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Development and Evaluation of Methods to Infer Biosynthesis and Substrate Consumption in Cultures of Cellulolytic Microorganisms

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ABSTRACT: Concentrations of biosynthate (microbial biomass plus extracellular proteins) and residual substrate were inferred using elemental analysis for batch cultures of *Clostridium thermocellum*. Inferring residual substrate based on elemental analysis for a cellulose (Avicel)-grown culture shows similar results to residual substrate determined by quantitative saccharification using acid hydrolysis. Inference based on elemental analysis is also compared to different on-line measurements: base addition, CO_2 production, and Near Infra Red optical density (OD_{850}). Of these three on-line techniques, NIR OD_{850} has the best correlation with residual substrate concentration and is the most practical to use. Both biosynthate and residual substrate concentration demonstrate typical sigmoidal trends that can easily be fitted with a five-parameter Richards curve. The sigmoidal character of the inferred concentrations and on-line data, especially the CO_2 production rate, suggest that there is a maximum in cell-specific rates of growth and substrate utilization during batch fermentations of crystalline cellulose, which is not observed during grown on cellobiose. Using a sigmoidal fit curve, the instantaneous specific growth rate was determined. While soluble substrate grown cultures show a constant growth rate, cultures grown on solid substrate do not. Features of various approaches are compared, with some more appropriate for rapid general indication of metabolic activity and some more appropriate for quantitative physiological studies.

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KEYWORDS: *Clostridium thermocellum*; anaerobic cellulose fermentation; elemental analysis; biomass determination

Introduction

Cellulolytic microorganisms are responsible for the largest flow of fixed carbon in the biosphere, play central roles in the rumen, ensiling, and composting, and could potentially be used for industrial production of fuels and chemicals. They also possess specialized and distinctive features responsive to their ecological niche. These include production of suites of proteins involved in plant cell wall solubilization that often number 50 or more distinct molecules (Bayer et al., 2007; Demain et al., 2005; Wilson, 2011) and occupy a substantial fraction of cellular resources (Zhang and Lynd, 2005), as well as features that are associated with adhesion of cells to the surface of insoluble substrates and likely to play a role in capture of hydrolysis products, enhanced solubilization rates, and inter- and intra- species competition (Lu et al., 2006; Weimer et al., 2006). Notwithstanding the considerable impetus to study cellulolytic microbes, documentation, and understanding of foundational aspects of microbial physiology—for example, absolute and cell-specific rates of growth and substrate utilization, cell yield and maintenance energy requirements, the extent of metabolic coupling, etc.—are generally at nascent stages of development for these organisms.

Commonly used methods for quantifying cell synthesis in soluble substrate fermentations—for example, optical density, viable cell counts, cell dry weight—are in most cases impractical, inaccurate, or both for fermentation of solid substrates (Lynd et al., 2002). As reviewed by Kennedy et al. (1992b), and Lynd et al. (2002), alternative approaches to the estimation of cell and substrate concentrations for microbial cultures growing on cellulose and other insoluble substrates include inference of cell growth based on indicators of metabolic activity, inference based on pellet composition, and physical approaches to discriminate microbial cells and residual substrate. Inference of substrate utilization based on metabolic activity—"metabolic methods"—has been suggested using off-gas analysis (Schofield et al., 1994), calorimetry (Dermoun and Belaich, 1985), volumetric addition of reagents for pH control, production of fermentation

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products, and measurement of substrate either directly, for example, by laborious quantitative saccharification (Saeman et al., 1945; Sluiter et al., 2008), or indirectly via (on-line) techniques (Kennedy et al., 1992a; Hernandez and Marin, 2002; Hernandez et al., 2000). Metabolic methods are often rather convenient, many of them can be implemented on-line, and under many circumstances they can provide a good indication of substrate consumption. However, they cannot be used to estimate cell synthesis in the quite common circumstance when proportionality between metabolism and cell synthesis cannot be assumed.

Compositional approaches, typically applied to pellets containing cells and residual substrate, rely on analysis of a chemical component that is either specific to cells or present in different proportions in cells and the insoluble substrate of interest. Molecules or classes of molecules suggested in this context include protein (Bradford, 1976; Jensen et al., 2008), ATP (Fujinami et al., 2004; Saludes et al., 2007), RNA/DNA (Bergmann et al., 2010; De Mey et al., 2006), and phospholipids (Babechuk et al., 2009; Fletcher et al., 2011). Jensen et al. (2008) combined different methods to measure cellular biomass in the presence of cellulosic solids to conclude that protein assays are useful indicators of microbial growth. Elemental analysis can also be used, with nitrogen by far the most common (Dharmagadda et al., 2010; Izquierdo et al., 2010; Lynd et al., 1989; Weimer et al., 1991). Once the target molecule or element is quantified, cell mass can be estimated based on the measured or assumed fraction of that component in cells. Concentrations of residual substrate are commonly calculated based on the difference between total pellet dry weight and the estimated mass of cells in the pellet.

