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Cellulase and Sugar Formation by *Bacteroides cellulosolvens*, a Newly Isolated Cellulolytic Anaerobe†

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A newly isolated mesophilic anaerobe, *Bacteroides cellulosolvens*, has the ability to produce cellulase and to degrade cellulose to cellobiose and glucose. It does not utilize glucose, and it lacks β-glucosidase activity. This anaerobe appears to degrade cellulose to cellobiose by cellulase action, and the presence of cells appears necessary for the formation of glucose.

Some cellulolytic anaerobes possess the unique ability to hydrolyze cellulosic materials to cellobiose, in higher quantities than they utilize, and to glucose, which they are unable to use at all. Consequently, these sugars accumulate during cellulolysis (3). These anaerobes are potentially useful in coculture in the single-step conversion of cellulose to sugars or to other useful end products (1, 7, 19). A study of the cellulase system of these anaerobes and the involvement of this system in the saccharification of cellulose is important for improving sugar yields.

During a study of the bacteria involved in a methanogenic culture enriched on a synthetic medium containing cellulose (6), three different mesophilic cellulolytic bacteria that produce sugars from cellulose were isolated (3). Among these isolates, *Bacteroides cellulosolvens* was shown to have a cellulose-degrading ability that is equal to that of *Clostridium thermocellum* (3). This paper describes the sugars and cellulolytic enzymes produced by *B. cellulosolvens*.

The isolation and characterization of *B. cellulosolvens* (NRC 2944) have been described elsewhere (13). In brief, this isolate is a non-spore-forming, mesophilic anaerobe, capable of growing in a synthetic medium containing cellulose or cellobiose as the sole carbon source. It produces large amounts of acetic acid, CO_2, and H_2 and small amounts of ethanol and lactic acid as the end products. It does not require yeast extract for growth and does not utilize glucose or xylose as a carbon source. The organism was maintained in a synthetic medium (10) containing tissue paper as a carbon source.

All tests were conducted in 60-ml serum vials containing 10 ml of prereduced medium as described earlier (8), cellulose powder (CF-11; Whatman, Inc., Clifton, N.J.) or delignified ball-milled pulp (Solka-floc, BW 200; Brown Co., Berlin, N.H.) at a 5% (wt/vol) concentration, and $CaCO_3$ at a 2% (wt/vol) concentration as a buffering agent. A 3-day-old culture was used for the inoculum (2 to 5% [vol/vol]). All cultures were incubated under an N_2-CO_2 (80%-20%) gas atmosphere at 37°C on a shaker operated at 200 rpm. All tests were made in triplicate and on two different occasions.

After incubation for a desired length of time, cultures were centrifuged for 20 min at 3,000 × *g*. The supernatant liquid was assayed for sugars and extracellular cellulolytic activities. The pellet was washed three times with acetate buffer and assayed either for cellulose content (9) or for cell-associated cellulolytic activities. For enzymatic assay, the pellet was suspended in a small amount of acetate buffer, and the cells were disrupted by sonication at 4°C (16). The cell debris was removed by centrifugation at 31,000 × *g* for 15 min. The cell extract thus obtained was assayed for cell-associated activities. The reducing sugar content of the culture supernatant liquid was measured by the colorimetric method with dinitrosalicylic acid reagent (12) and by liquid chromatography with a polypore (PB) carbohydrate column (Brownlee Laboratories Inc., Santa Clara, Calif.). The column temperature was kept at 80°C, and water was used as a mobile phase at a flow rate of 0.3 ml/min.

For cellulolytic enzyme assay, the culture supernatant liquid or the cell extract was incubated with one of the following substrates: carboxymethyl cellulose (Sigma Chemical Co., St. Louis, Mo.), a substrate used for determining endoglucanase activity; crystalline cellulose (Avicel, PH-101; FMC Corp., Philadelphia, Pa.), a substrate used for determining exoglucanase activity; Whatman no. 1 filter paper, a substrate used for determining combined endo- and exoglucanase activities; xylan (larchwood; Sigma), a substrate used for determining xylanase activity; or salicin (Sigma), a substrate used for determining β-glucosidase activity. The hydrolysis of these substrates was measured in 0.2 M acetate buffer (pH 5.0) at 50°C by a standard method (16). In these tests, reducing sugars were estimated colorimetrically with dinitrosalicylic acid reagent (12). The β-glucosidase activity was also determined with cellobiose as a substrate, and the glucostat enzyme assay method (15) was used for the estimation of glucose released. A 15-min incubation time was used for the carboxymethyl cellulose and xylan substrates, and a 1-h incubation time was used for all other substrates. The protein contents were determined by the method of Lowry et al. (11) with bovine serum albumin (Sigma) as a standard.

