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SHORT COMMUNICATION

A defined growth medium with very low background carbon for culturing *Clostridium thermocellum*

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Abstract A growth medium was developed for cultivation of *Clostridium thermocellum* ATCC 27405 in which "background" carbon present in buffers, reducing agents, chelating agents, and growth factors was a small fraction of the carbon present in the primary growth substrate. Background carbon was 1.6% of primary substrate carbon in the low-carbon (LC) medium, whereas it accounts for at least 40% in previously reported media. Fermentation of cellulose in LC medium was quite similar to Medium for Thermophilic Clostridia (MTC), a commonly used growth medium that contains background carbon at 88% of primary substrate carbon. Of particular note, we found that the organism can readily be cultivated by eliminating some components, lowering the concentrations of others, and employing a tenfold lower concentration of reducing agent. As such, we were able to reduce the amount of background carbon 55-fold compared to MTC medium while reaching the same cell biomass concentration. The final mass ratios of the products acetate:ethanol:formate were 5:3.9:1 for MTC and 4.1:1.5:1 for LC medium. LC medium is expected to facilitate metabolic studies involving identification and quantification of extracellular metabolites. In addition, this medium is expected to be useful in studies of cellulose utilization by anaerobic enrichment cultures obtained from environmental inocula, and in particular to diminish complications arising from metabolism of carbon-containing compounds other than cellulose. Finally, LC medium provides a starting point for industrial growth media development.

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Introduction

Clostridium thermocellum is of interest for fundamental and applied studies of microbial cellulose utilization [8]. Since isolation and cultivation of this organism was first reported by McBee in 1948 [9, 10], a variety of growth media formulations have been employed [2, 3, 6, 7, 11, 13, 18]. As presented in Table 1, these media contain substantial amounts of carbon-containing compounds other than the primary growth substrate. In particular, "background" carbon present in reducing agents, chelating agents, buffers, and complex components such as yeast extract represents 40% or more of the carbon present in the primary growth substrate.

We report here the development of a low-carbon (LC) medium for *C. thermocellum* ATCC 27405, and compare LC medium with 'Medium for Thermophilic Clostridia' (MTC) [6] a standard medium for *C. thermocellum,* which has been published elsewhere [11, 14, 15, 19, 20].

Materials and methods

Organism and culturing conditions

Clostridium thermocellum ATCC 27405 was obtained from the American Type Culture Collection (Manassas, Virginia). Inocula for experiments in MTC medium were from a single colony isolate that was maintained at −80°C in 4-ml aliquots containing 20% glycerol. Inocula for experiments with LC media (below) came from an MTC

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Table 1 Overview of selected *Clostridium thermocellum* media with background carbon-to-substrate carbon ratio

Medium[a]	Background carbon/ substrate carbon ratio[b]	Reference
D58 (Chemical defined[e,f])	1.35 (92.56)	[2, 13]
CM3[d]	0.43	[18]
GS	5.03	[3]
GS-2	3.50	[7]
MJ	2.37	[7]
RM	1.23	[11]
MTC (Complex)[c]	2.06 (2.96)	[6]

Only major carbon contributing components are considered

[a] Some media have yeast extract; this contains 0.4 g carbon/g determined by elemental analysis of a 0.1 g/l Sigma 'Select Yeast Extract' solution

[b] Based on 5 g/l Avicel PH105 solution of 2.11 g carbon/l. Under ambient conditions, Avicel has an average moisture content of 5%

[c] MTC has many variations, this is the original recipe from Hogsett's thesis [6] including MOPS buffer and with optional yeast extract. See also Table 2 for a detailed list of medium components

[d] The culturing gas mixture contains CO_2

[e] 70 g/l L-cysteine is listed as part of amino acids to replace yeast extract, but this is also a reducing agent

[f] Chemically defined D58 contains high concentrations of amino acids

single-colony isolate grown on LC media, and after two transfers frozen at −80°C in 2-ml aliquots. Freezer stocks were cultured on either MTC or LC medium at 60°C and after two transfers used as a 5% fresh inoculum for the bioreactor experiments.

