APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 203–206
0099-2240/89/010203-04$02.00/0

Vol. 55, No. 1

Cellulose Fermentation by an Asporogenous Mutant and an Ethanol-Tolerant Mutant of *Clostridium thermocellum*

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Received 6 June 1988/Accepted 26 October 1988

Two mutants of *Clostridium thermocellum* were isolated after UV light mutagenesis. Mutant A1, selected as asporogenous, exhibited a fermentation pattern similar to that of the wild type. However, at pH 6.5, the mutant degraded 12% more cellulose than did the wild type, leading to enhanced ethanol production. Mutant 647, selected as ethanol tolerant, was able to grow in medium containing 4% ethanol. During the early stage of the exponential growth phase, ethanol was produced as the main product, up to a concentration of about 9 g/liter. After 3 days of culture, 48.3 g (89% of the initial amount) of degraded cellulose per liter was fermented into 12.7 g of ethanol per liter.

Clostridium thermocellum, a gram-positive, thermophilic, and anaerobic bacterium can produce ethanol directly from cellulose. However, low ethanol tolerance and poor ethanol conversion yields make the process unpractical for industrial purposes, unless *C. thermocellum* is genetically modified for improved ethanol production. In the accompanying report (5), we describe a mutant of *C. thermocellum* with a pleiotropic phenotype. The ethanol-tolerant and asporogenous mutant LD1 was able to produce 14.5 g of ethanol per liter from 63 g of degraded cellulose. Both characteristics may contribute to ethanol hyperproduction. Ethanol tolerance may prevent growth inhibition at low ethanol concentration, and the absence of spore formation may allow nongrowing cells to proceed with fermentation instead of undergoing total arrest of metabolism. Furthermore, the growth of mutant LD1 displayed a 2-day lag period after transfer from a starter culture grown in the presence of cellobiose to a fermentor-containing cellulose as the carbon source.

It was important to analyze separately the contribution of each characteristic to enhance ethanol production and to find a strain growing with no delay upon transfer from the starter culture to the fermentor. Two other mutants were analyzed. Mutant A1 is asporogenous and ethanol sensitive, and mutant 647 is ethanol tolerant and sporogenous. Growth and cellulose fermentation properties of both mutants were compared with one another and with those of the wild type.

MATERIALS AND METHODS

Organism. *C. thermocellum* NCIB 10682 was used. Mutagenesis of spores by UV irradiation is described in the accompanying paper (5).

Isolation of asporogenous mutant A1. Irradiated spores were allowed to germinate in CM3-3 medium containing 0.5% cellobiose. When forespores were observed, a sample (10% [vol/vol]) was transferred to minimal medium (5) containing 0.5% cellobiose. After 12 transfers under the same conditions, no spore could be detected by microscopic examination of the culture. Single-colony isolates were obtained on minimal cellobiose agar medium, and their sporulation rate was determined. The A1 mutant was chosen for its low sporulation rate.

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Isolation of ethanol-tolerant mutant 647. After germination of the irradiated spores in CM3-3 medium containing 0.5% cellobiose, cells were transferred into the same medium containing 1.0% ethanol. The process was repeated with increasing ethanol concentrations up to 4.0% by steps of 0.5%. Since growth was obtained with 3% ethanol but not with 3.5%, spores adapted to 3% ethanol were irradiated with UV light to obtain a higher level of resistance. After germination in full-nutrient medium, the ethanol concentration was increased to 4%. Since growth with 5% ethanol was very slow, cells grown in the presence of 4% ethanol were plated onto solid medium containing the same concentration of inhibitor. After 3 days of incubation, some well-developed colonies were tested for resistance to 4% ethanol in liquid medium. Mutant 647 was studied further. Media, culture conditions, analytical procedures, and calculation methods were as described in the accompanying paper (5).

RESULTS

Asporogenous phenotype of mutant A1. Mutant A1 presented no sporulation septum even after 3 h of stationary phase. Thus, sporulation was blocked at an early stage, as it was for mutant LD1 (5). The sporulation rate of mutant A1 was $<10^{-9}$, compared with about 0.15 for the wild type and for mutant 647.

