0273-2289/96/5758—0599$6.50

Cellulose Degradation and Ethanol Production by Thermophilic Bacteria Using Mineral Growth Medium

HYUNG-JUN AHN AND LEE R. LYND*

Thayer School of Engineering, Dartmouth College, Hanover, NH 03755

ABSTRACT

Growth of thermophilic cellulase-utilizing bacteria in a vitimin-free growth medium is reported for both a previously described strain, *Clostridium thermoclelum* 31549, and now isolates HJA1 and HJA2. Formation of fermentation products with and without vitamins was similar for strains HJA1 and HJA2 as well as for the enrichment cultures from which these stains were derived. Strain HJA2 was maintained in continous culture on a vitamin-free mineral medium with Avicel as the carbon source for over a week. At a 38 h residence time, Avicel conversion was higher (81%) at pH 6.42 than at pH 6.97 (73%) or at 6.01 (58%). Ethanol and acetate were produced in significant amounts by strain JHA2 at all pH values tested (6.97, 6.42, 6.01). Lactic acid was the primary fermentation product at pH 6.97, but was not a significant product at both the lower values. Efforts to grow thermophilic, cellulose-utilizing bacteria at pH < 6.0 were unsuccessful for described strains, new isolates, and enrichment cultures.

Index Entries: Thermophilic; cellulolytic; low pH; vitamins; nutrition; mineral medium.

INTRODUCTION

Production of ethanol from cellulosic biomass via enzymatic hydrolysis involves four biologically mediated events: cellulase production, cellulose hydrolysis, fermentation of hexoses, and fermentation of pentoses. Biological conversion, comprised of these four events, is the most costly and least technologically mature of the steps involved in biomass ethanol production *(1)*. A key factor in distinguishing biomass ethanol process options is the degree of consolidation of the biologically mediated events. The direct microbial conversion (DMC) process concept represents the ultimate degree of consolidation, in that all four events are carried out by a single microbial community in a single-unit operation *(2)*. Microorganisms that produce both cellulase and ethanol are of particular importance in DMC processes, with the thermophile *Clostridium thermocellum* perhaps the most studied organism in this category.

Several issues need to be resolved before *C. thermocellum* and other DMC organisms will receive serious consideration for use in commercial ethanol production processes. These include developing strains that exhibit high ethanol selectivity and the ability to produce ethanol at recoverable concentrations. In addition, it is also important to demonstrate that DMC organisms can grow robustly under conditions typical of large-scale production.

*Author to whom all correspondence and reprint requests should be addressed.

All previous studies of *C. thermocellum* known to us have observed growth at pH ± 6.0 in the presence of either complex growth factors (e.g., yeast extract) or mixtures of at least four vitamins. However, operation at pH <6.0 would likely be beneficial in a process context, since it would decrease the risk of contamination while reducing or eliminating the quantity of caustic necessary to counter the acidic nature of carbonic acid produced in the course of fermentation. Commercial operation with addition of specific vitamins would likely be cost prohibitive, and some biomass materials (e.g., waste paper) are not likely to contain vitamins. Even for biomass materials that do contain required vitamins, it has not been shown that these remain biologically active after exposure to the rather severe conditions characteristic of pretreatment processes. Addition of vitamin supplements, such as corn steep liquor, is a possibility and is very likely to be the most cost-effective approach currently. However, such addition would represent an added cost in an endeavor requiring very low processing costs. In addition, the scale of production of all conceivable vitamin sources would be dwarfed by the requirements of a significant biomass fuel industry.

Motivated by the aforementioned considerations, this article seeks to determine whether existing and/or new isolates of thermophilic cellulose-degrading bacteria are capable of robust growth at low pH and/or with reduced or eliminated addition of exogenous vitamins.

METHODS

Sources of Organisms and Chemicals

Strains HJA1 and HJA2 were isolated from enrichment cultures. Inoculum sources were bark mulch from a local nursery (HJA1) and silage from a local dairy farm (HJA2), collected from locations that were warm to the touch (temperature was not measured). The agar roll tube method was applied to isolate a single colony from enrichments. Agar cultures containing 4% agar and 0.5% Avicel were inoculated with enrichment cultures in vitamin-free MTC medium. Clear zones with a distinct colony in the middle were evident after 3 d of incubation at 60°C. Single colonies were picked with sterile hypodermic needles and inoculated into liquid medium to examine whether the cell growth and the product formation were consistent with enrichment results. *C. thermocellum* strains 27405, 31549, and 35609 were obtained from the ATCC (Bethesda, MD). Strain Tex 5 was derived from strain 27405 in our laboratory as previously reported (*2*). All chemicals are from Sigma (St. Louis, MO) unless otherwise noted.

