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Organism Development and Characterization for Ethanol Production Using Thermophilic Bacteria

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ABSTRACT

This article provides an overview and evaluation of our recent interdisciplinary work having a bearing on ethanol production using thermophilic bacteria. Based on steady-state effluent substrate concentrations in continuous culture, the ratio of the ethanol-inhibited growth rate and the uninhibited growth rate $(\mu_I/\mu_0)_S$ was determined for *Clostridium thermosaccharolyticum* grown on xylose at both 55 and 60°C. $(\mu_I/\mu_0)_S$ exhibited an approximately linear pattern in relation to ethanol concentration. Based on least square linear fits to the data, ethanol concentrations corresponding to $(\mu_I/\mu_0)_S$ = 0.5 were 29 g/L ethanol at 60°C, and 36 g/L ethanol at 55°C, and 31 g/L for the data combined for both temperatures. It is concluded that ethanol inhibition is unlikely to constrain utilization of *C. thermosaccharolyticum* in processes for ethanol production from cellulosic biomass. Growth at high substrate concentrations using a defined medium has been achieved for *C. thermocellum* in continuous culture with essentially complete substrate utilization at 54 g/L cellobiose concentration. We also include work aimed at elucidating the molecular genetics of *C. thermocellum* with the ultimate goal of pathway manipulation. Antibiotics effective against *C. thermocellum* include chloramphenicol, thiamphenicol, and erythromycin at 125, 10, and 40 μg/mL, respectively. Summarized is work describing a restriction system of *C. thermocellum* and protection against it by methylation. Demonstration of restriction protection and antibiotic sensitivity provides two elements likely to be useful in the transformation of *C. thermocellum*.

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Index Entries: *Clostridium thermocellum; Clostridium thermosaccharolyticum;* ethanol production.

INTRODUCTION

The ethanol-producing thermophilic bacterium *Clostridium thermocellum* produces an active cellulase enzyme complex at high levels (*1,2*). This feature allows rapid hydrolysis and subsequent fermentation of cellulosic materials, for example, >85% utilization of dilute-acid pretreated mixed hardwood in a chemostat with a residence time of 12 h (*3*). *Clostridium thermosaccharolyticum,* as well as some other thermophiles, is capable of rapidly utilizing xylose, which is an important component of many biomass substrates. Continuous cultures of *C. thermosaccharolyticum* have been reported to achieve essentially complete substrate utilization in a 4 h residence time (*4*).

As discussed by Lynd et al. (*5*) and Hogsett et al. (*6*), the above features could be of significance in the context of ethanol production from cellulosic biomass. High conversion rates can be expected to lower the cost of the fermentor(s) in which cellulose and xylose conversion occur. Combined cellulose production and fermentation to ethanol eliminate the need for dedicated enzyme production, with consequent savings because of reduced capital costs and eliminated substrate diversion to enzyme production. The high temperature and completely anaerobic metabolism of thermophiles eliminates the need for oxygenation, required for enzyme production by more traditional aerobic processes, and also refrigerated water for fermentor cooling. These anticipated reductions in required utilities can have significant economic impacts (*7*).

The factors preventing currently available thermophilic strains from being seriously considered for ethanol production include:

1. A lack of consensus in the literature on the ethanol tolerance of thermophiles.
2. Little or no data under conditions of practical interest including high substrate concentrations, an economical growth medium, and potential inhibitors (e.g., from pretreatment) likely to be present in a real-world process.
3. Low ethanol yields associated with production of fermentation products other than ethanol.

In this article data are presented having relevance to each of these three factors. We present results from investigation of the ethanol tolerance of *C. thermosaccharolyticum,* growth medium formulation supporting growth of *C. thermocellum* at high substrate concentrations, and initial studies aimed at elucidating the molecular genetics of *C. thermocellum* in the context of future pathway manipulation.

MATERIALS AND METHODS

Organisms

Clostridium thermocellum strain ATCC 27405 was used for molecular manipulations and continuous operation. Strain TEX 5, used in medium studies, is a single colony isolate from a stable continuous culture of ATCC 27405 grown at 5 g/L cellobiose. *Clostridium thermosaccharolyticum* strain HG8 was used throughout the study. Both ATCC 27405 and HG8 strains were kindly supplied by Arnold Demain (Massachusetts Institute of Technology). Experiments were initiated with stock cultures stored at −20°C in 50% glycerol and 50% medium. Glycerol stocks were prepared from single colonies as described previously (*8*). *Escherichia coli* strains used in the methylation experiments and preparation of stocks and competent cells are described in work in preparation (*9*).

