end products and degree of hydrolysis. After three successive treatments with fresh enzyme-buffer mixture added after every 24 h, 62% of the acid-swollen cellulose was degraded (Table 4).

Microcrystalline cellulose (Avicel) was hydrolyzed to a lesser extent by the extracellular enzyme preparation (Fig. 6C). After 29 h incubation, only 4% of the Avicel was degraded, and glucose accounted for more than 90% of the reducing sugar produced. Repeated treatments of the Avicel with enzymes resulted in only about 7% hydrolysis (Table 4).

Growth of strain B527 on the products of hydrolysis of acid-swollen cellulose. As indicated earlier, neither strain B527 nor strain 824 used acid-swollen cellulose as a carbon source. However, as reported previously (16), the endoglucanase(s) of strain B527 was induced by cellobiose. In the light of these results, attempts were made to grow B527 on acid-swollen cellulose supplemented with a low concentration of cellobiose (0.05% [wt/vol]). Growth, measured by the increase in microbial proteins, ceased after the depletion of cellobiose.

The lack of growth of B527 on acid-swollen cellulose could have been due to the nonfermentable nature of its hydrolysis products. To preclude this possibility, the reducing sugar produced by the hydrolysis of acid-swollen cellulose with

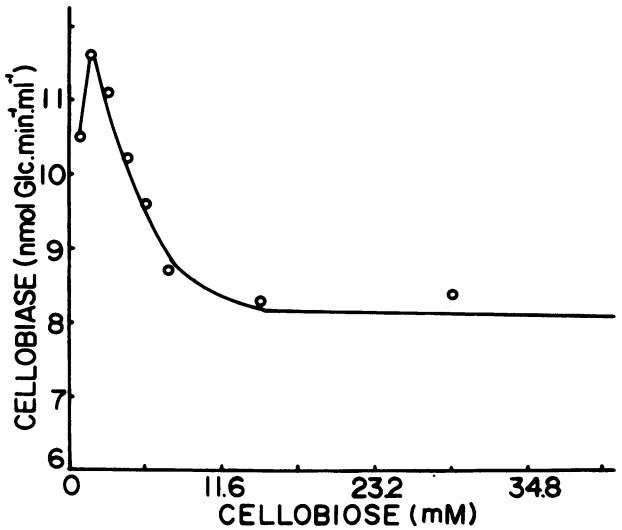
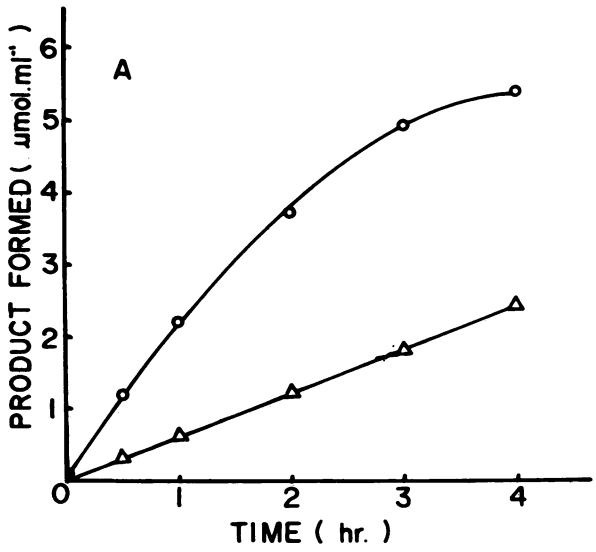
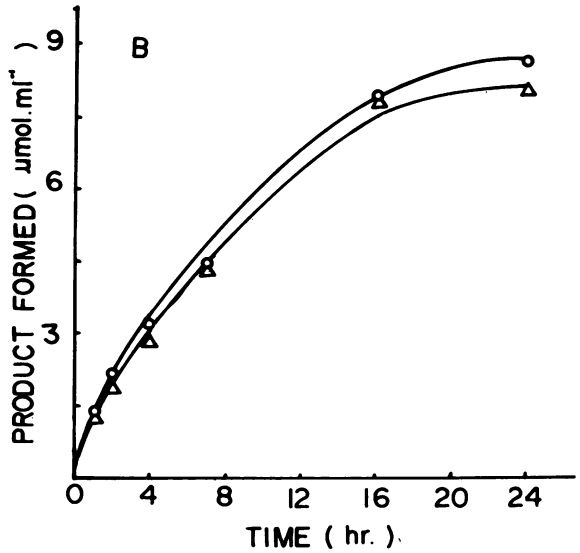


FIG. 5. Effect of substrate concentration on cellobiase activity of *Clostridium acetobutylicum* ATCC 824. Assay was conducted in 0.1 M sodium acetate buffer, pH 5.2, at 39°C.