Growth Media

Growth media used 5 g/L cellulose (Avicel, PH-105, FMC, Philadelphia, PA) and were based on MTC medium containing, in final concentration: 5 g/L mops Na salt, 5 g/L carbohydrate, 1.0 mg resazurin, 2.0 g citric acid potassium salt, 1.25 g/L citric acid monohydrate, 1.0 g/L Na_2SO_4, 1.0 g/L KH_2PO_4, 2.5 g/L $NaHCO_3$, 5.0 g/L urea, 1.0 g/L $MgCl_2h{\cdot}6H_2O$, 0.2 g/L $CaCl_2{\cdot}2H_2O$, 0.1 g/L $FeCL_2{\cdot}4H_2O$, and 1.0 g/L L-cysteine hydrochloride monohydrate. The medium was supplemented with vitamins and trace minerals by the addition of stock solutions described below at the rate of 20 and 1 mL/L final solution respectively. The 50X stock vitamin solution used contained per liter distilled H_2O: 1 g pyridoxamine dihydrochloride,

200 mg *p*-aminobenzoic acid, 100 mg *d*-biotin, and 100 mg vitamin B12. The 50X stock trace mineral solution contained per liter distilled H_2O: 10 g/L citric acid monohydrate pH adjusted to 7.0 using KOH, 25 mg $MnCl_2 \cdot 4H_2O$, 25 mg $CoCl_2 \cdot 6H_2O$, 10 mg $ZnC1_2$, 2.5 mg $CuC1_2 \cdot 2H_2O$, 2.5 mg H_3BO_3, 2.5 mg $Na_2MoO_4 \cdot 2H_2O$, and 2.5 mg $NiC1_2 \cdot 6H_2O$.

Enrichment cultures and cultivation of isolates derived therefrom used MTC with no added vitamins. Silage enrichments were carried out in the absence of Na_2SO_4, which prevented formation of a strong black color (presumably sulfide precipitates). For cultivation at 6.4, 6.0, and 5.8, 5 g/L MES (2[-N-Morpholino]-ethane-sulfonic acid, pK_a = 6.1 at 25°C) buffer replaced MOPS, and medium pH was adjusted prior to autoclaving using 50% HCl. All media were prepared under nitrogen gas.

Cultivation

Enrichment cultures were carried out in 75-mL serum vials (Bellco Biotechnology, Vineland, NJ) containing 30 mL of medium. Isolates were obtained using anaerobic roll tubes (Bellco). Growth rate measurements were made using crimp-seal anaerobic culture tubes (internal diameter 1.4 cm, Bellco). Batch and continuous cultivation were also undertaken using a 1.5-L, 750-mL working volume, fermenter (Applikon, Foster City, CA), with peristaltic feed delivery and agitation at 500 rpm as described previously (*3*). All results were obtained at 60°C.

Measurement of Growth Rate and Product Concentrations

Cell growth rates on Avicel were measured by light scattering at 660 nm in the presence of substrate using a Bausch and Lomb 21D-model spectrophotorrleter as follows: At low substrate concentrations (0–0.5 g/L), there is a linear relationship between OD and the insoluble substrate concentration:

$$S = k_1 \cdot OD_s \text{ where } k_1 \approx 0.8, S \text{ in g/L} \quad (1)$$

Similarly, at low cell concentrations:

$$X = k_2 \cdot OD_X \text{ where } k_2 \approx 1.1, X \text{ in g/L} \quad (2)$$