Media and Culture Apparatus

Apparatus for both batch and continuous cultivation of *C. thermocellum* and *C. thermosaccharolyticum* and methods for inoculation, startup, and sampling were as described previously (*3,6,8*). *Clostridium thermocellum* defined medium for continuous growth contained: cellobiose, at concentration described in text, 2.00 g/L potassium citrate, 1.25 g/L citric acid (monohydrate), 1.00 g/L Na_2SO_4, 1.00 g/L KH_2PO_4, 2.50 g/L $NaHCO_3$, 5.00 g/L urea, 1.00 g/L $MgCL_2 \cdot 6H_2O$, 0.20 g/L $CaCl_2 \cdot 2H_2O$, 0.10 g/L $FeCl_2 \cdot 4H_2O$, 1.00 g/L cysteine $HCl \cdot H_2O$, 20 mg/L pyridoxamine dihydrochloride, 4 mg/L *P*-aminobenzoic acid, 2 mg/L D-biotin, 2 mg/L vitamin B12; resultant pH is approx 7.0. GBG medium is prepared in three solutions: A solution (per liter distilled water): 0.3 g NaH_2PO_4, 0.7 g K_2HPO_4, 10 g MOPS buffer, 5 g yeast extract, 0.6 mL 0.2% Resazurin (Kodak, Rochester, NY); B solution in a 10X concentration (per 100 mL distilled water): 0.2 g $MgCl_2 \cdot 6H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.4 g cysteine HCl, 0.01 g $FeSO_4$; and C solution in a 10X concentration (per 100 mL distilled water): 1.3 g $(NH_4)_2SO_4$. After autoclaving, A, B, and C solutions are combined with a resultant pH of approx 7.0. For *C. thermocellum* batch experiments, cellobiose was added to the A solution at a concentration of 5 g/L prior to autoclaving. *Clostridium thermosaccharolyticum* xylose substrate was prepared as a tenfold concentrated solution and added to the A solution after autoclaving to a final concentration of 5 g/L for batch cultivation. In continuous culture, GBG medium contains the following modifications from above: 2.5 g/L MOPS, 1.92 g/L $(NH_4)_2SO_4$, and 0.1 g/L $FeSO_4$. To prevent precipitation, the level of phosphate containing compounds in GBG was decreased by 1/3 during the exogenous ethanol addition experiments and the medium contained 10 g/L xylose. Measurement of substrate, fermentation product, and cell mass were by HPLC and as described previously (*8*). Antibiotics were obtained

from Sigma Chemical Company (St. Louis, MO). All chemicals were of reagent grade. Absolute ethanol, 200 proof, was used in the tolerance experiments.

RESULTS AND DISCUSSION

Ethanol Tolerance

Reported estimates for the ethanol concentration resulting in 50% inhibition (P_{50}) vary by over an order of magnitude for thermophilic bacteria (*8*). Development of increased tolerance upon exposure to ethanol (referred to herein as acclimation) may be partially responsible for the disparity of tolerance estimates in the literature. Older studies of the ethanol tolerance of nonthermophilic organisms suggest a difference between the inhibitory effect of endogenously produced and exogenously added ethanol (*10–13*). However, this differential response has been questioned more recently based on experimental (*11,14–16*) and theoretical (*11,17*) grounds. Further, apparent differences between endogenous and exogenous ethanol with respect to both concentration (*10,12,18,19*) and inhibition (*20*) have been attributed to transient conditions not expected in a steady-state continuous culture. All studies of ethanol tolerance known to us have found inhibition to be noncompetitive with respect to substrate (*13,21–27*), with tolerance increasing with decreasing temperature (*28–33*).

We have undertaken an investigation of the tolerance of *C. thermosaccharolyticum* to exogenously added ethanol with respect to growth on xylose. Continuous cultures were used in our work because of the inherent reliability of steady-state measurements, and because cultivation over many weeks in the presence of ethanol affords maximum opportunity for acclimation. Our methodology for evaluating ethanol tolerance relies on measurement of effluent substrate concentration. The basis of this method is that introduction of ethanol at a particular dilution rate, and hence growth rate, results in an increased effluent substrate concentration that compensates for the noncompetitive inhibitory effect of ethanol. Details of this method will be presented in a manuscript currently in preparation.

