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Closing the carbon balance for fermentation by *Clostridium thermocellum* (ATCC 27405)

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ABSTRACT

Our lab and most others have not been able to close a carbon balance for fermentation by the thermophilic, cellulolytic anaerobe, *Clostridium thermocellum*. We undertook a detailed accounting of product formation in *C. thermocellum* ATCC 27405. Elemental analysis revealed that for both cellulose (Avicel) and cellobiose, ≥92% of the substrate carbon utilized could be accounted for in the pellet, supernatant and off-gas when including sampling. However, 11.1% of the original substrate carbon was found in the liquid phase and not in the form of commonly-measured fermentation products – ethanol, acetate, lactate, and formate. Further detailed analysis revealed all the products to be <720 da and have not usually been associated with *C. thermocellum* fermentation, including malate, pyruvate, uracil, soluble glucans, and extracellular free amino acids. By accounting for these products, 92.9% and 93.2% of the final product carbon was identified during growth on cellobiose and Avicel, respectively.

1. Introduction

Clostridium thermocellum is a thermophilic, obligately anaerobic bacterium that ferments cellulose and products of cellulose solubilization, but not pentoses. This organism solubilizes cellulose primarily using a multi-protein complex termed a cellulosome that is bound to the cell surface and thus is also thought to mediate surface attachment (Bayer et al., 1983; Lu et al., 2006). Cellulose is hydrolyzed extracellularly into soluble cellodextrins, which are taken up by the cell and phosphorolytically cleaved by cellodextrin and cellobiose phosphorylase to form glucose and glucose-1-phosphate which enters glycolysis. Ethanol, acetic acid and CO_2 are the dominant fermentation products, with lactic acid formed under some conditions, and formate reported in some studies (Islam et al., 2006; Levin et al., 2006; Magnusson et al., 2009; Rydzak et al., 2009; Weimer and Zeikus, 1977). *C. thermocellum* has received extensive study as a model system for the cellulosome (Bayer et al., 1983, 1994; Lamed and Bayer, 1988) and microbial cellulose utilization (Lu et al., 2006; Zhang and Lynd, 2005; Raman et al., 2009) and as a potential industrial catalyst for conversion of cellulosic biomass to fuels or chemicals (Lynd et al., 2005; Carere et al., 2008).

Closing a carbon balance, by accounting for carbon in the form of substrate and products, is a cornerstone for fundamental studies of microbial physiology and is also a prerequisite to achieving high product yields in an applied context. Ascertaining the fractional recovery of substrate carbon in fermentation products, R^C, can be undertaken at various levels of detail. The most common approach in reported studies of *C. thermocellum* by our group and others has been to use of the following equation (Lynd et al., 1989).

$$R^C_{C2+C3} = \frac{3 \times ([A] + [E] + [L])}{6 \times ([S_o] - S_f])} \quad (1)$$

where, R^C_{C2+C3} = carbon recovery based on concentrations of C2 + C3 products, $[A]$ = molar concentration of acetic acid formed during the fermentation, $[E]$ = molar concentration of ethanol formed during the fermentation, $[L]$ = molar concentration of lactic acid formed during the fermentation, $[S_o]$ = the molar concentration of glucosyl residues prior to fermentation, $[S_f]$ = the molar concentration of glucosyl residues after fermentation. The factor of 3 in the numerator is consistent with the expectation that one mole of CO_2 and/or formate is formed per mole of acetic acid and ethanol.

In studies of *C. thermocellum* ATCC 27405 carried out in our lab and by other investigators have consistently found that the carbon balance using the C2 + C3 method does not close and indeed is less than 70% in defined medium (Table 1). We also find substantial evidence for missing carbon in *C. thermocellum* fermentation data

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Table 1
Values of R^{C}_{C2+C3} for studies of *C. thermocellum* (ATCC 27405) undertaken in the Lynd lab[a].