Growth properties of the mutants. Figure 1 illustrates the identical ethanol sensitivities of the A1 mutant and of the wild type, and the ethanol-tolerant phenotype of mutant 647. The specific growth rate was higher for both mutants than for the wild type. Mutant 647 was able to grow in medium containing 4% ethanol.

Fermentation properties of the wild-type strain, the asporogenous mutant A1, and the ethanol-tolerant mutant 647. With all strains, ethanol conversion yields from cellulose and from fermented glucose equivalent were better at pH 7.2 than at pH 6.5 (Table 1). At both pH levels, fermentation product ratios of the wild type and mutant A1 showed little difference. At both pH levels, acetate yields from fermented glucose equivalent were somewhat higher from the A1 mutant. In contrast, ethanol yields from mutant 647 were significantly higher, and acetate and lactate yields were lower than those from the wild type.

With all strains, cellulose degradation was more efficient at pH 6.5 than at pH 7.2 (Fig. 2). At pH 6.5, mutant A1

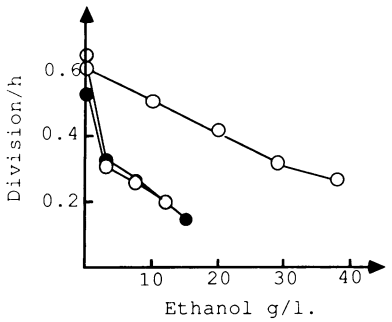

FIG. 1. Specific growth rate of *C. thermocellum* grown in CM3-3 medium containing cellobiose as a carbon source and various concentrations of ethanol. Symbols: ●, wild type; ◕, asporogenous mutant; ○, ethanol-tolerant mutant.

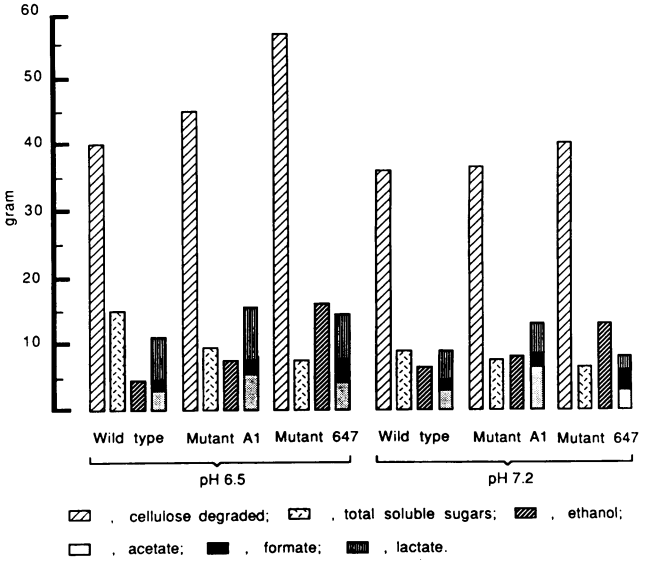

FIG. 2. Bioconversion of cellulose into end products by *C. thermocellum*. Fermentation time: 5 days.

degraded 12% more cellulose and mutant 647 degraded 41% more cellulose than did the wild type. Furthermore, with the wild-type strain, about 37% of the cellulose degraded remained as nonfermented soluble sugars. With mutants A1 and 647, the accumulation of nonfermented soluble sugars was reduced to 21 and 14% of the cellulose degraded, respectively. Accordingly, glucose concentrations accumulated in culture media of the wild type and the mutants A1 and 647 were 9.7, 4.5, and 1.7 g/liter, respectively, and cellobiose concentrations were 1.6, 0.7, and 0.7 g/liter, respectively.

At pH 7.2, the three strains displayed less variation in the amount of cellulose degraded (36.1 to 40.3 g) and total accumulation of nonfermented sugars (6.7 to 9.1 g). Likewise, levels of glucose accumulated were similar (2.1 to 2.6 g/liter), whereas cellobiose concentrations were reduced from 2.6 g/liter (wild type) to 1.6 and 1.2 g/liter, respectively, for the mutants A1 and 647.