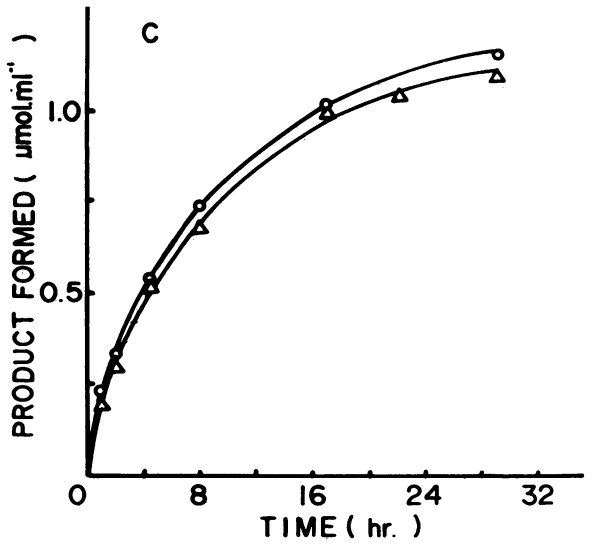


FIG. 6. Hydrolysis of carboxymethylcellulose, phosphoric acid-swollen cellulose, and Avicel by extracellular enzymes of *Clostridium acetobutylicum* B527. Shown are the amounts of reducing sugar (○) and glucose (△) formed in the following reactions: 12.5 U of endoglucanase activity per ml (98 μg of protein per ml) incubated with 1.25% (wt/vol) carboxymethylcellulose (A), and 49.7 U of endoglucanase activity per ml (390 μg of protein per ml) incubated with either 0.5% (wt/vol) acid-swollen cellulose (B) or 0.5% (wt/vol) Avicel (C).

the extracellular enzymes from strain B527 was tested as a carbohydrate source for growth of this organism. Strain B527 grew readily on the hydrolysis products (Fig. 7). Neither glucose nor reducing sugar was detected in the culture medium after growth stopped (26.5 h).

DISCUSSION

The model for the complete depolymerization of crystalline cellulose by fungi, which is often referred to in bacterial studies, is thought to involve synergistic action of at least two cellulase components, namely endo-1,4-β-glucanase and exo-1,4-β-glucanase, the latter often being called cellobiohydrolase (28). The endoglucanases initiate the degradation process, creating new chain ends from which the exoglucanases release cellobiose or glucose, depending upon the type of exoglucanase concerned. The cellobiose released is further hydrolyzed to glucose by the third component of the cellulase system, the cellobiase (β-glucosidase). The results reported here clearly indicate that *Clostridium acetobutylicum* B527 and ATCC 824 possess endoglucanase and cellobiase activities but lack enzymatic activities necessary for the hydrolysis of crystalline cellulose. Cellulolytic enzymes, however, are not widely distributed among the solvent-producing strains of *Clostridium* spp., and of the 21 strains tested in this study, only B527 and ATCC 824 were found to possess them.

Maximum endoglucanase and cellobiase activities were produced at pH 5.2 and 4.8, respectively (Table 1). Shifting the culture pH away from these values resulted in a decrease in total activity. These changes in levels of activity can, of course, be due to either the influence of pH on the synthesis of the enzyme protein or its effect on the stability of the enzyme molecule itself. The endoglucanase of strain B527 was stable on the alkaline side but unstable on the acidic side of pH 5.2 (Fig. 4A and B). The reduction in total endoglucanase activity below pH 5.2 is, therefore, probably due to the instability of the enzyme. The decrease in activity above pH 5.2 is probably due to the effect of pH on the regulation of synthesis of the enzyme. In the case of the cellobiase of B527, instability was observed on both the acidic and alkaline sides of pH 5.2 (Fig. 4A). Reduction in enzyme production at culture pH values either above or below 5.2 is most probably due to the inherent instability of the enzyme, although an effect of pH on the regulation of enzyme synthesis cannot be excluded.