Physical approaches warrant consideration, but have not been shown to be effective for microbial cultures growing on solid substrates. For example Neves et al. (2000) evaluated capacitance to discriminate between cells and residual substrate, but we found this approach to be ineffective at the low concentrations of cells typically encountered in fundamental studies of cellulolytic anaerobes (unpublished).

We report here work aimed at validating and comparing methods for inferring substrate and biosynthate concentrations for non-filamentous cellulolytic microorganisms. Experiments are carried out using *Clostridium thermocellum* ATCC 27405, an actively cellulolytic, thermophilic, obligately anaerobic microbe that is among the most widely-studied (Bayer et al., 2007; Demain et al., 2005; Lynd et al., 2002).

Materials and Methods

Organism, Culturing Conditions and Media Composition

The organism, media composition (low carbon medium (LC)) and the specific culturing conditions in bioreactors are described in detail elsewhere (Holwerda et al., 2012).

All data presented in this paper come from cultures of *C. thermocellum* grown on either 5 g/L crystalline cellulose (Avicel PH105) or 5 g/L cellobiose in LC medium. Experimental data points are averages of triplicate samples. Error bars represent ± 1 standard deviation.

On-Line Data Collection

Carbon dioxide produced during fermentation experiments was measured by a LI- 820 CO_2 analyzer (Li-Cor Biosciences, Lincoln NE) as a rate in ppm/min. A custom built LabVIEW®-based control system (National Instruments, Austin TX) recorded data (ppm/min) every 15 min. For the cumulative CO_2 plots in Figures 1B, 2B, and 5 the CO_2 rate data are integrated using numerical quadrature (trapezoidal rule) between two subsequent time points. Base addition data (4 N KOH) were collected by the MFSC/DA program included in the software accompanying the Sartorius Aplus bioreactor system (Sartorius Stedim, Bohemia, NY). Near Infrared Optical density (OD_{850}) data were obtained using the BE2100 "Bugeye" non-invasive biomass monitor by Buglab LLC Danville, CA. NIR optical density data from the BE2100 is shown as "OD_{850}." Four different on-line Optical Density systems were tested for measuring optical density and compared to off-line analysis data (elemental analysis/quantitative saccharification). The optical density signal was much better correlated with off-line data for the ex situ system reported herein than probes we tested that were immersed in the fermentation broth.

The base addition interval (5 min), the NIR OD interval (1 min), the CO_2 sampling interval (15 min) and the CO_2 sampling line residence time ($\sim$4 min), are an order of magnitude smaller than the time constant characterizing the dynamics of the system ($1/\mu$, at least 180 min) and thus meets the criterion for on-line measurement as specified by Olsson et al. (1998).

Analytical Methods

Fermentation products acetate, formate, ethanol, glucose and residual cellobiose were determined by HPLC (Waters, Milford MA), using an Aminex HPX-87H column (Bio-Rad, Hercules CA) and refractive index detection with a 5-mM sulfuric acid solution eluent. The residual Avicel PH105 concentration was quantified by Quantitative Saccharification as described by Holwerda et al. (2012).

The amounts of nitrogen and carbon in sample pellets taken from bioreactors were determined using a Shimadzu TOC-Vcph Total Organic Carbon analyzer with added Total Nitrogen unit and ASI-V autosampler (Shimadzu Scientific Instruments, Columbia MD), with an Avicel suspension as standard for carbon determination and an acidified glycine standard for nitrogen determination as described by Holwerda et al. (2012).

Biosynthate Dry Weight (BDW)

For cellobiose-grown cultures the biosynthate dry weight in g/L was determined by weighing the retentate on 0.22 μm polycarbonate filters (47 mm GTTP, Millipore, Billeria MA)

