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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.

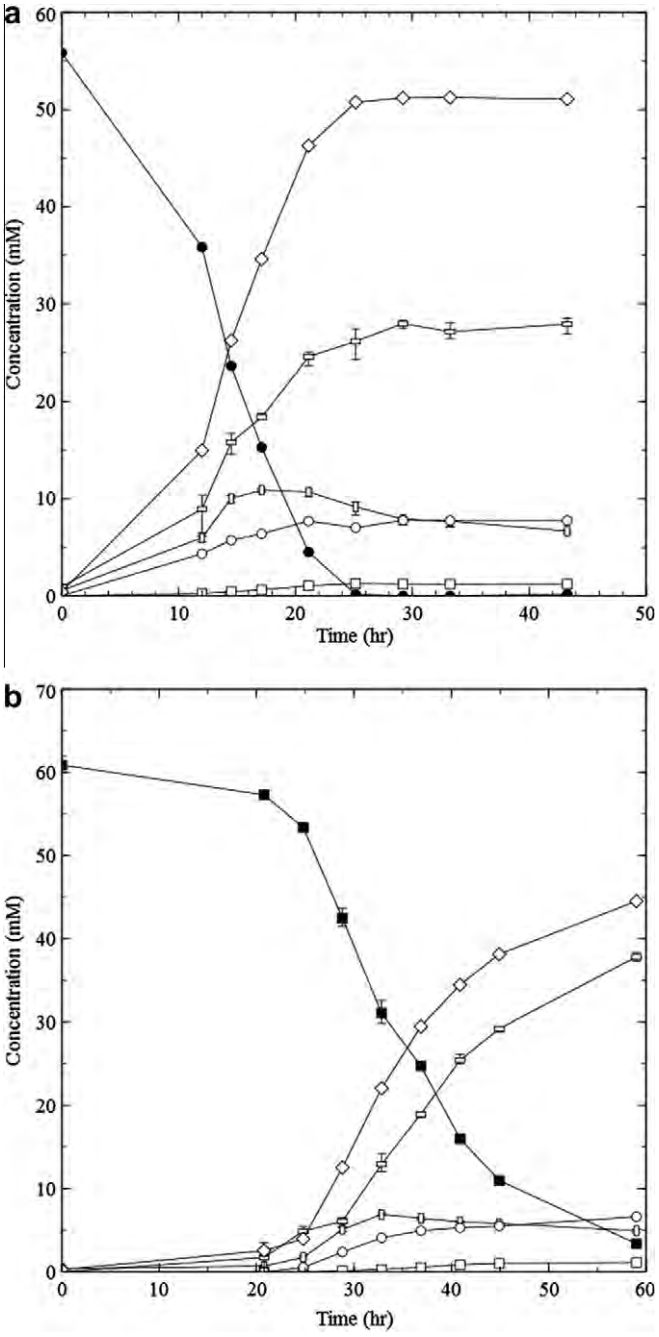

Fig. 1. Growth curves of *C. thermocellum* on cellobiose (a) and Avicel (b). Products concentrations of acetate (◇), ethanol (▭), formate (○), lactate (□), pellet nitrogen (▯), cellobiose (●), and Avicel (■), given in mM. Cells concentrations inferred from pellet nitrogen. Cellulose and cellobiose given in mM glucose equivalents.

Cell carbon concentrations in fermentations of Avicel were inferred with the difference between the carbon found in the washed pellet by TCN and that of hydrolysable carbon found by quantitative saccharification.