For a small initial cell concentration ($X_0 = 0$):

$$X = Y_{x/s}(Si - S) \quad (3)$$

For growth on 0.5 g/L Avicel with a cell yield $Y_{x/s}$ = 0.10, complete substrate utilization results in a cell suspension having an OD of ~0.06, which is 10-fold less than the OD of the initial substrate concentration. By neglecting the contribution of cell mass to absorbance readings, a conservative estimate of growth rate can be determined by combining (1) and (3):

$$X = Y_{x/s}(Si - S) = Y_{x/s}\, k_1\, (ODi - OD) = c\, (ODi - OD) \quad (4)$$

The cell growth can be calculated by:

$$\mu = \ln[(ODi - OD_2)/(ODi - OD_1)]/(t_2 - t_1) \quad (5)$$

where X = cell concentration in g/L, S = substrate concentration in g/L, t = time in hours, μ = specific growth rate in h^{-1}, $Y_{x/s}$ = cell yield coefficient, OD = optical density at 660 nm, k and c = constants. Ethanol, acetate, and lactate were measured

Table 1
Product Concentrations in Enrichment Culture
With and Without Exogenously Added Vitamins[a]

Inoculum source	Vitamins added	Product concentration, g/L		
		Lactate	Acetate	Ethanol
Bark mulch	–	0.52	0.49	0.87
Bark mulch	+	0.43	0.60	0.70
Silage	–	0.59	0.55	0.83
Silage	+	0.49	1.06	1.27

[a]All values were the average of more than four tubes after five transfers. Repeated unsuccessful attempts were made to establish enrichment cultures at pH 6.0 or less, both in the presence and absence of yeast extract.

Table 2
Product Concentrations in for Single-Colony Isolates
With and Without Exogenously Added Vitamins[a]

Isolated source	Vitamins added	Product concentrations, g/L		
		Lactate	Acetate	Ethanol
HJA1	–	0.24 (0.09)	0.71 (0.06)	0.84 (0.08)
HJA1V	+	0.52 (0.10)	0.64 (0.14)	0.71 (0.03)
HJA2	–	0.47 (0.08)	0.52 (0.08)	0.85 (0.21)
HJA2V	+	0.55 (0.14)	0.50 (0.08)	0.93 (0.25)

[a]All values are the average of three cultures after three transfers. Values in parentheses represent standard deviation of each concentrations.

via high-performance liquid chromatography using a Bio-Rad (Hercules, CA) HPX-87H column.

RESULTS

Enrichment Cultures

Since many laboratory strains have been maintained for years if not decades in complex media, we elected to include enrichment cultures and associated isolates in our study. All enrichments developed a yellow color characteristic of *C. thermocellum*. Enrichment cultures in the presence and absence of vitamins showed comparable growth after more than five successive transfers. As presented in Table 1, product concentrations in enrichment cultures were very similar in both the presence and absence of exogenously supplemented vitamins.

Isolated Strains

Strain HJA1 was isolated from bark mulch enrichment cultures, and HJA2 strain was isolated from silage enrichment cultures, in both cases without exogenously added vitamins. HJA1V and HJA2V strains were isolated and grown in the presence of vitamins.

From Tables 1 and 2, it may be seen that the trends of product formation of pure isolated cultures are for the most part consistent with those of enrichment

Table 3
Batch Growth of *C. thermocellum* Strains
in Relation to pH and Vitamin-Containing Supplements[a]

C. thermocellum strains	Growth, after 10 transfers			
	pH 6.25 + vitamins	pH 6.25 + yeast extract	pH 7.0 – vitamins	pH 7.0 + vitamins
27405	–	–	–	+
31549	–	–	+	+
35609	–	–	–	+
Tex 5	–	–	–	+
DWT	–	–	–	+
HJA2	–	–	+	+

[a]Vitamins: 0.02 g/L pyridoxamine hydrochloride; 0.004 g/L P-aminobenzoic acid; 0.002 g/L D-biotin; 0.002 g/L vitamin B-12; yeast extract; 2.5 g/L.

mixed cultures, and there is no significant difference in product concentrations between enrichments obtained in the absence and presence of exogenously added vitamins.

Comparison with Previously Described Strains

Repeated batch culture of different strains without exogenously supplemented vitamins were undertaken at pH 7.0. As presented in Table 3, two strains, *C. thermocellum* 31549 and HJA2, continued to grow after more than 10 transfers. Other strains did not show any growth after third transfers. No strain grew in batch culture at pH 6.25 either with vitamins or with yeast extract, whereas all the controls at pH 7.0 showed active growth. Exponential growth was observed for both *C. thermocellum* strain 31549 and strain HJA2, with associated growth rate constants, μ, of 0.03/h and 0.06/h, respectively (data not shown).