Growth of *B. cellulosolvens* on Solka-floc caused the accumulation of cellobiose, glucose, and xylose (Table 1). Over 60% (wt/vol) of the total sugars produced in 14 days was glucose; xylose was produced only in small amounts. The largest increase in total sugar accumulation occurred between days 4 and 7 of incubation, at a rate of ca. 1.8 g/liter per day. During 14 days of incubation, ca. 50% of the cellulose initially supplied in the medium was degraded, and more than 50% of the cellulose degraded was recovered in the form of free sugars. The remaining portion of the degraded cellulose was presumably converted to acetic acid, CO_2, ethanol, lactic acid, and biomass.

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TABLE 1. Accumulation of sugars by *B. cellulosolvens* in medium containing Solka-floc[a]

Incubation time (days)	Cellulose degraded (% of initial concn)	Sugar produced (g/liter)		
		Cellobiose	Glucose	Xylose
2		0.4	0.6	0.1
4	27	1.2	1.3	0.6
7	40	3.6	4.1	1.0
14	50	3.9	8.2	1.2

[a] Medium contained 50 g of Solka-floc and 20 g of $CaCo_3$ per liter.

B. cellulosolvens also produced extracellular and cell-associated cellulases (Table 2). Although the enzymes were produced in much lower concentration by this anaerobe than by many fungi (16), they possessed endo- and exoglucanase and xylanase activities and were able to degrade filter paper in situ. This enzyme system lacked, however, β-glucosidase activity; all attempts to detect β-glucosidase activity during 1 to 14 days of growth with cellobiose or salicin as substrate, as well as in cultures obtained by growing this anaerobe in media containing Avicel, cellulose powder, Solka-floc, or cellobiose, were unsuccessful.

The cellulolytic enzymes produced by *B. cellulosolvens* had an optimum pH of 5.0, and optimum temperatures of 50°C for endoglucanase and between 37 and 50°C for filter paper activity (Fig. 1). The effects of ascorbic acid, dithiothreitol, and EDTA on endoglucanase and filter paper activity are shown in Fig. 2. The presence of EDTA inhibited both of these enzymes and had higher inhibitory effects on filter paper activity than on endoglucanase. On the other hand, the presence of ascorbic acid and dithiothreitol increased these activities. Ascorbic acid had a much greater stimulatory effect than dithiothreitol and affected endoglucanase activity more than filter paper activity. The possibility of ascorbic acid interfering in the determination of reducing sugars was eliminated by running proper controls and by confirming the results by liquid chromatography. Dithiothreitol has also been shown to stimulate cellulolytic activity of *C. thermocellum* (5). The stimulatory effect of these reagents on the enzyme system produced by *B. cellulosolvens* may explain the discrepancies in the poor cellulolytic activity as measured by the conventional method, which is conducted in the presence of air, and the relatively high cellulose-degrading ability. In view of these findings, the tests reported below were carried out under strict anaerobic conditions.

TABLE 2. Distribution of cellulolytic enzyme activities in the culture supernatant liquid and the cell extract from *B. cellulosolvens*[a]

Enzyme (substrate)	Cellulolytic activity (%)[b]	
	Culture supernatant	Cell extract
Endoglucanase (carboxymethyl cellulose)	84	16
Exoglucanase (Avicel)	78	22
β-Glucosidase (cellobiose)	Tr	Tr
β-Glucosidase (salicin)	Tr	Tr
Xylanase (xylan)	80	20

[a] Medium contained 50 g of Solka-floc and 20 g of $CaCO_3$ per liter. Incubation time, 14 days.
[b] Calculated as percentage of the total enzymatic activity produced per vial.