2.6. Carbon dioxide and carbonates

Carbon dioxide was measured using an infrared Li-820 CO_2 analyzer manufactured by LiCor Biosciences (Lincoln, NB). Gas concentrations were determined by calibration to known concentrations of carbon dioxide. Total carbon dioxide was measured by integrating concentration measurements (trapezoidal rule) in a known flow rate of a nitrogen carrier gas.

$$n_{CO_2} \cong \frac{P}{2RT} v_{N_2} \sum_{i=1}^{i=t} (t_i - t_{i-1}) \left[\frac{x_i}{1-x_i} + \frac{x_{i-1}}{1-x_{i-1}} \right] \quad (2)$$

where n_{CO2} is the amount of moles of carbon dioxide and x_i is the mole fraction of carbon dioxide in the offgas stream at time point i. The concentration of dissolved CO_2 in the liquid phase was calculated using Henry's Law with values of the Henry's Law constant determined with temperature adjustment as described by Sander (1999). Estimation of carbonates was based on the following reaction mechanisms for ionization from dissolved carbon dioxide, as described by Ho and Sturtevant (1963) and Johnson (1982).

$$CO_2(aq) + H_2O \underset{K_1}{\Longleftrightarrow} H_2CO_3 \quad (3)$$

$$H_2CO_3 + H_2O \underset{K_2}{\Longleftrightarrow} H_3O^+ + HCO_3^- \quad (4)$$

$$HCO_3^- + H_2O \underset{K_3}{\Longleftrightarrow} H_3O^+ + CO_3^{-2} \quad (5)$$

$$CO_2(aq) + H_2O \underset{K_4}{\Longleftrightarrow} H_3O^+ + HCO_3^- \quad (6)$$

Values used for constants were as follows: $K_1 = 2.16 \times 10^{-3}$ (Garg and Maren, 1972), $K_2 = 4.46 \times 10^{-7}$ (Goldberg et al., 2002), $K_3 = 4.69 \times 10^{-11}$ (Goldberg et al., 2002), and $K_4 = 7.60 \times 10^{-7}$ (Johnson, 1982).

2.7. Size exclusion chromatography

Fermentation broth was centrifuged (20 min at 3000×*g*) to remove solids. Supernatant was spin filtered with a 0.2 μm filter and stored at −20 °C. A GE healthcare (Barre, VT) column was filled with HW55S resin (Toyopearl, Tessenderlo, Belgium) using 0.1% trifluoroacetic acid as the mobile phase. Refractive index was monitored continuously and fractions were collected at two minute intervals. These fractions were tested for neutral sugars using the phenol sulfuric acid colorimetric assay described by Masuko et al. (2005). Retention times of samples were compared to several known standards of maltohexaose, glucose, cellobiose, and a mixture of maltodextrins.

2.8. Carbon recovery

The carbon recovery, R^C, was calculated based on:

$$R^C = \frac{\sum_m \sum_{i=0}^{n} C_{C,i} \times V_{S,i} + v_{offgas} \int_0^\tau C_{CO_2} dt + V_{R,t=end} \sum_m C_{C,t=end}}{V_{R,t=0} \sum_m C_{C,t=0}} \quad (7)$$

where $C_{C,t}$ = concentration of carbon in component i at time t (either $t = 0$ or t = end), $V_{S,i}$ = volume of the ith sample, n = number of samples taken, and m = the number of measured carbon compounds, v_{offgas} = flow rate of the off gas stream, C_{CO2} = concentration of carbon dioxide in the off gas stream, and $V_{R,t}$ = is the volume in the reactor at time t.

Similarly, the nitrogen recovery, R^N, was calculated using

$$R^N = \frac{\sum_p \sum_{i=0}^{n} C_{N,i} \times V_{S,i} + V_{R,t=end} \sum_p C_{N,t=end}}{V_{R,t=0} \sum_p C_{N,t=0}} \quad (8)$$

where $C_{N,i}$ = concentration of nitrogen at the ith sample, and p = the number of measured nitrogen compounds.

All error bars presented in figures are the sum of the standard deviations of the measurements.