Substrate	Medium	CSTR/ Batch	Replicates	Average (%)	Standard deviation (%)
Avicel	GBG (complex)	CSTR	6	74.4	3.9
Pretreated Hardwood	GBG (complex)	CSTR	5	82.5	8.7
Avicel	MTC (defined)	CSTR	20	58.1	10.2
Avicel	MTC (defined)	Batch	5	63.8	2.6
Cellobiose	MTC (defined)	Batch	9	51.7	4.5
Avicel	LC (defined)	Batch	5	66.7	8.6
Cellobiose	LC (defined)	Batch	8	63.7	10.3

[a] All data reported here for the first time except those in GBG medium which are from Lynd et al. (1989).

in most reports from other labs (Dharmagadda et al., 2010; Magnusson et al., 2009; Islam et al., 2008; Islam et al., 2006; Levin et al., 2006; Lu et al., 2006; Lynd et al., 1989; Tailliez et al., 1989; Kurose et al., 1988; Lamed and Zeikus, 1980). This study was undertaken to identify the carbon that is missing from the previous carbon balances in *C. thermocellum* ATCC 27405. In the course of these studies, the nitrogen balance was also investigated.

2. Methods

2.1. Microbial cultures and chemicals

C. thermocellum ATCC 27405 was purchased from ATCC. A single colony isolate was transferred twice on LC medium (see below) with Avicel PH-105 (FMC Corp., Philadelphia, PA) as the substrate in order to dilute non-substrate carbon found in MTC medium and stored in 5 mL serum vials at −80 °C until needed. All chemicals were reagent grade and unless otherwise stated were purchased from Sigma Chemical Co. (St Louis, MO). All gases were purchased from Airgas East (White River Junction, VT).

2.2. Growth media

C. thermocellum was cultured with either cellulose (Avicel) or cellobiose as the growth substrate using either low carbon (LC) medium. In medium for thermophilic clostridia (MTC) medium, the concentration of carbon in compounds other than the substrate (MOPS buffer, citric acid, L-cysteine, and vitamins) exceeds the concentration of carbon in the substrate. By contrast, the concentration of non-substrate carbon in LC medium was 2% of the concentration of substrate carbon in LC medium. Thus, LC medium had a much smaller background signal for total carbon analysis and was the primary medium used. The concentration and preparation of MTC was followed as described by Zhang and Lynd (2003).

LC media is composed of 10 g/L carbohydrate (for bottles, 5 g/L), 2 mg/L resazurin (optional), 0.0 g/L (for bottles 2.0 g/L) 3-(N-morpholino)propanesulfonic acid (MOPS), 3.0 g/L K_2HPO_4, 2.0 g/L KH_2PO_4, 2.0 g/L NH_4Cl, 0.2 g/L $MgCl_2{\cdot}6H_2O$, 0.5 g/L $CaCl_2{\cdot}2H_2O$, 0.0025 g/L $FeCl_2{\cdot}4H_2O$, 0.1 g/L L-cysteine hydrochloride monohydrate, 0.002 g/L Pyridoxamine dihydrochloride, 0.004 g/L P-aminobenzoic acid (PABA), 0.002 g/L D-biotin, 0.002 g/L Cobalamin, 0.00125 g/L $MnCl_2{\cdot}4H_2O$, 0.0005 g/L $ZnCl_2{\cdot}6H_2O$, 0.000125 g/L $CoCl_2{\cdot}6H_2O$, 0.000125 g/L $NiCl_2{\cdot}6H_2O$, 0.000125 g/L $CuSO_4{\cdot}5H_2O$, 0.000125 g/L H_3BO_3, and 0.000125 g/L Na_2MoO_4. Components are made in six separate solutions, A through F. Solution A contains desired carbohydrate (final concentration 5 g/L for bottles and 10 g/L for reactors) and DI water. Solution B contains K_2HPO_4 and KH_2PO_4 at 25× concentration. Solution C contains NH_4Cl at 50× concentration. Solution D contains $MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}2H_2O$, $FeCl_2{\cdot}4H_2O$, and L-cysteine hydrochloride monohydrate at 50× concentration. Solution E (light sensitive) contains the vitamins: Pyridoxamine dihydrochloride, P-aminobenzoic acid (PABA), D-biotin, Cobalamin at 50× concentration and is stored at 4 °C. Solution F contains trace elements: $MnCl_2{\cdot}4H_2O$, $ZnCl_2{\cdot}6H_2O$, $CoCl_2{\cdot}6H_2O$, $NiCl_2{\cdot}6H_2O$, $CuSO_4{\cdot}5H_2O$, H_3BO_3, and Na_2MoO_4 at 500× concentration. Solution A was autoclaved for 90 min and purged with nitrogen until mixing of the components. Solutions B, C, and D were made separately in sealed serum vials, purged with nitrogen and autoclaved for 25 min. Solutions E and F were filter sterilized into a nitrogen purged and sterile serum vial. Solutions B, C, vitamins and trace elements were added to the medium, then purged with nitrogen gas overnight. Solution D was then added.

