APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1986, p. 710–714
0099-2240/86/040710-05$02.00/0

Vol. 51, No. 4

Symbiotic Relationship of *Bacteroides cellulosolvens* and *Clostridium saccharolyticum* in Cellulose Fermentation†

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Received 4 November 1985/Accepted 22 January 1986

In coculture, *Bacteroides cellulosolvens* and *Clostridium saccharolyticum* fermented 33% more cellulose than did *B. cellulosolvens* alone. Also, cellulose digestion continued at a maximum rate 48 h longer in coculture. *B. cellulosolvens* hydrolyzes cellulose and supplies *C. saccharolyticum* with sugars and a growth factor replaceable by yeast extract. Alone, *B. cellulosolvens* exhibited an early cessation of growth which was not due to nutrient depletion, low pH, or toxic accumulation of acetic acid, ethanol, lactic acid, H_2, CO_2, cellobiose, glucose, or xylose. However, a 1-h incubation of *B. cellulosolvens* spent-culture medium with *C. saacharolyticum* cells starved for growth factor allowed a resumption of *B. cellulosolvens* growth. The symbiotic relationship of this naturally occurring coculture is one of mutualism, in which the cellulolytic microbe supplies the saccharolytic microbe with nutrients, and in turn the saccharolytic microbe removes a secondary metabolite toxic to the primary microbe.

Early attempts to study cellulolytic microorganisms were hindered by the inability of investigators to isolate pure cultures. Cellulolytic cultures were usually composed of a cellulolytic microbe and one or two closely associated noncellulolytic contaminants (5). The impossibility of isolating a cellulolytic microbe and the fact that contaminants were so closely associated with cellulolytic microorganisms led Pochon (23) to postulate that cellulose fermentation by a single bacterial species was impossible and that noncellulolytic associated forms were required. Not until the development of anaerobic isolation techniques by Hungate (10, 11) were cellulolytic cultures of unquestioned purity obtained (5). Enebo (6) isolated *Clostridium thermocellulaseum* from a mixed cellulolytic culture containing three microbial species. In pure culture, the cellulolytic activity of *C. thermocellulaseum* was greatly reduced. Hungate (10) suggested that the beneficial effects of associated microbes on cellulose digestion were not the consequence of direct influence on the cellulase system but rather occurred through synthesis of essential nutrilites, establishment of a suitable anaerobic environment, or removal of toxic metabolites.

A naturally occurring mesophilic coculture was used in our laboratory to carry out the single-step conversion of cellulose to ethanol (14). This coculture was isolated from a cellulose enrichment culture started from sewage sludge and contained a cellulolytic microbe, *Bacteroides cellulosolvens* (20), and a saccharolytic microbe, *C. saccharolyticum* (19). A pure culture of *C. saccharolyticum* was obtained by heating the coculture to 90°C for 10 min. This noncellulolytic sporeformer would grow only if autoclaved coculture liquid or yeast extract was added to the synthetic medium. This indicated that in coculture *C. saccharolyticum* depended upon *B. cellulosolvens* for the production of sugars from cellulose and the production of a growth factor replaceable by yeast extract (19).

Unlike the saccharolytic microbe, *B. cellulosolvens* could not grow in glucose-yeast extract broth and therefore was amenable to isolation by penicillin counterselection (20). *B. cellulosolvens* grew well by itself in cellulose-synthetic medium and showed no direct need for the presence of *C. saccharolyticum*. The relationship of these two microbes in cellulose fermentation was studied further and is discussed here.

MATERIALS AND METHODS

Microorganisms and media. *B. cellulosolvens* ATCC 35603 and *C. saccharolyticum* ATCC 35040 were used in this study. These microbes were maintained in the simplified synthetic medium developed for *B. cellulosolvens*. A detailed description of this medium and its preparation was reported earlier (18). For the growth of *C. saccharolyticum* alone, this medium was supplemented with 0.5% (wt/vol) yeast extract.

All tests were conducted in 60-ml serum vials containing 10 ml of medium. The cellulosic substrate used was Solka Floc (delignified ball-milled pulp; Brown Co., Berlin, N.H.). Soluble substrates, glucose and cellobiose, were sterilized separately and added to the medium with a hypodermic syringe. Extra buffering capacity was achieved by the addition of 50 g of Ca^{2+}-charged cation-exchange resin (Amberlite IRC-50; Rohm & Haas Co., Milwaukee, Wis.) per liter to the medium (25). To observe coculture growth on solid synthetic medium, agar was stab inoculated and overlayed with sterile filter paper.

