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Characteristics and Adaptability of Some New Isolates of *Clostridium thermocellum*

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Six strains of *Clostridium thermocellum* isolated from various environments were characterized as to growth rate, production of reducing sugars, ethanol, and acetic acid from cellulose, base composition of DNA, and the abilities to adapt to ethanol and to grow at 45°C. Five of the six new isolates produced 7 to 15% more ethanol and two produced about 45% more reducing sugars than a standard reference strain. One strain (MC-6) adapted more readily to growth in 2% ethanol than the others.

The initial objectives of this research were to identify strains of *Clostridium thermocellum* which are more efficient than existing parental isolates in the bioconversion of cellulose or more adaptable to practical fermentation conditions and to thereby increase the available gene pool for further strain selection and development. Several *Clostridium* strains have been investigated for their potential ethanol production and cellulose degradation (1, 3, 4, 14). Thus far, however, application of this microbial technology has proved elusive. Failure has been in part attributable to the low ethanol production and tolerance of this organism. The feasibility of utilizing thermophilic anaerobes in the bioconversion of cellulose to ethanol has been extensively reviewed by Zeikus (15).

The extreme thermophile *C. thermocellum* holds a unique position in this technology. It ferments cellobiose or glucose to ethanol, and its cellulase hydrolyzes both cellulose and hemicellulose substrates to their monomeric components. However, even though xylans are depolymerized, xylose is not fermented. Cellobiose is the principal sugar taken up by *C. thermocellum*. The following improvements must be made before *C. thermocellum* can be effectively employed: (i) increase the cell growth rate, (ii) improve the cellulase activity, and (iii) increase the ethanol tolerance and yield. Additionally, ability to ferment xylose would be useful. Some progress has been made in meeting these objectives, especially with regard to items ii and iii (1, 12). Less has been accomplished in improving the growth rate, probably because growth rate is a function of the overall genome, rather than any particular genetic trait. This research assessed the range of growth rates, cellulase activities, and ethanol production and tolerance occurring in several independent new isolates.

MATERIALS AND METHODS

Isolation and cultivation of strains. Soil, compost, or leachate samples (10 g each) were pasteurized at 90°C for 10 min, flushed with a mixture of 10% hydrogen, 5% carbon dioxide, and 85% nitrogen, and placed in 100 ml of CM3 medium (13) with cellulose (Whatman no. 2 strips) as a carbon source. The cultures were incubated at 55 to 60°C for 3 to 5 days, subcultured, pasteurized again, and incubated for an additional 3 days. If digestion of cellulose occurred, the subculture was streaked on cellulose agar plates and incubated under anaerobic conditions. The isolates were streaked for purity and inoculated into cellulose medium. Isolation and subculturing procedures were repeated three times. Purity of the isolates was verified by scanning electron microscopy and by inoculation of the strain into anaerobic medium with xylose as a carbon source. Inability to grow on xylose is consistent with the characteristics previously identified for *C. thermocellum* (10). This technique was used to verify that certain other thermophilic organisms which might contaminate the *C. thermocellum* cultures at 60°C were not present. The strains were cultured at 60°C in anaerobic tubes on CM3 medium containing 15 g of cellulose (Whatman no. 2 filter paper) per liter as a carbon source.

Assays. Growth rates were determined by measuring the optical density (1.0 cm) of suspended cells at 525 nm (OD_{525}) (10). OD measurements were correlated with protein production; the amount of protein was determined with Coomassie blue (2).

Reducing sugar concentrations in supernatant fractions were determined by the colorimetric dinitrosalycilic acid reagent method of Miller (9). Glucose solutions were used as standards.

The amount of residual cellulose was determined by centrifuging 8 ml of whole broth at 20,000 × *g* for 15 min, suspending the pellet in 5 ml of 8% formic acid to effect cell lysis, filtering the residue through membrane filters (pore size, 0.45 μm; Millipore Corp.), and measuring the dry weights of residual cellulose on the filter (13).

Ethanol and acetic acid concentrations were determined by direct aqueous injection gas chromatography (Varian model 2860-30 gas chromatograph with flame ionization detector) with a Teflon column (180 by 0.63 cm) packed with 3% Carbowax (20 M) and 0.1% H_3PO_4 on 80/100 mesh Carbopack C. Ethanol was separated with a Teflon column (180 by 0.63 cm) packed with 80/100 mesh Porapak Q. The initial oven temperature was 97°C with a hold time of 5 min, followed by an increase to 108°C in increments of 2°C/min. Nitrogen carrier gas was delivered at 25 ml/min. The injector temperature was 225°C; the detector temperature was 350°C.

DNA from 2 to 3 g (wet weight) of cells was isolated by the procedures of Marmur (7). Percent guanine plus cytosine/adenine plus thymine (G+C/A+T) content of DNA was determined by thermal denaturation (8) and optical reassociation (5, 11) with a Gilford 2500S recording spectrophotometer fitted with a thermal programmer.