3. Results and discussion

C. thermocellum ATCC 27405 was cultivated in batch culture on a medium having a very low carbon background (non-substrate

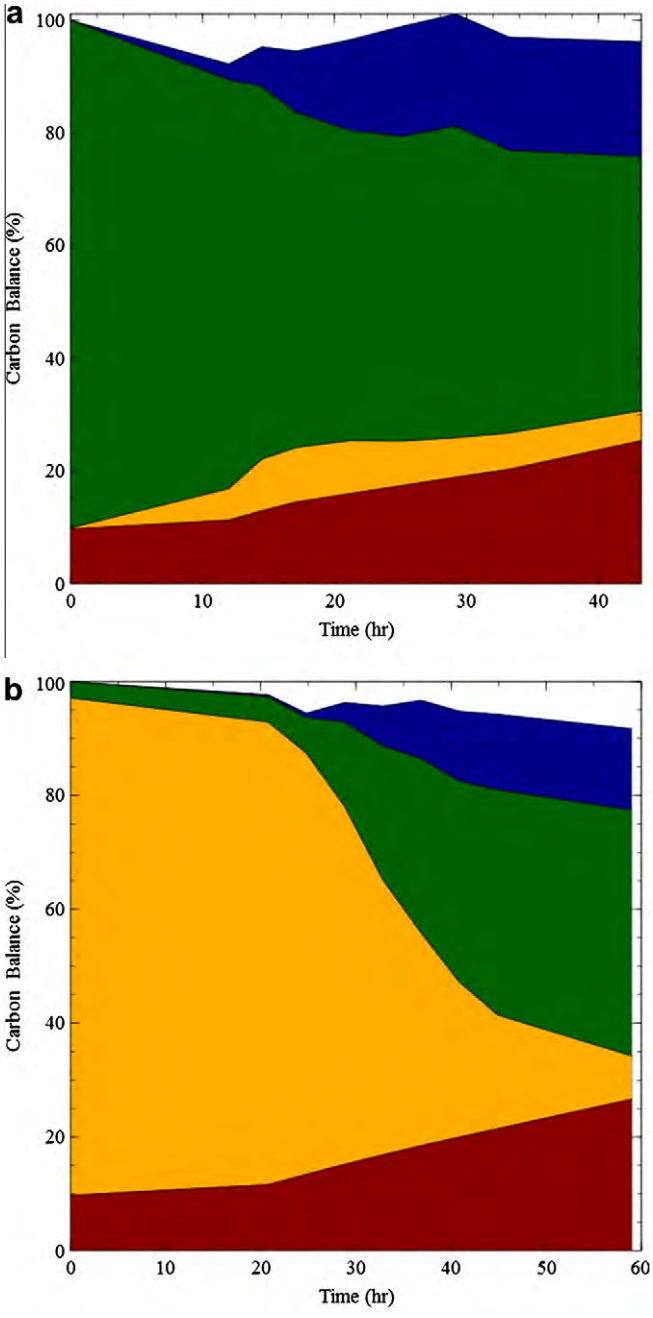

Fig. 2. Carbon balance applied to *C. thermocellum* fermentations of cellobiose (a) and Avicel (b) in terms of solids, liquids, carbon dioxide and sampling. Total sample removed (red), solids (orange), liquids (green), and carbon dioxide (blue).