Electron micrographs. For electron microscopy, the coculture was grown for 48 h in synthetic medium containing 20 g of Solka Floc per liter. After dilution in distilled water, the cells were placed in Formvar films and dialyzed overnight. The films were placed on carbon-coated grids, shadowed with Pd-Au, and examined with a Siemens electron microscope, model 101.

Analytical techniques. The amount of cellulose degraded by *B. cellulosolvens* alone and in coculture with *C. saccharolyticum* was calculated from dry weight determination of residual cellulose. Cells and protein were dissolved and removed from the cellulose by NaOH treatment. An equal volume of 2 N NaOH was added to the culture, which was then heated for 10 min in boiling water and then centrifuged at 12,000 × *g* for 15 min. The dissolved biomass was either discarded or used for protein determination. The residual

† National Research Council of Canada paper number 25357.

cellulose pellet was suspended in water, collected by membrane filtration (pore size, 0.22 μm; Millipore Corp., Bedford, Mass.), washed free of NaOH with 100 ml of water, and dried to constant weight at 80°C. The amount of cellulose degraded was calculated as the difference between the amount degraded in test vials and in similarly treated uninoculated control vials. The accuracy of this method was verified by the anthrone procedure of Herbert et al. (8).

Growth on soluble substrates was measured as A_{650} of the culture in a cuvette with a 10-mm light path. Protein concentration, determined by the method of Lowry et al. (16), was used to quantify growth on cellulose. Growth was also indirectly estimated by product formation. Ethanol and acetic acid were assayed by gas chromatography by the method of Ackman (1) for GLC of volatile fatty acids. Gas volume was measured with a gas manometer, and the gas composition (N_2, CO_2, and H_2) was determined by gas chromatography by the method of van Huyssteen (27). The volumes and composition of the gases produced were corrected for initial headspace gas. Lactic acid was determined enzymatically by the method of Olson (21). The dinitrosalicylic acid method of Miller (17) was used to measure concentrations of cellobiose and total reducing sugars, and glucose was assayed enzymatically with glucose oxidase (kit number 510; Sigma Chemical Co., St. Louis, Mo.).

Test conditions. Tests were performed to identify the heat-stable growth factor required by *C. saccharolyticum*. Yeast extract was replaced by the following medium components (final concentrations): (i) a purine-pyrimidine mixture of cytosine, 5-methylcytosine, xanthine, uracil, uridine, adenine, guanine, and thymidine (10 mg/liter); (ii) a mixture of 22 amino acids (10 mg/liter); (iii) a mixture of the alcohol-soluble vitamins A, D, E, and K (1 mg/liter), added by the procedure of Holdeman et al. (9) for vitamin K; (iv) a mixture of water-soluble vitamins consisting of *p*-aminobenzoic acid, ascorbic acid, biotin, cyanocobalamin, folic acid, lipoic acid, myoinositol, niacinamide, nicotinic acid, pantothenic acid, pyridoxal hydrochloride pyridoxamine, pyridoxine, riboflavin, and thiamine (0.02 mg/liter); (vi) casein (5 g/liter); (vii) Casamino Acids (5 g/liter; Difco Laboratories, Detroit, Mich.); (viii) peptone (5 g/liter); (ix) betaine (1 mg/liter); and (x) hemin (5 mg/liter). Rumen fluid, sewage sludge liquid, and *B. cellulosolvens* spent-culture medium were also tested as replacements for yeast extract. They were clarified by centrifugation under anaerobic conditions, and each was added to synthetic medium at 30% (vol/vol) concentration. To ensure that these growth factors were not added at toxic levels, a parallel set of media was prepared which contained yeast extract (5 g/liter) and the replacement compound.

The ability of *B. cellulosolvens* to degrade cellulose (Solka Floc, 50 g/liter) alone and in coculture with *C. saccharolyticum* was determined. Cellulose degradation, production of ethanol, acetic acid, and reducing sugars, and culture pH were monitored for 21 days.

The possibility that end product accumulation inhibited the growth of *B. cellulosolvens* was investigated by the addition of ethanol, acetic acid, lactic acid, and reducing sugars, at concentrations equal to those found in 10-day fermentation broths (60 mM acetate, 50 mM ethanol, 7 mM lactic acid, 25 mM cellobiose, 13 mM glucose, and 10 mM xylose), to fresh medium. To determine whether growth cessation was due to nutrient depletion, spent-culture medium was filter sterilized under anaerobic conditions and used to rehydrate fresh medium components. This refreshed medium contained new cellulose, nutrients, and an 80% N_2–20% CO_2 (vol/vol) headspace and had a pH of 6.8.