2.3. Fermentation studies

Fermentations were carried out in 3.0 L (2.0 L working volume) BIOSTAT A-plus autoclavable, round-bottom, single-wall bioreactors, Sartorious (Goettingen, Germany) using a small (2.5 to 5% v/v) innocula. *C. thermocellum* fermentations were maintained at 60 °C using a resistive heat blanket. The pH was monitored by a Mettler (Columbus, OH) pH probe and maintained at a pH of 6.95 by addition of filter sterilized 4 M KOH. Mixing was accomplished by two flat blade impellors at 200 rpm. The head space was continuously purged with 95 mL/min ultra pure grade nitrogen. Innocula were grown in 260 mL serum bottles using LC media with the addition of 2 g/L MOPS buffer.

2.4. Liquid phase analysis

Liquid phase carbon and nitrogen were measured after removing solids by centrifuging samples for 5 min at 21,000×*g* and combusted using total carbon and nitrogen (TCN) analyzer, Shimatzu Scientific Instruments (Columbia, MD). The TCN analyzer measures carbon and nitrogen using the conventional combustion catalytic oxidation method.

Acetic acid, cellobiose, ethanol, formic acid, glucose, lactic acid, malic acid, pyruvic acid, uracil, and xylose were measured using HPLC. Solids were removed from fermentation broth samples via centrifugation (2 min at 21,000×*g*). The supernatant was then acidified with 50 μL of 10% ($^{wt.}/_{wt.}$) H_2SO_4 to 1 mL of sample and subsequently analyzed on a Bio-Rad HPX-87H column (Hercules, CA) operated at 60 °C with 0.01% ($^{vol.}/_{vol.}$) H_2SO_4 mobile phase using refractive index and ultra violet (λ = 210 nm) detectors. Free amino acids and lipids were measured by AminoAcids.com (St Paul, MN) after broth samples were centrifuged to remove solids (5 min at 21,000×*g*) then filtered using a 0.2 μm spin filter. Samples were then sent for analysis while frozen and packed in dry ice. Soluble glucan and xylan were measured by dilute acid hydrolysis. Solids were removed by centrifugation (20 min at 3000×*g*) then samples were acidified with H_2SO_4 to a concentration of 2.5% $^{wt.}/_{wt.}$ and autoclaved for 60 min. Samples were then filtered using a 0.2 μm spin filter. Monomeric glucose and xylose were then measured by HPLC. Ammonium chloride was measured using a nitroprusside and phenol colorimetric assay (Weatherburn, 1967). Supernatant protein was measured using the Bradford assay (Bradford, 1976).

2.5. Solid phase analysis

Solids were centrifuged (5 min at 21,000×*g*) and washed twice with DI water before being resuspended to the original volume. Solid slurry solutions were combusted using TCN analysis. Cellulose concentration was measured by quantitative saccharification, as described by Lynd et al. (1989), with a fourfold reduction in scale.