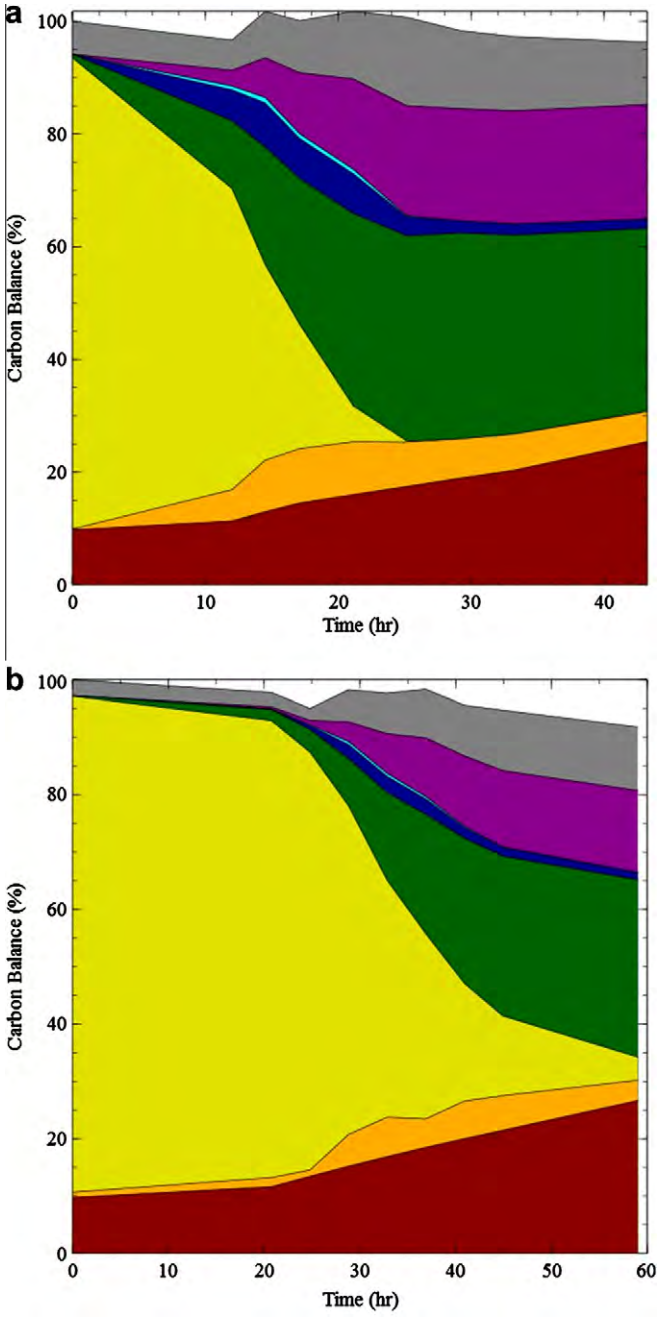

Fig. 3. Carbon balance applied to *C. thermocellum* fermentation on cellobiose (a) and Avicel (b) having accounted for total sample removed (red), cells (orange), substrate (yellow), ethanol, acetate and lactate (green), carbonates and formate (blue), dissolved CO_2 (light blue), CO_2 (purple), unidentified liquid carbon (grey). There is a large portion of unidentified liquid carbon for both substrates.

carbon 1.6% of substrate carbon) with microcrystalline cellulose (Avicel) or cellobiose as the growth substrate at an initial concentration of about 10 g/L. Only a small amount of carbon ($\leq$1% of substrate carbon) was detected when fermentation off gas was passed through a cold trap, indicating that loss of volatile products was negligible under the conditions tested (data not shown).

Batch growth curves were carried out for cellobiose (Fig. 1a) and Avicel (Fig. 1b) with reported products and cultivation conditions typical of those in the literature. The substrate initially present is consumed completely in the case of cellobiose, and nearly completely (95%) in the case of Avicel, with pellet nitrogen (indicative of biosynthesis) peaking and declining before the substrate is exhausted. Acetate, ethanol and lactate were produced in relative proportions (mass basis) of 2.38:1.00:0.09 for cellobiose and 1.53:1.00:0.06 for Avicel.

3.1. Carbon balances

Carbon was quantified in the solid phase in the bioreactor (washed pellet), the liquid phase in the bioreactor (supernatant), the gas phase leaving the bioreactor (by integrating the mass flow of CO_2 over time), and samples removed from the bioreactor (containing both solids and liquid) over the course of batch cultivation. At the end of fermentation, the carbon content of these four streams accounted for >92.3% and 91.4% of the carbon initially

present in the substrate for cellobiose and Avicel, respectively (Fig. 2).

If the carbon recovery is calculated based on concentrations of products and neglecting cells as per Eq. (1), rather low values are obtained (72.1% for cellobiose and 72.6% for Avicel), consistent with the results in Table 1. Somewhat higher values, 85.2% for cellobiose and 80.7% for Avicel, are calculated if cell mass and carbon removed in sampling are accounted for as in Eq. (7). However, the total carbon in the liquid phase exceeds the sum of carbon in the measured products by over 10% for both cellobiose and Avicel, implying byproduct formation (Fig. 3).