Media composition

All chemicals were reagent grade and obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) unless indicated otherwise. All solutions were made with water purified using a MilliQ system (Millipore, Billerica, MA), and were purged extensively with ultra high purity grade nitrogen gas (Airgas Northeast, White River Junction, VT) and either autoclaved or filter sterilized (0.22-μm filter, Millipore, Billerica, MA). Cellobiose or Avicel PH105 (FMC Corporation, Philadelphia, PA) were used as growth substrates at a concentration of 5 g/l. The identity and concentrations of LC and MTC media are presented in Table 2 for carbon-containing compounds and major inorganic components. Both media also contained trace elements with final concentrations as follows: $MnCl_2*4H_2O$ 0.00125 g/l, $ZnCl_2$ 0.0005 g/l, $CoCl_2*6H_2O$ 0.000125 g/l, $NiCl_2*6H_2O$ 0.000125 g/l, $CuSO_4*5H_2O$ 0.000125 g/l, H_3BO_3 0.000125 g/l and $Na_2MoO_4*2H_2O$ 0.000125 g/l. Stock solutions for both media were prepared as described by Zhang and Lynd [19].

Bioreactor experiments

Bioreactor experiments were carried out in Sartorius Aplus 2.5-l vessel units (Sartorius Stedim, Bohemia, NY) at 60°C at 200 rpm stirrer speed with a working volume of 2 l. The headspace was purged continuously with nitrogen controlled by a mass flow controller (Omega Engineering, Stamford, CT) at 100 ml/min. The water in the cooling-bath for the off-gas condenser was kept at 4°C (Cole Parmer, Vernon Hills, IL) to minimize evaporation due to gas purging. Purging of the bioreactor headspace was initiated before the first 5 ml of base was added. The pH was maintained at 6.95–7.00 by automatic addition of 2 N KOH and checked manually when samples were taken.

Analytical methods

Acetate, formate, ethanol, glucose and residual cellobiose were determined by HPLC (Waters, Milford, MA) with refractive index detection using an Aminex HPX-87H column (Bio-Rad, Hercules CA) with a 5-mM sulfuric acid solution eluent.

Pellet nitrogen was determined using a Shimadzu TOC-Vcph Total Organic Carbon analyzer with added Total Nitrogen unit (Shimadzu Scientific Instruments, Columbia, MD), calibrated using an acidified glycine standard. One-mL samples were spun down at 21,000 × *g* for 5 min, the supernatant was discarded and the pellet was rinsed twice using equal volumes of MilliQ water.

Residual Avicel PH105 concentration was quantified by quantitative saccharification as described by Saemen et al. [16] and adapted by Sluiter et al. [17]. Samples of 10 ml were centrifuged at 2,800 × *g* for 10 min, rinsed with MilliQ water and centrifuged again. Supernatant was discarded and pellets were placed in a freeze dryer (Labconco Corp, Kansas City, MO) for at least 24 h before analysis.

Supernatant protein was determined with the Bradford assay (Thermo Scientific, Rockford IL) with BSA as a standard. Amino acid concentrations were determined by Aminoacids.com (St. Paul, MN) by 'Free Amino Acid' analysis from the supernatant of centrifuged fermentation samples (5 min at 21,000 × *g*).

The carbon dioxide produced during fermentation experiments carried out in bioreactors was measured by a LI- 820 CO_2 analyzer (Li-Cor Biosciences, Lincoln, NE). A custom-built LabVIEW®-based control system (National Instruments, Austin, TX) recorded data (ppm/min) every 15 min.