Spent-culture medium was also treated with *C. saccharolyticum* cells to see whether the saccharolytic microbe might be able to remove any toxic compound(s). *C. saccharolyticum* was grown for 24 h in medium containing 2% cellobiose and 0.5% yeast extract. The cells were then washed to free them of yeast extract. Half of the cells were suspended in synthetic medium without yeast extract to starve the cells for growth factor. The other half were suspended in medium containing an excess of yeast extract (2.5%). After 24 h of incubation, the two cultures were harvested and suspended to equal cell concentrations in filter-sterilized spent-culture medium. Treatment with *C. saccharolyticum* cells was carried out for 1 h at 35°C (longer treatment periods resulted in high acetic acid production). These two treated spent-culture media were again filter sterilized and used to rehydrate fresh medium components, which were then used in the same manner as described above. All tests were performed in triplicate and on two separate occasions.

RESULTS

Electron micrographs of the coculture showed that *B. cellulosolvens* and *C. saccharolyticum* exist in close physical contact (Fig. 1). Cells of these two species were often observed to be touching and, in many cases, to be aligned along part of their lengths (14). Coculture colonies had a glistening, shiny appearance and grew out from the point of inoculation on filter paper (Fig. 2). When the colonies were touched with an inoculating loop, a viscous slime thread followed the loop.

Attempts to identify the heat-stable growth factor required by *C. saccharolyticum* were unsuccessful. It did not grow in media containing any of the defined synthetic mixtures. However, it grew well in medium with yeast extract and, to a lesser extent, in medium supplemented with *B. cel-*

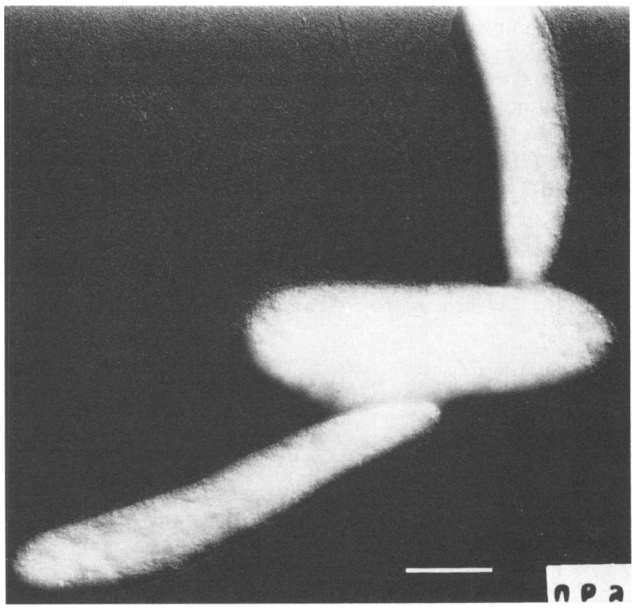

FIG. 1. Electron micrograph of the coculture grown for 48 h on Solka Floc. The shorter, thicker rod is *B. cellulosolvens*; *C. saccharolyticum* is the longer, spindle-shaped rod. Bar = 1.0 μm.

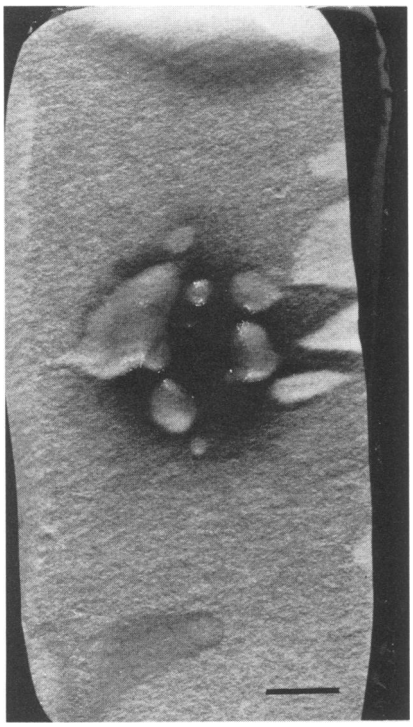

FIG. 2. Coculture colony growth on filter paper at day 6. Bar = 1 cm.

lulosolvens culture broth or sewage sludge liquid. Light growth occurred in the presence of peptone (Table 1).