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TABLE 1. Comparison of isolated strains of *C. thermocellum*

Strain	Source[a]	Final ethanol concn (g/liter)	Final mole ratio of acetic acid/ethanol[b]	Base ratio[c] (% G+C)	Doubling time (h most rapid period)[d]	Maximum reducing sugars (g/liter)	Cellulose solubilized[b] (g/liter)	Final OD
Standard strain	J. G. Zeikus	0.89	1.27		14	3.04	7.62	0.41
MC-1	Composting cow manure	0.96	1.15	38.3	10	4.50	6.56	0.55
MC-2	Pony scat	1.04	1.29	37.1	14	1.15	6.35	0.53
MC-3	Paper landfill leachate	1.00	1.02	38.5	6	2.18	8.70	0.88
MC-4	Cotton compost	0.95	1.28	ND[e]	10	4.36	7.28	0.63
MC-5	Pond mud	1.04	1.29	38.0	14	1.80	5.29	0.34
MC-6	Acid bog mud	0.89	1.28	ND	17	2.69	5.84	0.61

[a] Geographic origins of strains: MC-1, MC-2, and MC-3, Atlanta, Ga.; MC-4, Alabama; MC-5 and MC-6, North Carolina.
[b] After 96 h.
[c] Average of two determinations of G+C.
[d] First 24 h for all strains except for strains MC-3, MC-5, and MC-6, which were between 24 and 48 h.
[e] ND, Not determined.

Adaptation. To adapt cells to growth at lower temperatures, log-phase cultures of each strain were inoculated into fresh medium and placed at 45°C. When the first evidence of growth was observed (approximately 1 month after inoculation), the cultures were transferred into fresh medium. Transfers were repeatedly made at the first evidence of growth at 45°C for a 2-month period.

To adapt strains to higher ethanol concentration, 10 ml of each isolate grown to stationary phase (OD_{525}, 0.6 to 0.7) was irradiated with UV (254 nm) for 8 s at a distance of 25 cm from the UV source and placed in medium with 0.2% ethanol. The strains showing the most rapid growth were transferred stepwise into 0.4, 0.6, and 0.8% ethanol. One strain was adapted to growth in 2% ethanol.

Electron microscopy. For electron microscopy, 3 ml of cells was washed three times with 0.1 M MOPS (morpholinepropanesulfonic acid) buffer (pH 7.5) and fixed with 3% glutaraldehyde. The micrographs were taken with a Perkin-Elmer scanning electron microscope.

RESULTS AND DISCUSSION

Six strains were isolated and identified as *C. thermocellum* by the following characteristics (10): (i) motile rods producing terminal spores; (ii) growth at 60°C with cellulose carbon source; filamentous growth in liquid medium; (iii) production of yellow pigment; (iv) lack of growth on xylose; (v) base ratio between 37.1 and 38.5% G+C; (vi) similar morphology in electron micrographs; (vii) production of ethanol and acetic acid in an approximately 1:1 ratio. No isolate was obtained which was capable of utilizing xylose and which exhibited the other traits characteristic of *C. thermocellum*.

The six characterized isolates (MC-1 through MC-6) exhibited distinct patterns of growth and solubilization of cellulose (Table 1). Generally, ethanol and acetic acid concentrations were low. Final ethanol concentrations ranged from 0.89 g/liter for the standard strain to 1.04 g/liter for MC-2 and MC-5. Almost all of the ethanol and acetic acid production occurred in the first 24 h. The acetic acid-to-ethanol ratio for all strains was relatively consistent, ranging from 1.02 for MC-3 to 1.29 for MC-2 and MC-5. The highest levels of reducing sugars (3 to 4.5 g/liter) were attained by MC-1, MC-4, and the standard strain. The cellulose solubilized in 96 h ranged from 5.29 g/liter for MC-5 to 8.7 g/liter for MC-3. The relatively high rate of cellulose degradation for MC-3 did not result in a higher level of ethanol production or in the accumulation of reducing sugar but rather in apparent cell growth. It is possible that production of the yellow pigment, reportedly an affinity factor in the cellulolytic system (6, 10), interfered with growth determinations by OD_{525}. Increases in OD_{525} correlated with increases in protein and cellulose solubilized. The correlation coefficient for the OD_{525} value and the amount of cellulose solubilized was 0.68. The cell growth in 96 h (OD_{525}) ranged from 0.34 (MC-5) to 0.88 (MC-3). MC-3 also showed a high level of cellulose solubilized (8.70 g/liter) in 96 h; however, the product was not utilized but accumulated as reducing sugar.

The irradiated MC-6 strain grew more rapidly than the other irradiated strains in 0.8% ethanol. However, the growth rate in 0.8% ethanol was only one-half that of the original strain without ethanol present in the medium. After additional selection with this strain, some growth was evident in 2% ethanol, but it continued to be extremely slow after a 96-h lag period.

Adaptation to 45°C was attempted to see whether an organism might be obtained which could be cocultured with *Saccharomyces cerevisiae*. After a 2-month selection period, growth rates at 45°C increased by a factor of 3. However, rates at 60°C were still three to four times those at 45°C. The latter temperature is still well outside the range of most yeasts.

Although considerable differences were observed among these strains in growth and ability to solubilize cellulose, they were all remarkably similar in ethanol and acetic acid production. They were all notably poorer than adapted laboratory strains in ethanol production (1, 3), but they were all similar to a standard laboratory strain of this species. Low concentrations of ethanol inhibited growth of these strains, but they adapted to higher ethanol tolerance relatively easily.

From the variability observed among these strains, it seems likely that continued screening for natural isolates could lead to the selection of better stocks for further strain improvement. Incorporation of low concentrations of ethanol into soil enrichments might be useful in selecting for strains with greater ethanol tolerance. Selection for higher growth rates and cellulase activities might be achieved through continuous enrichment culture with cellulose as a sole carbon source. Subsequent strain selection and adaptation in the laboratory could be used to amplify these traits.

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