Figure 1 presents data for a continuous xylose-fed culture of *C. thermosaccharolyticum* maintained at a constant dilution rate of 0.22 h^{-1}. At time zero, a step increase in the concentration of exogenously added ethanol was made such that the steady-state concentration changed from 15 to 20 g/L. In response, the culture exhibited a pronounced transient increase in effluent substrate concentration and a decreased cell concentration ($O.D._{660}$), both indicative of inhibition. However, after a period of about 50 h (11 residence times), a new steady-state was reached with a much lower effluent substrate concentration than those during the transients.

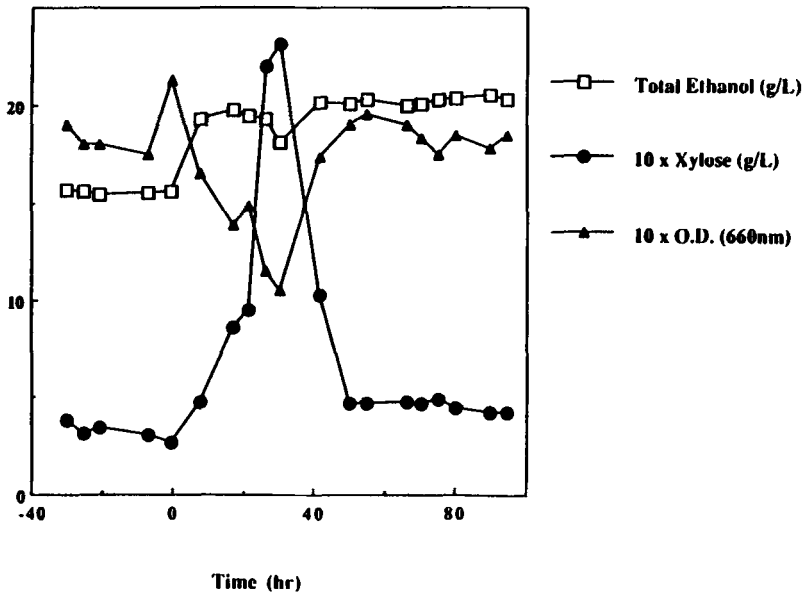

Fig. 1. Continuous fermentation at 60°C of *C. thermosaccharolyticum* HG8 with addition of exogenous ethanol. Data at D = 0.22 h^{-1}, 10 g/L xylose. Exogenous ethanol concentration was raised from 15 to 20 g/L at time zero. Concentrations shown include both endogenously produced and exogenously added ethanol.

We take the transiently high effluent substrate concentrations followed by a return to a steady state at near the prestep xylose concentration to be indicative of adaptation to ethanol. Apparent acclimation such as exhibited in Fig. 1 was observed repeatedly for successive step increases in the exogenous ethanol concentration up to 36 g/L (data not shown).

Based on a compilation of thirty steady-states such as those at the beginning and end of the transient depicted in Fig. 1, we have evaluated the ethanol tolerance of *C. thermosaccharolyticum* grown on xylose at both 55 and 60°C. Results are presented in terms of the ratio of the inhibited growth rate to the uninhibited growth rate at constant substrate concentration $(\mu_I/\mu_0)_S$, in relation to the total ethanol concentration. Data at both temperatures exhibit an approximately linear trend, with 50% inhibition of the growth rate occurring at 31 g/L ethanol. Based on best-fit lines, the P_{50} value for the 55°C data is 36 g/L, and that for the 60°C data is 29 g/L; however, we are not at present prepared to claim that this difference is significant. The data we have to date are consistent with a model for the growth of *C. thermosaccharolyticum* as follows:

$$\mu = \mu_{max} (S / k_s + S) (1 - P / 2^*P_{50}) \quad (1)$$

Table 1 compares the ethanol tolerance of *C. thermosaccharolyticum* to that of *Saccharomyces cerevisiae* and *Zymomonas mobilis*. It may be seen that the

Table 1
Comparative Ethanol Tolerance for Ethanol-Producing Organisms

	Optimal growth Temperature				Suboptimal growth Temperature			
Organism	P_{50}	T	System	Ref	P_{50}	T	System	Ref
C. thermosaccharolyticum	29	60°C	CSTR	This work	36	55°C	CSTR	This work
S. cerevisiae	25[a]	37°C	Batch	33	~50	30°C	CSTR	22,23
Z. mobilis	28	37°C	Batch	34	~50	30°C	CSTR	17,25

[a] Value calculated from the maximum ethanol concentration allowing growth assuming linear inhibition.