Table 2 Comparison between MTC and LC media for carbon content of components, with background carbon to substrate carbon as a percentage

Ingredient[a]		MTC		LC medium	
Name	Chemical formula	Concentration (g/l)	Concentration carbon (g carbon/l)	Concentration (g/l)	Concentration carbon (g carbon/l)
Carbon and energy source					
Avicel PH105[b]	$[C_6H_{10}O_5]_n$	5.0	2.11	5.0	2.11
Cellobiose	$C_{12}H_{22}O_{11}$	5.0	2.10	5.0	2.10
Buffers, chelating agents and salts					
Morpholinopropane sulfonic acid (MOPS)[c,d]	$C_7H_{14}NNaO_4S$	(5.0)	(1.82)	(2.0)	(7.27E−01)
Resazurin	$C_{12}H_6NNaO_4$	1.0E−03	5.73E−04	0.0	0.0
Monopotassium phosphate	KH_2PO_4	0.0	0.0	2.0	0.0
Dipotassium phosphate	K_2HPO_4	1.0	0.0	3.0	0.0
Citric acid tripotassium salt	$C_6H_5O_7K_3$	2.0	4.44E−01	0.0	0.0
Citric acid monohydrate	$C_6H_8O_7.H_2O$	1.25	4.28E−01	0.0	0.0
Sodium sulfate	Na_2SO_4	1.0	0.0	0.0	0.0
Sodium bicarbonate	$NaHCO_3$	2.5	3.57E−01	0.0	0.0
Nitrogen source					
Ammonium chloride	NH_4Cl	1.5	0.0	2.0	0.0
Urea	CH_4N_2O	2.0	4.00E−01	0.0	0.0
Salts and reducing agent					
Magnesium chloride hexahydrate	$MgCl_2*6H_2O$	1	0.0	0.2	0.0
Calcium chloride dihydrate	$CaCl_2*2H_2O$	0.2	0.0	0.05	0.0
Ferrous chloride tetrahydrate	$FeCl_2*4H_2O$	0.1	0.0	0.0	0.0
Ferrous sulfate hepta hydrate	$FeSO_4*7H_2O$	0.0	0.0	0.0035	0.0
L-cysteine hydrochloride monohydrate	$C_3H_7NO_2S*HCl*H_2O$	1.0	2.05E−01	0.1	2.05E−02
Vitamins					
Pyridoxamine Dihydrochloride	$C_8H_{12}N_2O_2*2HCl$	0.02	7.96E−03	0.02	7.96E−03
PABA	$C_7H_7NO_2$	0.004	2.45E−03	0.004	2.45E−03
D biotin	$C_{10}H_{16}N_2O_3S$	0.002	9.82E−04	0.002	9.82E−04
Vitamin B-12	$C_{63}H_{88}CoN_{14}O_{14}P$	0.002	1.12E−03	0.002	1.12E−03
Total background carbon content in g carbon/l (with MOPS)			1.85 (3.66)		0.03 (0.76)
Percent background carbon to carbohydrate carbon[e] (with MOPS)			87.52 (173.60)		1.56 (36.00)

[a] Trace elements do not contain any carbon and therefore are not included in this table

[b] Avicel PH105 has average moisture content of 5%

[c] MOPS is recommended to use in MTC, but has been not been used as the bioreactor experiments were done under actively controlled pH conditions with 2 N KOH

[d] MOPS is recommended to use for LC medium when there is no active pH control i.e. culturing in serum bottles or Balch tubes

[e] Based on 5 g/l Avicel PH105 solution for carbohydrates

Results and discussion

Medium development in serum bottles

LC medium was developed for cultivation of *C. thermocellum* ATCC 27405 by reducing or eliminating carbon-containing compounds present in MTC medium in a step-wise fashion using batch cultures in sealed serum vials. Cell growth and complete utilization of 5-g/l substrate (Avicel or cellobiose) were verified with each step.