Clostridium acetobutylicum produced extracellular endoglucanase with a specific activity similar to those of *Bacteroides succinogenes* (10), *Clostridium thermocellum* (20), and *Cellulomonas fimi* (15). However, the specific activity of the endoglucanase of *Clostridium acetobutylicum* was much lower than that of *Trichoderma reesei* QM6a (30). The total specific activity of the cellobiase of *Clostridium acetobutylicum* was about three times higher than that of *B. succinogenes* (10), and only one-fifth or one-third of those of *Cellulomonas fimi* (38) and *Acetivibrio cellulolyticus* (18), respectively.

TABLE 4. The extent of hydrolysis of phosphoric acid-swollen cellulose and Avicel after successive treatments with an extracellular enzyme preparation of *Clostridium acetobutylicum* B527

Treatment no.[a]	% Hydrolysis of [b]:	
	Acid-swollen cellulose	Avicel
1	25.0	3.0
2	50.0	4.4
3	62.0	5.7
4	ND[c]	6.8

[a] 0.78 mg of enzyme protein was incubated with 10 mg of cellulose in 2.0 ml of 0.1 M sodium acetate buffer, pH 5.2, with 50 μg of chloramphenicol at 39°C. At the end of each treatment (24 h), the cellulose was sedimented by centrifugation at 10,000 × *g* at 22°C and resuspended in fresh enzyme-buffer-chloramphenicol mixture. The supernatant was heated for 15 min, and samples were removed for sugar determinations.

[b] Percent hydrolysis was calculated as (micromoles of reducing sugars formed/micromoles of cellulose as glucose) × 100.

[c] ND, Not determined.

Clostridium acetobutylicum B527 and ATCC 824 produced endoglucanase with similar specific activities when grown on cellobiose in a chemostat at pH 5.2 (Tables 1 and 2). The endoglucanases of these two strains exhibited similar properties with respect to pH optimum, pH stability, and cellular distribution. However, the cellobiases produced by these two strains differed in several ways. (i) When the organisms were cultivated under glucose limitation, strain B527 produced no detectable cellobiase (16), whereas strain 824 produced detectable amounts (Table 2). (ii) The cellobiase of 824 had a broader pH activity spectrum than that of B527. (iii) The cellobiase of B527 was most stable at pH 5.2 and was unstable at pH values on the acidic and alkaline side of this value (Fig. 4A), in contrast to the cellobiase of 824, which was stable over the pH range of 4.8 to 6.5 (Fig. 4B). The cellobiases produced by these two strains were subject to substrate inhibition (Fig. 5; reference 16) and showed similar distribution, i.e., mainly cell bound.

The endoglucanase and cellobiase activities of *Clostridium acetobutylicum* B527 and 824 differed in several respects from the corresponding activities of strain P270, as reported by Allcock and Woods (1). Strains B527 and 824 exhibited both extracellular and cell-bound endoglucanase and cellobiase activities, whereas only extracellular activity was shown by strain P270. The endoglucanases of strains B527 and 824 exhibited higher pH and temperature optima than the enzyme of strain P270. Strain B527 also exhibited some activity against Avicel, whereas strain P270 did not. Strains B527 and P270 were both able to degrade but unable to grow on acid-swollen cellulose.

The synthesis of microbial cellulases is generally regulated by the induction-repression mechanism. For instance, in *Cellvibrio gilvus* (4), *Cellulomonas uda* (33), and *Pseudomonas fluorescens* var. *cellulosae* (41), the cellulases are formed during growth on cellulose or carboxymethyl cellulose, but not during growth on glucose, cellobiose, or other readily metabolizable sugars. Wood et al. (40) have reported recently that the yield of cellulases in *Thermomonospora curvata* is directly related to the concentration of cyclic AMP inside the cells, thus indicating control by catabolite repression. Results from this study suggest that the biosynthesis of the cellulase enzymes of *Clostridium acetobutylicum* may also be subject to catabolite repression (cf. Tables 2 and 3), although the precise mechanism by which glucose represses cellulase synthesis in *Clostridium acetobutylicum* is not known. It is interesting that in *Clostridium perfringens* and related genera, cyclic AMP has not been detected (29). If cyclic AMP is also absent in *Clostridium acetobutylicum*, glucose may inhibit the synthesis of cellulase at the translational level by affecting the translation of cellulase mRNA (as has been shown to be the case in *Trichoderma viride* [21]), or by other catabolite repression mechanism, through a catabolite modulator factor(s) (37). In contrast to these extensively regulated cellulases, that of *Clostridium thermocellum* is constitutive in nature (24).