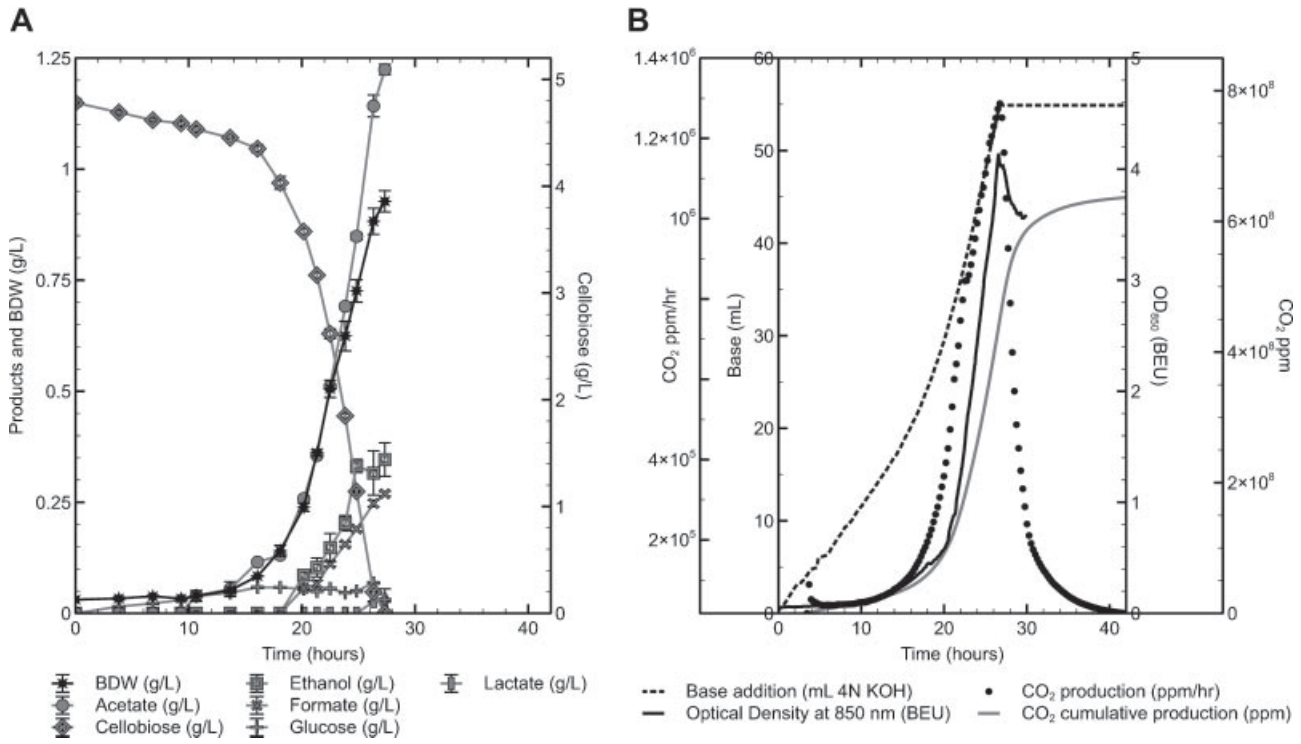

Figure 1. Fermentation time course for *C. thermocellum* grown on cellobiose in LC medium. **A**: Biosynthate dry weight (BDW), products, and residual substrate concentration. **B**: On-line signals: base addition, CO_2 production rate, cumulative CO_2 production, and OD_{850}.

after washing once with a volume of MilliQ water equal to the volume of sample filtered, and drying at 60°C for at least 72 h.

Curve Fitting, Rate Calculation and Data Normalization

Microsoft's Excel Solver add-in and Mathworks®'s MATLAB with its symbolic toolbox were used to fit parameters to exponential and sigmoidal models by minimizing the sum of squared errors.

For cellobiose-grown cultures, curves of biosynthate versus time were fit to experimental data using the exponential growth model:

$$X = X_0 \mathrm{e}[\mu t] \tag{1}$$

where X_0, initial biosynthate concentration (g/L); μ, growth rate (h^{-1}); t, time (h).

The specific growth rate for the exponential fit curve of the cellobiose grown cultures was calculated according to Equation (2):

$$\mathrm{d}X/\mathrm{d}t/X(t) = \frac{\mu X_0 \mathrm{e}[\mu t]}{X_0 \mathrm{e}[\mu t]} = \mu \tag{2}$$

The residual cellobiose concentration data in Figure 5 was calculated by solving the cell yield equation. We have used a yield value of 0.2 (Holwerda and Lynd 2013):

$$S = S_0 - \frac{[X - X_0]}{Y_{X/S}} \tag{3}$$

where S, concentration of substrate (g/L); S_0, initial substrate concentration (g/L); X, biosynthate concentration (g/L); X_0, initial biosynthate concentration (g/L); $Y_{X/S}$, yield of biosynthate over substrate (g/g).

For cellulose-grown cultures, data for biosynthate and residual substrate concentrations was fitted using the five parameter version of the Richards curve (Richards, 1959):

$$y(t) = A_0 + \frac{[A_t - A_0]}{[1 + \mathrm{e}[[t_0 - t]/\mathrm{sl}]]^{\mathrm{ap}}} \tag{4}$$

where A_0, lower horizontal asymptote; A_t, higher horizontal asymptote; t, time point (variable); t_0, inflection time parameter; sl, slope parameter; ap, asymmetry parameter.