P_{50} values are about equal for all three organisms at the optimal growth temperature, and that the value for *C. thermosaccharolyticum* is about 60% that of both the more conventional ethanol-producing organisms at suboptimal temperatures. The relative insensitivity of the *C. thermosaccharolyticum* P_{50} values to temperature awaits further corroboration and explanation.

From a practical point of view, our results suggest that ethanol tolerance is unlikely to significantly constrain the use of *C. thermosaccharolyticum* for ethanol production from cellulosic biomass. The most direct support for this statement comes by noting that the ethanol tolerance reported here for *C. thermosaccharolyticum* is essentially equal to that of yeast used in the state-of-the-art NREL process design employing simultaneous saccharification and fermentation (SSF) (*35*). In the NREL and other SSF designs, hydrolysis and fermentation are carried out at 37°C in order to maximize the rate of cellulose hydrolysis, which is rate-limiting, except when $(\mu_1/\mu_0)_S$ is very close to zero (*36*). As shown in Table 1, the P_{50} values for *C. thermosaccharolyticum* are comparable to that of *S. cerevisiae* at 37°C. In the NREL design, recovery of a final ethanol concentration of 4.4 wt% ethanol is a relatively minor cost factor, contributing 1.8 cents/gal in annualized capital and operating cost exclusive of steam, with steam (valued in terms of potential electricity revenue) accounting for an additional 4 cents per gallon (*7*). In addition, ethanol recovery does not prevent the process from having a decidedly favorable energy balance (*7,37*). Yet another perspective from which to evaluate the ethanol tolerance of *C. thermosaccharolyticum* involves consideration of the absolute rate of substrate utilization. According to the model represented by Eq. (1), a residence time of about 13 h is required to achieve essentially complete substrate utilization at the NREL base-case ethanol concentration of 4.4 wt%. This is a modest value in comparison to the 3- to 7-d reaction times required for conversion of cellulosic materials (*35*).

Growth Medium Formulation

Development of medium for thermophilic organisms has been primarily undertaken in batch culture, where substrate utilization is often measured in zero growth conditions. Figure 2 shows data obtained in continuous culture of *C. thermocellum* strain TEX5 at increasing cellobiose feed concentrations up to 54 g/L at a 9 h residence time. At each feed concentration, effluent cellobiose is <0.25 g/L at steady state. Medium published previously, (*6*), which utilized ammonium as the nitrogen source instead of urea, was less successful, with ammonium inhibition (indicated by increased effluent substrate concentration) apparent in an analogous experiment to that in Fig. 2.

Strain development probably contributes to the characteristic of complete substrate utilization at high concentration in continuous culture.

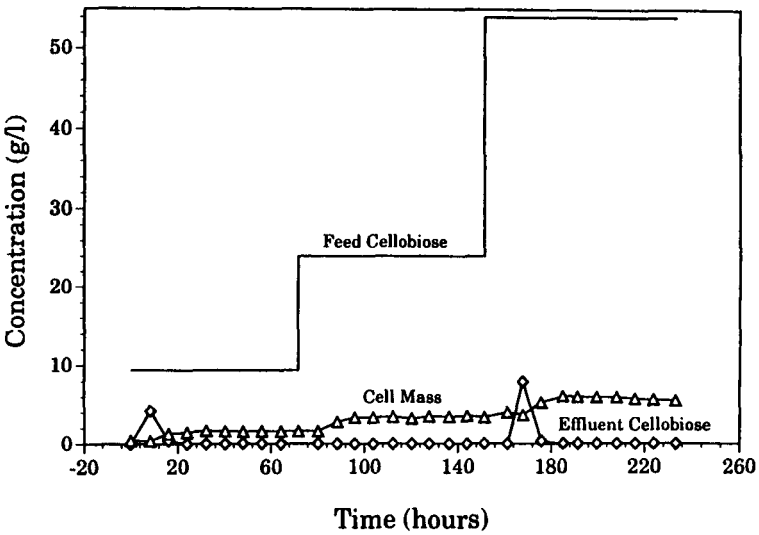

Fig. 2. Continuous fermentation of *C. thermocellum* TEX5 at increasing cellobiose concentrations. Data at D = 0.11 h^{-1}.