B. cellulosolvens and *C. saccharolyticum* grown on cellulose and glucose, respectively, both produced the metabolites CO_2, H_2, ethanol, acetic acid, and lactic acid, but in different proportions. Reducing sugars, which are a result of cellulase activity, were also produced by *B. cellulosolvens* grown on cellulose.

The rate of cellulose digestion by *B. cellulosolvens* alone and in coculture with *C. saccharolyticum* was the same for the first 4 days of the fermentation (Fig. 3). After this time, the rate of cellulose degradation by *B. cellulosolvens* decreased, and reducing sugars began to accumulate. This is in contrast to the coculture in which cellulose digestion continued at approximately the same rate for an additional 48 h, at which time the coculture had an acetic acid concentration of

TABLE 1. *C. saccharolyticum* growth in synthetic medium supplemented with yeast extract, peptone, *B. cellulosolvens* culture broth, or sewage sludge liquid

Medium supplement[a]	Growth (OD650)[b]	Product formation (mmol/l)		
		Total H_2 and CO_2	Ethanol	Acetic acid
Glucose	0.07	0	0	0
Yeast extract	0.07	0	4.8	4.1
Glucose-yeast extract	1.34	504.0	227.0	64.3
Glucose-peptone	0.24	57.0	21.0	26.0
Glucose-culture broth	0.63	107.0	50.5	54.2
Glucose-sewage sludge liquid	0.60	95.2	48.6	51.0

[a] Supplements were added at the following concentrations (grams per liter): glucose, 40; yeast extract, 5; and peptone, 5. *B. cellulosolvens* culture broth and the sewage sludge liquid were clarified by centrifugation and added at a 30% (vol/vol) concentration.

[b] OD_{650}, Optical density at 650 nm.

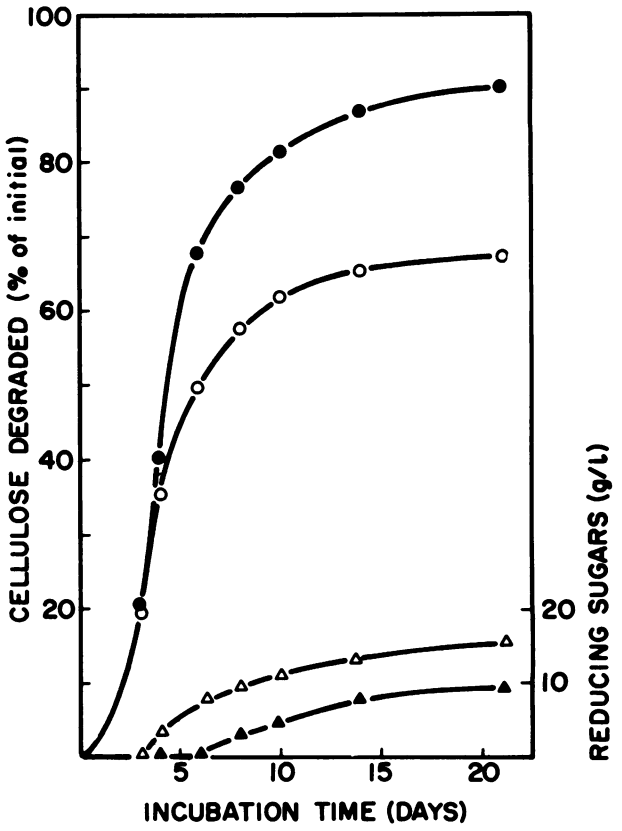

FIG. 3. Cellulose degradation (○ and ●) and sugar production (△ and ▲), by the *B. cellulosolvens-C. saccharolyticum* coculture (●, ▲) and by *B. cellulosolvens* alone (○, △).

97 mM, a level that has been found to inhibit *B. cellulosolvens* growth (unpublished data). On day 8, ethanol and acetic acid production ceased, and reducing sugars began to accumulate in the culture broth. Accumulation of sugar in the fermentation broth indicates that the microbes have stopped growing (7). However, the cellulases remain active and continue to saccharify cellulose until high cellobiose levels inhibit enzyme activity. The presence of *C. saccharolyticum* in the coculture, therefore, improves total cellulose degradation by keeping the level of reducing sugars low for a longer period of time. Also, the fact that cellulose digestion continued at an undiminished rate for an additional 48 h in coculture suggests that *C. saccharolyticum* exerts another beneficial effect, perhaps through the removal of metabolites toxic to *B. cellulosolvens*.