Continuous Culture

Continuous cultivation was carried out in a 1.5-L fermenter using strain HJA2 in MTC vitamin-free medium with a 0.5% Avicel feed at a ~38 h residence time and pH 7.0. The steady-state data (Table 4) show ~73% substrate utilization and a higher concentration of lactate than other products. The HJA2 strain was able to grow in continuous culture with no exogenously added vitamins for more than a week of continuous feeding at pH 7.0, further confirming the ability of this strain to grow in the absence of vitamins. Repeated batch cultivation was also undertaken in the 1.5-L fermenter (data not shown), resulting in conversion (% substrate utilized) >92% in time periods of 2–3 d and production of ethanol, lactate, and acetate in a ratio of approx 1:1:0.5 (mass basis).

As presented in Table 4, product formation by strain HJA2 in continuous culture was affected by the pH. Lactate was a main product at pH 7.0, but ethanol concentration was increased and lactate concentration was severely decreased at lower pH. At steady-state with pH-controlled 6.4 using MES buffer, the HJA2 culture showed higher substrate conversion than at pH 7 and lactate production was nearly eliminated. This strain also maintained growth at pH 6.0, resulting in 58% substrate conversion at steady state. At pH 6.0, no lactate was produced and the

Table 4

Continuous Culture of HJA2 in Mineral Medium at Different pH Values

pH	Avicel conversion	Number of data points at steady-state	Product concentration, g/L			Carbon recovery
			Lactate	Acetate	Ethanol	
6.97	73 ± 2%	6	0.83	0.64	0.73	0.94
6.42	81 ± 6%	4	0.04	0.63	1.26	0.96
6.01	58 ± 1%	5	0.00	1.04	0.75	1.06

[a]Residence time = ~38 h.

major product was acetate. However, the culture was washed out when the pH was changed from 6.0 to 5.8. Enrichment cultures and the HJA2 isolate were tested at different pH values in batch culture in defined medium, but no growth was observed at pH 6.2 and 5.5, whereas both grew at pH 7.0. This result suggests that low-pH cultivation may be more successful in continuous culture than batch culture.

CONCLUSION

We have demonstrated that isolates of *C. thermocellum*-like organisms can be obtained that are capable of growth in mineral medium containing no organic growth factors. For strain HJA2 at least, the rate of cellulose utilization appears to be sufficiently high so as not to prohibit utilization in a practical process, especially in light of the recalcitrant nature of Avicel compared to most pretreated substrates. It may be noted that the batch growth rate reported here on Avicel is 60% that obtained previously for the type 27405 strain under similar conditions, except with yeast extract supplementation *(3)*.

We attempted to establish thermophilic, cellulose-utilizing strains at pH <6.0, and we also attempted to grow previously described strains at these moderately low pH values. Both efforts were unsuccessful. This result is puzzling in light of both the prevalence of low pH, high-temperature, cellulose-rich environments in nature, as well as reports, such that as Chyi and Dague *(4)*, indicating that the optimum pH for cellulose degradation in undefined (although mesophilic) mixed cultures is 5.6. At this point, we suspect some pH-dependent reaction involving essential medium components. However, this matter awaits further study.

ACKNOWLEDGMENTS

This work was supported by subcontract No. XAE-3-13005-01 from the National Renewable Energy Laboratory, for which the authors are grateful. We thank David Hogsett, Steve Goodwin, and Susan Leschine for useful discussions.

REFERENCES

1. Lynd, L. R., Cushman, J. H., Nichols, R. J., and Wyman, C. E. (1991), *Science* **251**, 1318–1323.
2. Hogsett, D. A., Alm, H.-J., Bernardez, T. D., South, C. R., and Lynd, L. R. (1992), Direct microbial conversion: prospects, progress, and obstacles. *Appl. Biochem. Biotechnol.* **34/35**, 527–541.
3. Lynd, L. R., Grethlein, H. E., and Wolkin, R. H. (1989), *Appl. Environ. Microbiol.* **55(12)**, 3131–3139.
4. Chyi, Y. T. and Dague, R. R. (1994), *Water Environ. Res.* **66(5)**, 670–678.