Clostridium thermocellum ATCC 27405 was tested using the present defined medium or the complex medium (GBG) and showed good batch growth but poor growth in continuous culture. Single colony isolates from a stable continuous culture inoculum of ATCC 27405 having complete substrate utilization at 5 g/L cellobiose were examined for improved performance at higher substrate concentrations. With defined medium, the isolated strain TEX5 has been tested at growth rates from 0.04 to .22/h, and at temperatures from 50 to 60°C, with essentially complete substrate utilization in all cases where feed substrate concentration was <60 g/L.

Clostridium thermosacharolyticum media development has been described with respect to defined media (*38*) and for complex media supporting complete xylose utilization at 65 g/L (*8*).

Ethanol Yield

Table 2 presents a compilation of ethanol yield data for thermophilic bacteria with entries in chronological order, and including both naturally occurring strains and strains obtained by nonspecific mutagenesis. It may be seen that although high-yielding strains have been reported for each species, subsequent studies have reported lower yields, often with the same strains. Of particular note, exhaustive continuous culture studies with *C. thermosaccharolyticum* HG8 (*42*) and *Thermoanaerobacter ethanolicus* JW 200 (*64,65*) did not demonstrate consistently high yields because of unexplained variability.

One approach to improving ethanol yields involves manipulation of the culture environment either heuristically or based on hypotheses concerning metabolic control (*8*). Such hypotheses involve some speculation,

as the mechanism for regulation of thermophilic product yields is unknown (*66*). Using *C. thermosaccharolyticum*, we have studied two potential routes to environmentally based yield manipulation: mass action by ethanol removal (*8*), and variation of ATP supply and demand by nutrient limitation (*38*). Unfortunately, neither of these approaches led to a significant impact on steady-state product yields.

The data and experience described above have prompted us to consider molecular-level approaches that specifically impact genes associated with formation of catabolic end products. Two approaches are possible: the overexpression of enzymes involved in the desired pathway or the deletion of the undesired enzyme activity. Either tack necessitates the cloning of key catabolic enzymes and reintroduction of genes back into the cell. We are aware of no published reports of either the cloning of any fermentation end-product genes from *C. thermocellum* or *C. thermosaccharolyticum* or the transformation of either organism. Factors likely to be necessary in order to achieve transformation include: restriction compatibility, selective agents, and cloning vectors that are stably replicated in the cell (*67,68*). We have recently begun work aimed at providing these elements.

In studies with cell extracts, we have found restriction enzyme activity in *C. thermocellum* (*9*) that effectively digests all DNA tested to date. Isoschizomer generated DNA patterns confirmed the recognition sequence to be GATC. Furthermore, the Dam methylation system from *E. coli* (GA^mTC) has been shown to protect DNA from digestion at this sequence. This is also true of the related thermophile, *Clostridium thermohydrosulfuricum* (*69*), although *C. thermosaccharolyticum* does not exhibit endonuclease activity at GATC (data not shown). With DNA thus protected, we anticipate that the *C. thermocellum* restriction system is unlikely to prevent transformation.

A further requirement for genetic manipulation is selective agents used in cloning. Instability at the high temperatures of thermophilic growth (*70*) along with possible endogenous resistance are two of the reasons for failure of antibiotic activity. We have studied the effect of various antibiotics on *C. thermocellum* grown in batch tubes. Table 3 lists antibiotics investigated and the level at which sensitivity is observed. Antibiotics found to be ineffective against *C. thermocellum* are also listed. These data identify commonly available antibiotics that can be used as selective agents when cloning genes into *C. thermocellum* by selecting for antibiotic resistance markers.

CONCLUSIONS

Contributions of this work to the evaluation of the status of thermophilic bacteria are as follows:

1. The ethanol tolerance of *C. thermosaccharolyticum* appears to be sufficiently high that it is unlikely to constrain use of this organism in biomass ethanol processes.