3.2. More detailed liquid phase analysis

A more detailed analysis was undertaken in order to characterize the unidentified organic compounds in the liquid phase. To test for the presence of high molecular weight organic products, such as extracellular polysaccharides (EPS), supernatant samples from cellobiose and Avicel fermentations were separated by size exclusion chromatography. As seen in Fig. 4, no compounds with a molecular weight larger than 720 Da, corresponding to a glucan with a degree of polymerization (d.p.) = 4, were detected via refractive index. A phenol sulfuric acid assay, indicative of carbohydrates, confirmed the absence of high molecular weight polysaccharides above the limits of detection. The chromatography trace indicated a molecule with molecular weight corresponding to a 3–4 d.p. oligosaccharide, and dilute acid hydrolysis indicated the presence of soluble glucan (Avicel and cellobiose fermentations) and xylan (Avicel fermentation only).

The following small molecules were detected in fermentation broth supernatant: glucose, xylose (Avicel only), pyruvate, uracil, soluble glucan, soluble xylan (Avicel only), and free amino acids. Over 4% of the initial substrate carbon is converted into free amino acids. During growth on cellobiose, valine was the amino acid in greatest concentration, followed by alanine and proline, while growth on Avicel saw alanine in the greatest concentration, followed by valine and proline (Fig. 5).

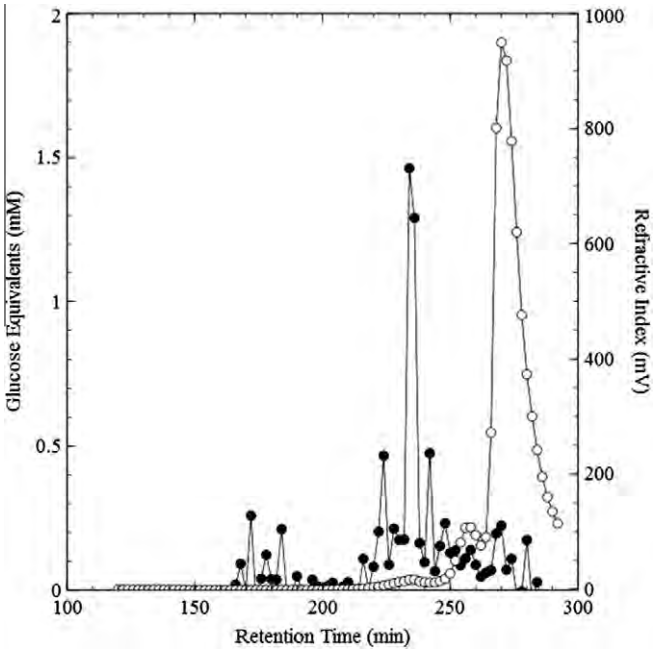

Fig. 4. Fermentation broth from an Avicel fermentation (taken at 59 h post–inoculation) was separated by size. Refractive index (○) and a colorimetric assay for carbohydrates (●) found no compound greater in length than an oligosaccharide of d.p. 3–4.