For batch experiments without pH control, we used MOPS to increase the medium buffering capacity sufficient to allow complete utilization of 5 g/l carbohydrate. In addition, concentrations of some inorganic components (Fe^{2+}, Ca^{2+}, and Mg^{2+}) were reduced to avoid precipitation in the absence of organic chelating agents [4]. We

found that cultures with cellobiose as the growth substrate were particularly useful for detecting precipitation of media constituents.

Replacing the reducing agent L-cysteine with the inorganic alternative $Na_2S.9H_2O$ proved problematic. Although $Na_2S.9H_2O$ has been used for culturing Clostridia and other obligate anaerobes [1, 5, 18] we found it to cause variability in lag phases with *C. thermocellum*, sometimes lasting several days. We thus investigated reducing the concentration of L-cysteine while intensifying purging with nitrogen gas for all solutions. Although growth at 0.01 g/l of L-cysteine was observed, we chose a concentration of 0.1 g/l because it required less stringent purging and still resulted in acceptably low background carbon levels.

We were able to reduce the amount of background carbon for LC medium to 1.6% of the primary substrate carbon, which represents a 55-fold reduction compared to MTC. A detailed comparison between the two media is given in Table 2.

Bioreactor cultivation

Growth of *C. thermocellum* 27405 in MTC and LC media was compared in pH-controlled bioreactors (Fig. 1a–d).