To determine whether *B. cellulosolvens* growth cessation was due to nutrient depletion, filter-sterilized spent-culture medium from a 10-day cellulose fermentation was used to reconstitute fresh, dried medium components and was inoculated with a fresh, actively growing culture of *B. cellulosolvens* (2.5%, vol/vol). The refreshed medium (pH 6.8) was unable to support *B. cellulosolvens* growth, indicating that neither nutrient limitation nor low pH had caused growth cessation. Similarly, toxic accumulation of the major fermentation products, acetic acid, ethanol, lactic acid, cellobiose, glucose, and xylose, did not cause growth cessation, because fresh inoculum grew well in medium supplemented with these compounds at concentrations normally found in 10-day-old culture broths, producing 26.3 mmol of ethanol per liter, 23.8 mmol of acetic acid per liter, and 0.37

mg of cell protein per ml. Also, H_2 and CO_2 accumulation alone did not cause total growth cessation. This was shown by the fact that spent-culture medium, which had undergone degassing during vacuum filtration and was bottled under fresh headspace gas, did not support the growth of fresh inoculum.

Although these major causes of growth inhibition do not appear to be applicable to *B. cellulosolvens*, the possibility of sensitivity to low-level accumulation of a secondary metabolite still existed and was investigated by treatment of filter-sterilized spent-culture medium with *C. saccharolyticum* cells. In spent-culture medium treated with cells starved for the growth factor, fresh *B. cellulosolvens* inoculum produced an additional 11.8 and 10.6 mmol of ethanol and acetic acid per liter, respectively. There was also a moderate increase (0.16 mg/ml) in cell protein. No growth occurred in spent-culture medium treated with cells not starved for the growth factor. *C. saccharolyticum*, therefore, takes up a compound(s) from spent-culture medium toxic to *B. cellulosolvens*. This removal of toxin would appear to be due to active uptake, not cell adsorption, because unstarved cells did not remove any toxic compound(s) from the medium.

The fact that *C. saccharolyticum* cells grown in 2.5% yeast extract did not remove toxin from spent-culture medium indicates that yeast extract contains this compound. If this is true, then the cellulolytic microbe should also be sensitive to yeast extract. This is indeed the case (Fig. 4). In cellobiose broth, *B. cellulosolvens* growth was 81% inhibited by 1% (wt/vol) yeast extract and completely inhibited by 2.5% (wt/vol) yeast extract. In contrast, *C. saccharolyticum* growth was stimulated by increased yeast extract concentrations.

DISCUSSION

In anaerobic environments in which cellulose is degraded, interdependent, symbiotic microbial associations have evolved (12). Microbes within the consortium depend on each other for nutrient flow, removal of toxic metabolites, pH balance, and establishment of a suitable oxidation-reduction potential. Mechanisms have been developed to guard or aid these relationships. *C. saccharolyticum* and *B. cellulosolvens* live in close physical proximity (Fig. 1). This closeness ensures that sugars and growth factor released from the cellulolytic microbe are quickly and efficiently delivered to the saccharolytic microbe. Also, the viscous, slimy nature of the coculture helps ensure that the two species remain together. The success of this mechanism has been shown by the inability of conventional isolation techniques to separate the two microbes (20).

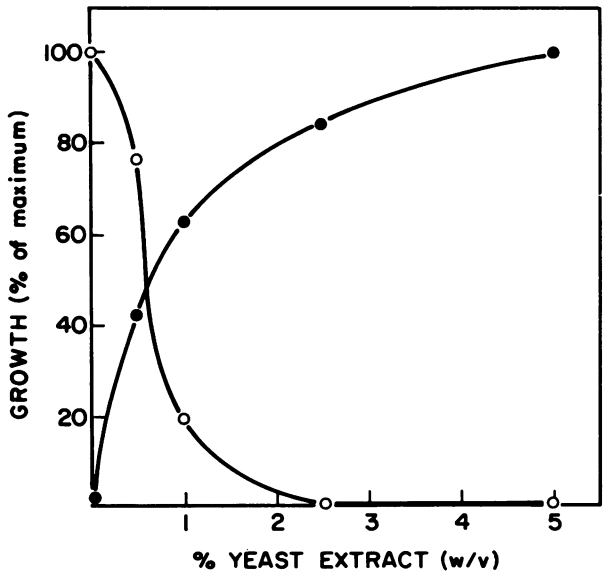

FIG. 4. The effect of yeast extract on *B. cellulosolvens* (○) and *C. saccharolyticum* (●) growth in cellobiose broth. Maximum optical densities at 650 nm attained were 1.25 and 3.2 for *B. cellulosolvens* and *C. saccharolyticum*, respectively.