The sum of glucose and cellobiose concentrations was not equal to the concentration of nonfermented soluble sugars detected by the anthrone method. Soluble sugars included no cellodextrins larger than cellobiose. As described in the accompanying paper (5), the difference may be due to the presence of glucose polymers other than cellodextrins which are not detected by the assays used.

As a result of the higher amount of substrate fermented, both mutants produced more ethanol than did the wild type at pH 6.5. For mutant 647, ethanol production was further enhanced by the fact that a larger fraction of the metabolized substrate was converted into ethanol (Fig. 2; Table 1).

As shown by the kinetics of cellulose degradation and fermentation product formation, growth of the asporogenous mutant at both pH levels was delayed by about 40 h, compared with that of the wild type and the ethanol-tolerant mutant (Fig. 3 and 4).

For both the wild type and the ethanol-tolerant mutant at the end of the growth phase, lactate was the main product; ethanol was the major product at the early stages of culture growth (Fig. 4). Lactate was produced later by mutant 647, in keeping with the absence of early growth inhibition caused by the accumulation of ethanol. Thus, mutant 647 was able to reach the highest ethanol concentration, i.e., 12.7 g/liter, from 48.3 g of degraded cellulose per liter. The asporogenous mutant A1 differed from the two other strains by producing lactate simultaneously with ethanol.

TABLE 1. Products of cellulose fermentation by *C. thermocellum* at two pH levels after 5 days of culture

Strain[a]	Amt produced (mmol/100 mmol of GEF[b]) of:						% Product yield[c]	% Carbon recovery[d]	O/R[e] index	% Yield[f]
	Ethanol	Acetic acid	Lactic acid	Formic acid	CO_2	H_2				
pH 6.5										
WT	61	32	44	19	73	44	68	79	0.99	20
A1	73	41	40	20	94	58	77	82	1.02	30
647	114	22	25	26	110	16	81	83	1.01	50
pH 7.2										
WT	85	31	26	20	96	34	71	78	1.04	33
A1	96	58	28	26	128	44	91	93	1.19	39
647	134	24	13	31	127	14	86	88	1.01	57

[a] WT, Wild type; A1, asporogenous mutant; 647, ethanol-tolerant mutant.
[b] GEF, Glucose equivalent fermented.
[c] Carbon from fermentation products/carbon from glucose equivalent fermented.
[d] Carbon from end products and soluble sugars/carbon from glucose equivalent liberated.
[e] O/R, Oxido-reduction balance.
[f] Conversion yield of cellulose into ethanol.

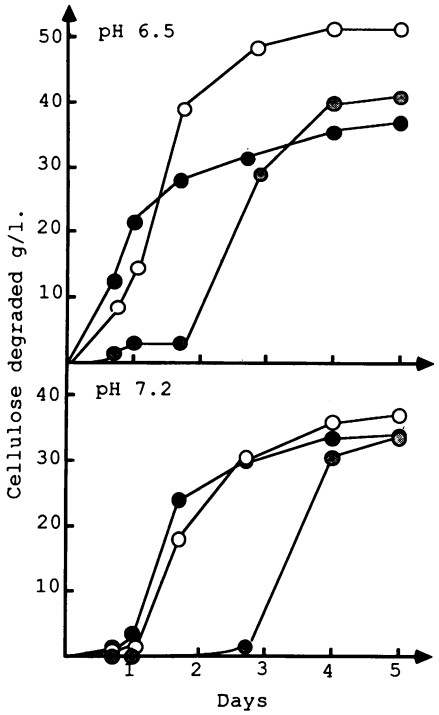

FIG. 3. Kinetics of cellulose degradation by *C. thermocellum*. Initial concentrations of cellulose were 54 g/liter at pH 6.5 and 41.6 g/liter at pH 7.2 for cultures of mutant 647, and 42 to 44 g/liter for the other cultures. Symbols: ●, wild type; ◔, asporogenous mutant; ○, ethanol-tolerant mutant.