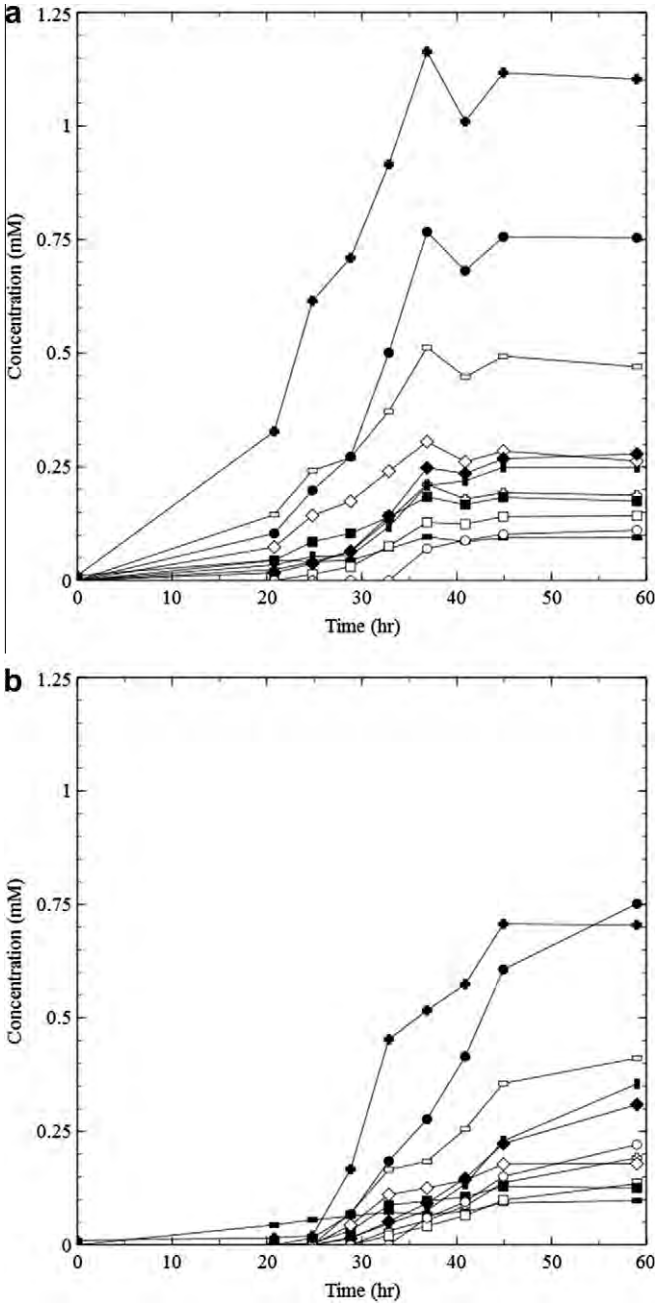

Fig. 5. Free amino acid profile found in the supernatant during fermentations on cellobiose (a) and Avicel (b). alanine (●), asparagine (○), glycine (♦), isoleucine (◇), leucine (■), lysine (□), phenylalanine (◔), proline (▭), serine (◆), threonine (▬), and valine (⬡).

To confirm these results, an Avicel fermentation was carried out without sampling during the course of fermentation. The carbon recovery based on elemental analysis was 97% relative to the substrate carbon initially present; by including the small molecules listed above and using Eq. (7), carbon recovery based on identified compounds was 93% of the substrate carbon initially present (Table 2).

Formic acid has previously been reported in many studies of *C. thermocellum* (Sparling et al., 2006; Rydzak et al., 2009; Levin et al., 2006; Islam et al., 2006; Magnusson et al., 2009), and extracellular pyruvate has been reported in a few studies (Rydzak et al., 2009; Levin et al., 2006; Islam et al., 2006). Soluble xylan and xylose found in the fermentation broth of Avicel fermentations is presumed to originate from the substrate and not metabolism, since

Table 2
Summary of carbon recovery (C2 + C3) and mass balance results for *C. thermocellum* grown on crystalline cellulose (Avicel). Ethanol, acetate, lacate, formate and carbon dioxide were abbreviated with E, A, L, F, and CO_2, respectively.

End point analysis, *C. thermocellum* fermentations		
Time	50	h
Substrate	Avicel	
Initial conc.	10.3	g Gluc equv.
Final conc.	0.9	g Gluc equv.
Solid		
Insoluble glycan	12.5%	gC/gC
Insoluble xylan	0.5%	gC/gC
Cells	11.0%	gC/gC
Liquid		
Cellobiose	0.0%	gC/gC
Acetate	18.1%	gC/gC
Ethanol	11.8%	gC/gC
Lactate	8.1%	gC/gC
Formate	5.1%	gC/gC
Glucose	0.0%	gC/gC
Xylose	0.7%	gC/gC
Pyruvate	1.0%	gC/gC
Malate	0.7%	gC/gC
Uracil	0.1%	gC/gC
Soluble glucan	1.5%	gC/gC
Soluble xylan	2.2%	gC/gC
Protein	2.8%	gC/gC
Free amino acids	2.4%	gC/gC
CO_2 (aq)	0.2%	gC/gC
Carbonate	1.4%	gC/gC
Gas		
CO_2 (g)	12.9%	gC/gC
Unidentified compounds		
Unknown liquids	3.8%	gC/gC
C2 + C3 Carbon balance (Eq. (1))		
E, A, L	62.6%	molC/molC
E, A, L, Cells	76.0%	molC/molC
Complete carbon balance (Eq. (8))		
E, A, L, F, CO_2	57.7%	gC/gC
E, A, L, F, CO_2, cells	68.7%	gC/gC
Identified products	93.2%	gC/gC
Total	97.0%	gC/gC