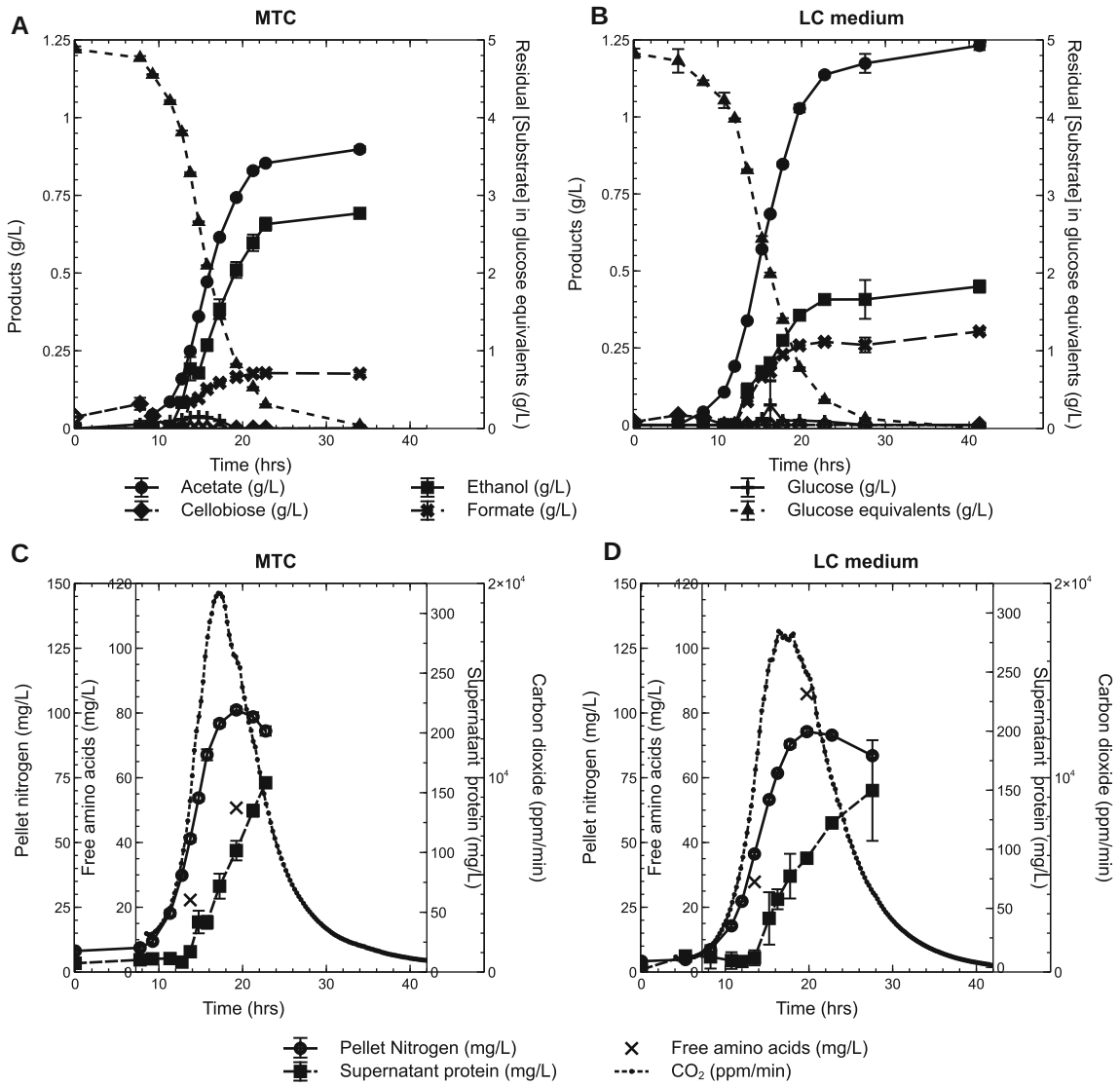

Fig. 1 Fermentation profile of *Clostridium thermocellum* ATCC 27405 for products, residual substrate, pellet nitrogen, supernatant protein, amino acids (cumulative) and the rate of carbon dioxide production on MTC and LC medium with 5 g/l crystalline cellulose (Avicel PH-105). All data points are triplicates except for 'residual substrate concentration in glucose equivalents' for LC medium $T = 0, 2, 5$ are duplicates and $T = 4$ is a single data point. *Error bars* represent 1 SD. CO_2 rate data is the average of 1 min of measuring. The concentration of amino acids is the cumulative concentration of all detectable amino acids with the values of L-cystine omitted, as L-cysteine is a component of both MTC and LC medium. Free amino acids samples are single data points

Pellet nitrogen, indicative of biosynthesis, is similar for growth in LC and MTC medium with an increase of 90 mg nitrogen/l (Fig. 1c, d). The size of inoculum differed by 4 mg nitrogen/l with an average of 8.1 ± 0.8 mg/l nitrogen for MTC and an average of 4.2 ± 0.5 mg/l of nitrogen for LC medium. The time required for biosynthesis and substrate exhaustion is equal for MTC and LC medium, at about 35 h (Fig. 1a, b). The peak rate of CO_2 production is only 10% higher for MTC medium than for LC medium (19,500 vs. 17,500 ppm/min). The similarity in results is remarkable since LC medium has five fewer components, a lower concentration of all components present except phosphate, and a tenfold lower concentration of reducing agent when compared to MTC.

Fermentation in LC medium resulted in somewhat higher concentrations of acetate and formate and somewhat lower concentrations of ethanol as compared to fermentation in MTC medium, with final mass ratios of acetate:ethanol:formate 5:3.9:1 for MTC and 4.1:1.5:1 for LC medium. Effects of media composition on the product ratio for Clostridia have been described before [5, 12], but here as elsewhere are not understood at a metabolic level. The concentration of free amino acids differs by 35 mg at the peak of the concentration of pellet nitrogen, while the free amino acid concentration at 50% of maximum pellet nitrogen is equal between the two media.

LC medium has far lower (>27-fold) background carbon than previously reported media for cultivation of *C. thermocellum*, and in general much lower background carbon than media used for cultivation of anaerobic cellulolytic microbes of which we are aware. This medium is expected to facilitate metabolic studies involving identification and quantification of extracellular metabolites. In addition, this medium is expected to be useful in studies of cellulose utilization by anaerobic enrichment cultures obtained from environmental inocula, and in particular to diminish complications arising from metabolism of carbon-containing compounds other than cellulose. Finally, LC medium provides a starting point for industrial growth media development.

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