The isolation of some cellulolytic microbes has been difficult because of their dependence for nutrients on other microorganisms of the same ecological niche. Khouvine (15) isolated *Bacillus cellulosae dissolvens* from fecal matter; however, it would grow only in medium containing fecal extract. Similarly, Cowles and Rettger (4) reported isolating a cellulolytic, anaerobic mesophilic sporeformer in medium which had been preincubated with an aerobic, nonsporeforming contaminant that was subsequently eliminated by heating. The cellulolytic microbe in this study is different, because *B. cellulosolvens* is capable of good growth in a defined synthetic medium (18) with cellulose as the sole carbon and energy source and $(NH_4)_2SO_4$ as the sole nitrogen source. However, it does supply *C. saccharolyticum* with nutrients, sugars from cellulose digestion, and an essential heat-stable growth factor (19). Besides yeast extract and *B. cellulosolvens* culture broth, sewage sludge liquid was found to be another source of the growth factor. It is interesting that although many microbes isolated from the rumen require rumen fluid for growth (12), rumen fluid did not support *C. saccharolyticum* growth. It appears, therefore, that separate cellulose-degrading niches which are distinct in their microbial flora and nutrient requirements have evolved.

The influence of *C. saccharolyticum* on cellulose degradation by another cellulolytic microbe, *Acetivibrio cellulolyticus*, has been reported (13). *A. cellulolyticus* supplied the saccharolytic microbe with sugars and growth factor; however, cellulose degradation was reduced by 40% in the presence of *A. cellulolyticus*. This inhibition was believed to be due to competition for available sugar substrate. In the present study, coculture cellulose fermentation continued at a maximum rate 48 h longer than cellulose fermentation by *B. cellulosolvens* alone, and approximately 33% more cellulose was degraded. It appears, therefore, that *B. cellulosolvens* derives benefit from its association with *C. saccharolyticum* and that it can exercise some degree of control over the growth of the potential competitor through nutrilite synthesis.

B. cellulosolvens, *A. cellulolyticus*, and *C. saccharolyticum* were all isolated from the same cellulose enrichment culture started from sewage sludge (19, 20, 22), and both of the cellulolytic microbes produce the growth factor required by the saccharolytic microbe. However, the fact that only *B. cellulosolvens* formed a stable coculture with *C. saccharolyticum* further indicates that *B. cellulosolvens* derives benefit from the association and is the partner responsible for the mechanism that ensures their continued association.

Besides the transfer of nutrients within a microbial community, symbiotic associations may be based on the removal of toxic metabolites (24). For example, Ben-Bassat et al. (2) reported that *Thermoanaerobium brockii* was inhibited by H_2 accumulation, but when it was grown in coculture with

Methanobacterium thermoautotrophicum, interspecies H_2 transfer alleviated the inhibition.

The early cessation of *B. cellulosolvens* growth was not due to low pH, nutrient depletion, or accumulation of major metabolites. However, the inability of refreshed spent-culture medium to support new growth indicated the presence of a toxic secondary metabolite. Many bacterial products, such as low-molecular-weight antibiotics, metabolites, lytic agents, enzymes, and bacteriocins, may be autotoxic as well as inhibitory to other susceptible species (26).

The growth exhibited by *B. cellulosolvens* in spent-culture medium treated with *C. saccharolyticum* cells starved for growth factor shows that the saccharolytic microbe is capable of removing from the medium any compound(s) toxic to the cellulolytic microorganism. The inability of *C. saccharolyticum* cells grown in 2.5% yeast extract to remove toxin indicates that yeast extract contains this compound. This suggestion is further substantiated by the fact that yeast extract inhibits the growth of *B. cellulosolvens*. Although the possibility is tempting, it cannot yet be said that the growth factor produced by *B. cellulosolvens* and replaceable by yeast extract is the same toxin removed by *C. saccharolyticum* and believed to be present in yeast extract. Such comparison must await the isolation and identification of these compounds. The symbiotic relationship of this naturally occurring, stable coculture, therefore, is one of mutualism in which *B. cellulosolvens* supplies *C. saccharolyticum* with nutrients and *C. saccharolyticum* removes any metabolite(s) toxic to *B. cellulosolvens*.

ACKNOWLEDGMENT

The expert technical assistance of Rosemary Weersink is gratefully acknowledged.

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