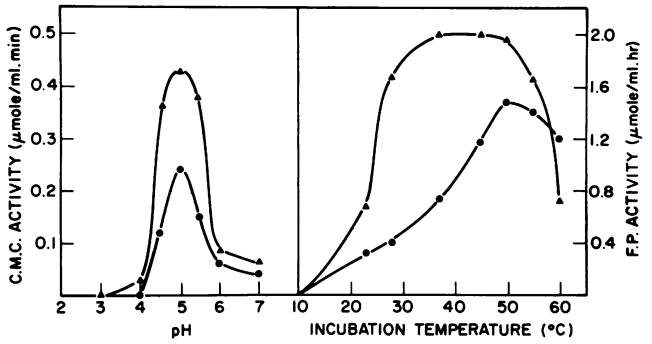

FIG. 1. Effect of pH and incubation temperature on endoglucanase (●; carboxymethyl cellulose [C.M.C.] activity scale) and filter paper (F.P.) (▲; F.P. activity scale) activities.

The results of tests that were made to investigate the involvement of extracellular cellulases produced by *B. cellulosolvens* in the formation of sugars showed that the sugars were mainly produced in the presence of cells (Fig. 3). In samples which contained both the bacterial cells and the culture broth, the formation of sugars continued between days 7 and 14 of incubation. On the other hand, in samples in which the cells were removed after day 7 of incubation and the culture broth was supplemented with fresh cellulose and incubated under pH, Eh (−350 mV), and temperature conditions identical to those of the control samples, the amounts of sugars formed between days 7 and 14 of incubation were much lower. These results indicate that the accumulation of sugars, particularly glucose, is not brought about by the extracellular enzyme system produced by *B. cellulosolvens* but by the bacterial cells with the help of cell-associated enzymes. Unlike many cellulolytic microorganisms (4), *B. cellulosolvens* does not appear to utilize cellobiose by hydrolyzing it to glucose with β-glucosidase. Alternately, for assimilation, cellobiose can be converted to

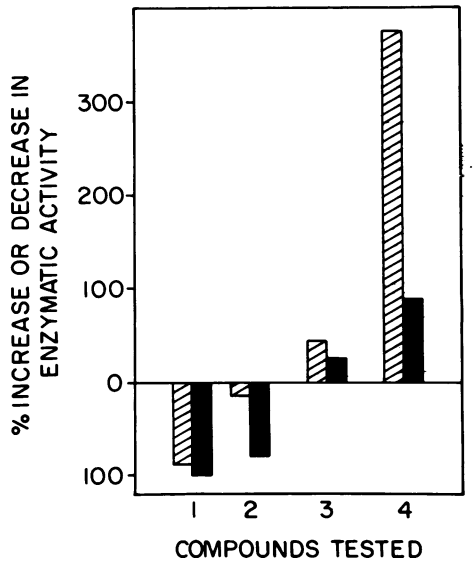

FIG. 2. Effect of 100 mM EDTA (column 1), 10 mM EDTA (column 2), 10 mM dithiothreitol (column 3), and 1 mM ascorbic acid (column 4) on endoglucanase (▨) and filter paper (■) activities.

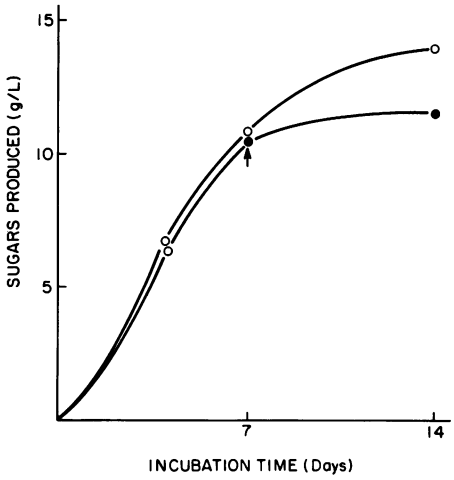

FIG. 3. Sugar formation by whole culture (○) and cell-free broth (●) obtained from *B. cellulosolvens*. Arrow indicates time of removal of bacterial cells from the broth and addition of Solka-floc.

glucose 1-phosphate and glucose by the action of cellobiose phosphorylase. This metabolic pathway has been demonstrated in some cellulolytic bacteria (2, 14, 17, 18) and would seem to be a reasonable possibility for the pathway used by this anaerobe. Further work is in progress to elucidate the mechanism of sugar formation by *B. cellulosolvens* and other mesophilic cellulolytic anaerobes.