xylan was found in the pellet at the initial time point. Consistent with this, about 2% of the total carbon initially present in Avicel-containing medium is in the form of insoluble xylan. Moreover, insoluble xylan concentration decreases significantly and the amount of xylose and soluble xylan increases over the course of fermentation, with the concentrations of xylose and soluble xylan essentially equal to the original concentration of insoluble xylan. Insoluble glucan was found to be present at concentrations as high as 2.63% of the initial substrate carbon during a cellobiose fermentation, indicative of either glucose-containing insoluble cellular components or perhaps synthesis of a glucan. Lynd et al. (1989) also detected residual glucan in cellobiose-grown cell pellets of *C. thermocellum*, and the cellulosome of this organism is known to be glycosylated (Bayer et al., 1994). The presence of storage carbohydrates, has been reported for other cellulolytic bacteria, including *Clostridium cellulolyticum* (Desvaux, 2006) and *Fibrobacter succinogenes* (Matheron et al., 1996).

Soluble glucan was found in fermentations of both Avicel and cellobiose, with size exclusion chromatography results consistent with an oligosaccharide of between 3 and 4 d.p. (Fig. 4). Cellotriose and cellotetraose synthesis from cellobiose was observed, as has been reported previously for *C. thermocellum* (Zhang and Lynd, 2005), *C. cellulolyticum* (Desvaux, 2006) and *F. succinogenes* (Matheron et al., 1996). Oligosaccharide synthesis is thought to be due to the cellobiose and cellodextrin phosphorylase activities present in *C. thermocellum*, for which equilibrium lies in the direction of chain lengthening (Nakai et al., 2010). Uracil was also found in the fermentation broth of *C. thermocellum*. Most surprising was the amount of free amino acids. There was a 1.1% and 1.6% yield of valine and 5.0% and 5.6% yield of all free amino acids from Avicel and cellobiose, respectively.

The secretion of pyruvate suggests a metabolic block after the pyruvate node of fermentation. Further investigation of this portion of metabolism will be a fruitful area for future research.

3.3. Nitrogen balance

Motivated by the high concentration of free amino acids found in the supernatant, a nitrogen balance was undertaken (Fig. 6). For the crystalline cellulose fermentation, 95.7% of the original nitro-