Table 2
Summary of Ethanol Selectivity and Yield Data for Thermophilic Bacteria

Organism/strain	Substrate/cultivation mode	Se[a]	Yield[b]	Reference
C. thermosaccharolyticum				
HG8	Xylose/fed-batch	14.7		(39)[c]
3 Strains	Glucose (5 g/L)/batch	0.76–1.25		(40)
HG6	Xylose (6 g/L)/continuous		≤0.3	(41)
HG8	Xylose (18 g/L)/continuous		0.32	(42)
HG8	Xylose (≤27.5 g/L)/continuous, recycle		0.33–0.51	(42)
HG8	Xylose (≤80 g/L)/continuous	2.0	0.31	(8)
C. thermohydrosulfuricum				
JW102	Glucose (5 g/L)/batch	1.23		(43)
E100-69	Glucose (5 g/L)/batch	0.98		(43)
39E	Cellobiose/batch	6.7		(44)
39E	Various (5 g/L)/batch	3.8–5.9		(45)
39E	Xylose (30 g/L)/continuous		0.33	(46)
39E	Glucose/fed-batch	≤3.66		(47)[c]
YM3	Starch (50 g/L)/batch	2.40		(48)
C. thermocellum				
LQRI	Cellobiose/batch	0.82		(44)
LQRI	Cellobiose (8 g/L)/batch	0.65		(49)
AS39	Cellobiose (8 g/L)/batch	1.30		(49)
HTD4	Cellobiose (8 g/L)/batch	0.74		(49)
S6	Cellulose (35 g/L)/batch	10.4		(50)
AS39	Cellobiose (2–20 g/L)/batch	1.2–2.8		(51)[c]
7 strains	Cellulose (10 g/L)/batch	1.02–1.29		(52)[c]

ATCC 27405	Cellulose (10 g/L)/batch	1.03		(53)
ATCC 31549	Cellulose (10 g/L)/batch	0.74		(53)
ATCC 27405	Cellulose (40 g/L)/batch	1.20		(54)
ATCC 27405	Cellulose (10 g/L)/batch	.54–.77		(55)
Mutant No. 647	Cellulose (10 g/L)/batch	1.80		(55)
YS	Cellulose (20 g/L)/batch 2.5 atm H_2	3.0		(56)[c]
I-1-B	Cellulose/fed-batch	3.62		(57)
ATCC 27405	Cellulose (5 g/L)/batch	2.35		(3)
ATCC 27405	Cellulose (5 g/L)/continuous	1.17		(3)
SS8	Cellulose (10–50 g/L)/batch		0.2–0.29	(58)[c]
ATCC 27405	Cellulose (47 g/L)/batch	0.80		(59)
Mutant LD1	Cellulose (47 g/L)/batch	3.14		(59)
T. ethanolicus				
JW200	Starch, Cellobiose, glucose (5 g/L)/batch		0.46	(60)
JW200 Fe(4)	Glucose (20 g/L)/batch		0.50	(61)
JW200 Fe(4)	Cellobiose (10 g/L)/batch		0.46	(61)
JW200	Xylose (10 g/L)/batch		0.44	(61)
JW200L-Fe(7)	Starch (31 g/L)/batch		0.47	(62)
Unspecified, from U. Georgia	Glucose (10 g/L)/batch		0.19	(63)
	Glucose (20 g/L)/batch		0.44	(63)
	Glucose (35 g/L)/batch		0.30	(63)
JW200L-Fe(7)	Xylose (4 g/L)/continuous	0.5–2.16		(64)
JW200L-Fe(7)	Xylose (20 g/L)/continuous		0.29	(65)

[a]Moles ethanol/(moles lactate + moles acetate).
[b]Mass ethanol produed/mass substrate fermented.
[c]Either acetate or lactate not reported and thus not included in the ratio; in most such cases it appears that production of the non-reported product was minor.

Table 3
Antibiotic Resistance Determination for *Clostridium thermocellum* ATCC 27405

Antibiotic	Mode of inhibition	Growth	Dosage
Chloramphenicol	Peptidyl transferase	–	125 μg/mL
Erythromycin	Peptide bond formation	–	25 μg/mL
Thiamphenicol	Peptidyl transferase	–	10 μg/mL
Kanamycin	Translation misreading	+	< 250 μg/mL
Bleomycin	DNA strand scission	+	< 40 μg/mL

Legend: – = increase in optical density at 660 nm < 0.07 over 48 h; + = increase in optical density at 660 nm > 0.50 over 48 h.

2. *C. thermocellum* is capable of vigorous growth at high substrate and cell concentrations in a properly formulated growth medium. Although formulation of an economically feasible medium awaits further study, we see no indication of intrinsic barriers to utilizing high substrate concentration such as the cell-density associated factor reported by Avgerinos (*71*).
3. Specific genetic approaches are attractive alternatives to environmental manipulation and nonspecific mutation as means to obtain high ethanol yields in thermophiles. Two key factors necessary to pursue such approaches are now available for *C. thermocellum:* antibiotic sensitivity such that cells transformed with an appropriate resistance determinant can be selected, and a means of protecting foreign DNA from attack by restriction enzymes.

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