LITERATURE CITED

1. **Avgerinos, G. C., H. Y. Fang, I. Biocic, and D. I. C. Wang.** 1980. A novel single-step microbial conversion of cellulosic biomass to ethanol, p. 119–124 *In* M. Moo-Young and C. W. Robinson (ed.), Advances in biotechnology, vol. 2. Pergamon Press, Inc., Elmsford, N.Y.
2. **Ayers, W. A.** 1958. Phosphorylation of cellobiose and glucose by *Ruminococcus flavefaciens*. J. Bacteriol. **76**:515–517.
3. **Giuliano, C., M. Asther, and A. W. Khan.** 1983. Comparative degradation of cellulose and sugar formation by three newly isolated mesophilic anaerobes and *Clostridium thermocellum*. Biotechnol. Lett. **5**:395–398.
4. **Halliwell, G.** 1979. Microbial β-glucanases. Prog. Ind. Microbiol. **15**:1–60.
5. **Johnson, E. A., M. Sakajoh, G. Halliwell, A. Madia, and A. L. Demain.** 1982. Saccharification of complex cellulosic substrates by the cellulase system from *Clostridium thermocellum*. Appl. Environ. Microbiol. **43**:1125–1132.
6. **Khan, A. W.** 1977. Anaerobic degradation of cellulose by mixed culture. Can. J. Microbiol. **23**:1700–1705.
7. **Khan, A. W., and W. D. Murray.** 1982. Single step conversion of cellulose to ethanol by a mesophilic coculture. Biotechnol. Lett. **4**:177–180.
8. **Khan, A. W., J. N. Saddler, G. B. Patel, J. R. Colvin, and S. M. Martin.** 1980. Degradation of cellulose by a newly isolated mesophilic anaerobe, *Bacteriodaceae* family. FEMS Microbiol. Lett. **7**:47–50.
9. **Khan, A. W., and T. M. Trottier.** 1978. Effect of sulfur-containing compounds on anaerobic degradation of cellulose to methane by mixed cultures obtained from sewage sludge. Appl. Environ. Microbiol. **35**:1027–1034.
10. **Khan, A. W., T. M. Trottier, G. B. Patel, and S. M. Martin.** 1979. Nutrient requirement for the degradation of cellulose to methane by a mixed population of anaerobes. J. Gen. Microbiol. **112**:365–372.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Miller, G. L.** 1959. Use of dinitrosalicylic acid reagent for the determination of reducing sugars. Anal. Chem. **31**:426–428.
13. **Murray, W. D., L. C. Sowden, and J. R. Colvin.** 1984. *Bacteroides cellulosolvens* sp. nov., a cellulolytic species from sewage sludge. Int. J. Syst. Bacteriol. **34**:185–187.
14. **Ng, T. K., and J. G. Zeikus.** 1982. Differential metabolism of cellobiose and glucose by *Clostridium thermocellum* and *Clostridium thermohydrosulfuricum*. J. Bacteriol. **150**:1391–1399.
15. **Raabo, E., and T. C. Terkildsen.** 1960. On the enzymatic determination of blood glucose. Scand. J. Clin. Lab. Invest. **12**:402–406.
16. **Saddler, J. N., and A. W. Khan.** 1980. Cellulase production by *Acetivibrio cellulolyticus*. Can. J. Microbiol. **26**:760–765.
17. **Sih, C. J., and R. H. McBee.** 1955. A cellobiose-phosphorylase in *Clostridium thermocellum*. Proc. Mont. Acad. Sci. **15**:21–22.
18. **Swisher, E. J., W. O. Storvick, and K. W. King.** 1964. Metabolic nonequivalence of two glucose moieties of cellobiose in *Cellvibrio gilvus*. J. Bacteriol. **88**:817–820.
19. **Weimer, P. J., and J. G. Zeikus.** 1977. Fermentation of cellulose and cellobiose by *Clostridium thermocellum* in the absence and presence of *Methanobacterium thermoautotrophicum*. Appl. Environ. Microbiol. **33**:289–297.