The hydrolysis of acid-swollen cellulose and Avicel by the extracellular enzyme preparation of *Clostridium acetobutylicum* B527 yielded D-glucose as the principal product (Fig. 6B and C). In contrast, the unpurified cellulase of *Clostridium thermocellum* yielded both cellobiose and D-glucose from cellulose MN 3000 (20) and Avicel (13); cellobiose was the major product in both cases. Notwithstanding the apparent absence of a β-glucosidase activity, a *Cytophaga* species digested cellulose to D-glucose (5). Similarly, the *B. succinogenes* extracellular cellulase, in the absence of measurable cellobiase activity, degraded acid-swollen cellulose to D-glucose, cellobiose, and cello-oligosaccharides (11). It is of interest here that the purified endoglucanase of *Trichoderma viride* hydrolyzed acid-swollen cellulose to give glucose, cellobiose, and cellotriose (31). In contrast to the cellulolytic activity of these organisms, the cellulase of *Ruminococcus flavefaciens* (25) and a *Cellulomonas* species (12) yielded only cellobiose from cellulose.

The substrate *p*-nitrophenyl-β-D-cellobioside was cleaved by an enzyme in the cell-free culture fluid of *Clostridium acetobutylicum*. The major sugar reaction product was cellobiose, indicating that cleavage occurred between the cellobiose residue and the *p*-nitrophenol, characteristic of the cellobiosidase type of cleavage mediated by an enzyme

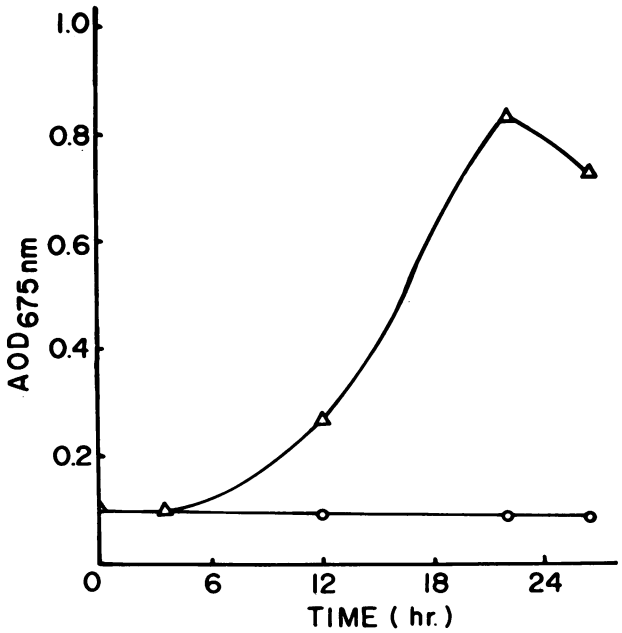


FIG. 7. Growth of *Clostridium acetobutylicum* B527 on hydrolysis products of acid-swollen cellulose. Cultures contained either hydrolysis products (2.7 mg of D-glucose per ml) as sole source of carbohydrate (△) or no hydrolysis products (○).

in the culture fluid of *Ruminococcus albus* (22). This contrasts with the activity of a cellular β-glucosidase from the same organism which mediated a cleavage wherein the glucose residues were reported to have been sequentially removed (23). Perhaps the same situation occurs in *Clostridium acetobutylicum*. The cellobiosidase activity of *Clostridium acetobutylicum* may be an endoglucanase activity, since it is reported that endoglucanases from *Trichoderma reesei* and *Sporotrichum pulverulentum* cleave the cellobioside (7).

The hydrolysis products of acid-swollen cellulose by the extracellular enzyme preparation of *Clostridium acetobutylicum* B527 were fermented by the bacterium (Fig. 7). However, the bacterium failed to grow on the cellulose in a chemically defined medium. The reason for this is obscure. It is possible that the organism requires some unknown but specific factors for the utilization of cellulose. The possibility of some fermentation products having an inhibitory effect on the cellulase activity during growth has not yet been excluded. Nevertheless, if the goal of producing solvents from cellulosic substrates by *Clostridium* fermentation is to be attained, the reasons for these phenomena must be determined.

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