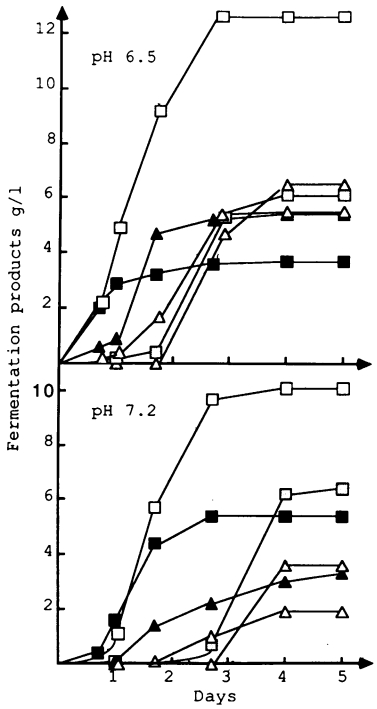

FIG. 4. Kinetics of ethanol (squares) and lactic acid (triangles) production from cellulose by *C. thermocellum*. Initial concentrations of cellulose were as indicated in Fig. 3. Closed symbols indicate wild type; stippled symbols indicate asporogenous mutant; open symbols indicate ethanol-tolerant mutant.

DISCUSSION

Despite the lack of tools available to characterize mutations of *C. thermocellum* genetically, the mutants described in this and the accompanying study (5) shed some light on the characteristics which may influence ethanol production.

Ethanol tolerance appears to be the major factor. Ethanol inhibition causes metabolism to stop prematurely. Furthermore, ethanol-tolerant mutants exhibit an extended early-growth phase, during which ethanol is produced at the highest rate, and therefore have better overall ethanol conversion yields. In this respect, the phenotypes of mutants LD1 (5) and 647 are strikingly similar.

The advantage of asporogenous strains is less obvious. In the case of mutant A1, the conversion into nongrowing cells instead of spores probably accounts for the somewhat higher substrate fermentation at pH 6.5, compared with that of the wild type. Like the wild type, however, mutant A1 was rapidly inhibited by fermentation products so that the increase in productivity was moderate.

Among ethanol-tolerant mutants, the sporogenous mutant 647 did not produce much less ethanol than did the asporogenous mutant LD1, except perhaps at late stages of the cultures during which mutant LD1 fermented the substrate at a reduced rate, whereas metabolism of mutant 647 stopped completely. It must be noted, however, that at late stages the LD1 mutant produced mainly lactate so that the conversion yield of substrate into ethanol fell dramatically (5). The results are in agreement with other reports concerning different asporogenous mutants of other sporogenous bacteria. In *Clostridium botulinum* (1), ethanol levels in cultures of the wild type and of an asporogenous mutant were identical during growth but continued to increase slightly in the asporogenous mutant during the late stationary phase. Other studies of asporogenous mutants of *Clostridium acetobutylicum* (3, 4) and *Clostridium thermosaccharolyticum* (2) showed that the asporogenous phenotype did not enhance significantly the production of solvents or ethanol.

Furthermore, both asporogenous mutants exhibited a longer lag period than did their sporeforming counterparts. There is no indication that the asporogenous phenotype is linked to a prolonged lag phase. Since both mutants may contain more than one mutation, it cannot be concluded that the asporogenous phenotype is linked to a prolonged lag phase. However, if delayed growth is a general feature of asporogenous mutants, it may be a serious limitation of the practical use of such mutants in industrial processes.

For all strains, ethanol conversion yields were better at pH 7.2 than at pH 6.5. However, with the exception of the wild type, this was compensated by a more efficient degradation of cellulose at pH 6.5, leading to enhanced bulk ethanol production.

ACKNOWLEDGMENTS

We express our gratitude to J.-P. Aubert for continuing support and for helpful discussions and critical reading of the manuscript. We thank A. Edelman for his help with the manuscript.

This work was supported by grant ATP 955355 from the Centre National de la Recherche Scientifique, grant EN3B-0082-F from the Commission of the European Communities, and by research funds from the University of Paris 7.

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