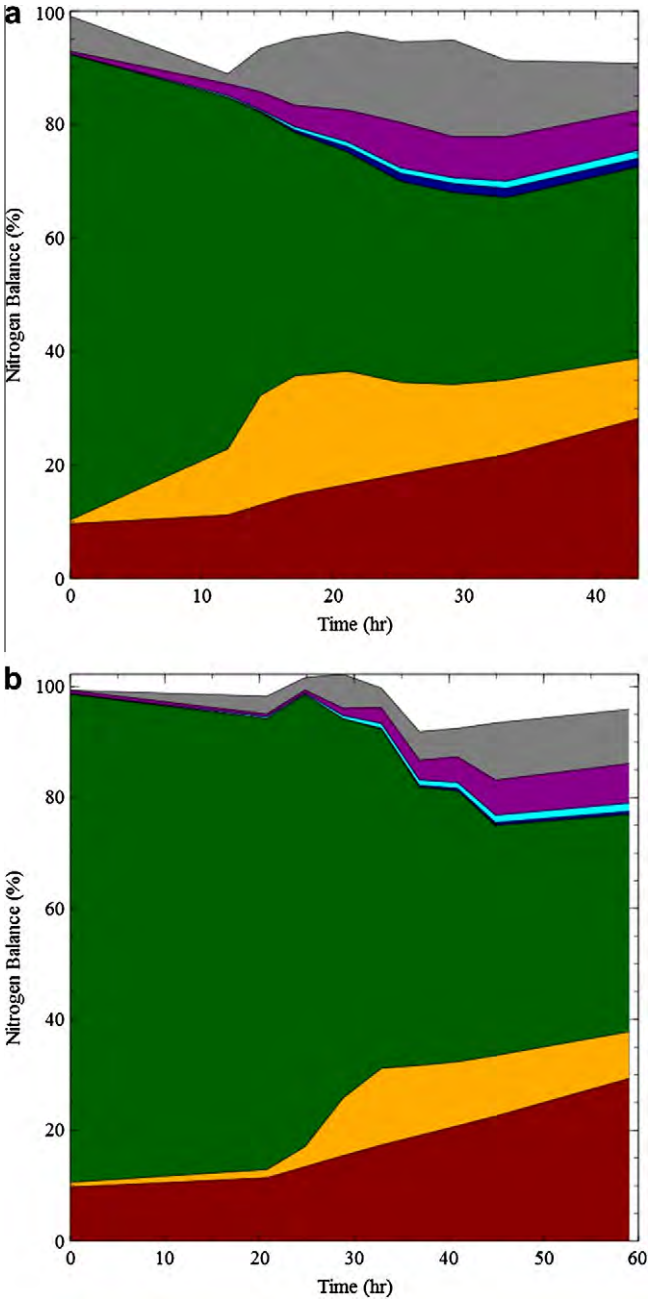

Fig. 6. Nitrogen balance for cellobiose (a) and Avicel (b) fermentations by *C. thermocellum*. Total sample removed (red), cells (orange), ammonium chloride (green), uracil (blue), protein (light blue), free amino acids (purple), and unidentified liquid nitrogen (grey).

gen is measured at the end of fermentation with 86.5% of the original nitrogen identified. For cellobiose, 91.6% of the original nitrogen is accounted for with 83.7% of the nitrogen identified.

The low carbon (LC) medium used here was developed to minimize carbon, but not nitrogen. In particular, LC medium contains 2.0 g/L NH_4Cl, which accounts for the majority of the nitrogen found in a nitrogen balance. The loss of about 10% of the nitrogen during the fermentation could be due to ammonia evaporation.

It is possible LC medium is affecting product formation such as free amino acid synthesis. It is important to note that the natural environments where cellulose is degraded by anaerobic clostridia are deficient in reduced nitrogen (Leschine et al., 1988). Thus, an abundant source of reduced nitrogen (2.0 g/L ammonium in LC medium) could significantly distort bacterial metabolism, as has been seen in *Clostridium hungatei* (Monserrate et al., 2001) and *Clostridium cellulolyticum* (Desvaux, 2006). Additionally, Lamed and Zeikus (1981), found an ammonium activated malic enzyme that could be part of a pathway (with PEP carboxykinase and malate dehydrogenase) from phosphoenolpyruvate to pyruvate, providing the NADPH needed for reductive amination of α-ketoglutarate to glutamate. This could explain the presence of malic acid found in the fermentation broth (Table 2) as well as the production of free amino acids.

4. Conclusions

C. thermocellum fermentations consistently exhibit low carbon recoveries. Using a mass balance we were able to account for 93% of the original carbon in solids, liquids and carbon dioxide. This analysis indicated a portion of unidentified carbon in the liquid phase, which consisted of low molecular weight compounds not commonly reported for *C. thermocellum*, such as, pyruvate, malate, uracil, soluble glucan, and free amino acids. Our results provide an improved fundamental foundation for future studies of *C. thermocellum* in both fundamental and applied contexts, and also illustrate general principles applicable to other organisms.

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