The first derivative of the sigmoidal curve, used to calculate the specific growth rate on cellulose is defined as:

$$\frac{\mathrm{d}y}{\mathrm{d}t} = \frac{-\mathrm{ap}\ \mathrm{e}[[t_0 - t]/\mathrm{sl}][A_0 - A_t]}{[\mathrm{sl}\ \mathrm{e}[[t_0 - t]/\mathrm{sl}] + 1]^{[\mathrm{ap}+1]}} \tag{5}$$

Division of Equation (5) by Equation (4) results in the specific growth rate for cellulose-grown cultures.

Table I. Comparative features of methods to study microbial cellulose utilization.

Measurement	Inference	Accuracy (this study)[a]	General applicability
On line, biosynthesis calculated from inferred cell concentration. The maximum time for X is set at 25 h and for S at 41.25 h			
Optical density	Substrate from predetermined correlation, perhaps adjusted using final OD	S: MSE = 1.4E−01, first over predicted then under predicted and over predicted again X: MSE = 6.5E−01, first under predicted then over predicted	All methods in this category: • Least effort required, suitable for multiple measurements & screening
CO_2 production	Substrate from assumed fermentation stoichiometry or predetermined correlation	S: MSE = 4.2E−01, under predicted X: MSE = 8.4E−01, over predicted	• Good semi-quantitative indicator of substrate consumption • Quality not sufficient for many kinetic analyses[b]
Base addition	Substrate from predetermined correlation or, perhaps, fermentation stoichiometry	S: MSE = 1.5E−01, over predicted X: MSE = 7.3E−02, first under predicted then over predicted	• Insensitive to changing biosynthate yield
Off line analysis of two elements			
C, N	Substrate and biosynthesis from elemental composition Cell concentrations may be inferred, but reporting biosynthate N involves fewer assumptions	NA	• More effort required compared to on-line measurements • High accuracy—for example, sufficient for growth rate determination • Sensitive to changing biosynthate yield • Insensitive to changing allocation of biosynthate between cells and cellulase
Off line analysis of two elements plus measurement of cellulase			
C, N, ELISA or proteomics	Similar to above	Not tested here	• Additional effort to measure cellulase • As above but sensitive to changing allocation of biosynthate between cells and cellulase

[a]Mean squared errors (MSE) are calculated relative to normalized elemental analysis data on or close to the same time points.
[b]Compared to elemental analysis and as implemented here.

For comparison of the different on-line data sets with sigmoidal fit curve and fermentation data, all data were normalized by setting the maximum value to 1, and the minimum value to zero:

$$N(t) = \frac{D(t) - D_{\min}}{D_{max} - D_{\min}} \quad (6)$$

where $N(t)$, normalized data at time t; $D(t)$ = data at time t; $D_{\min}$, minimal data value; $D_{\max}$, maximum data value.

Normalized data sets for on-line data (see Table I) were compared to data obtained by elemental analysis by calculating the "mean squared error" or MSE using the normalized sigmoidal fit curve as reference data set.

Within the comparison for the residual substrate concentration and normalized on-line data the maximum time point was set at 41.25 hrs. When the measured parameter (base, OD_{850} or cumulative CO_2) reached a stable value earlier, the data value from that time point was used as maximum.

Results

Biosynthate

The term "biosynthate" was used to denote the aggregate result of biosynthesis, and was conceived with the objectives of being both interpretable and measurable in the context of microbial fermentation of insoluble substrates. Conceptually, biosynthate refers to an aggregate measurement of cells including cell-bound proteins such as cellulases, and extracellular protein, which maybe present in the pellet or supernatant. Operationally, biosynthate was inferred for cultures grown on insoluble substrate (Avicel) based on elemental analysis using Equations (10)–(13). For cultures grown on soluble substrate (cellobiose), biosynthate was measured based on dry weight and elemental analysis.

Batch Fermentation of Soluble and Insoluble Substrates

Figures 1A and 2A present time courses for substrate and fermentation products for *C. thermocellum* grown on cellobiose and Avicel PH105, respectively. Figure 1A also includes the biosynthate dry weight (BDW). Figures 1B and 2B show on-line data for base addition, the rate of CO_2 production and optical density measured at 850 nm (OD_{850}) for the same fermentations as Figures 1A and 2A.

Clear differences between soluble and insoluble substrate fermentation data are evident from both the fermentation product profiles and on-line data. Cells grown on cellobiose exhibit accelerating substrate utilization until substrate is nearly exhausted (Fig. 1A). Consistent with this, all three on-line signals also have an accelerating trend until substrate exhaustion is approached, and change abruptly thereafter (Fig. 1B). By contrast, the time course for residual Avicel

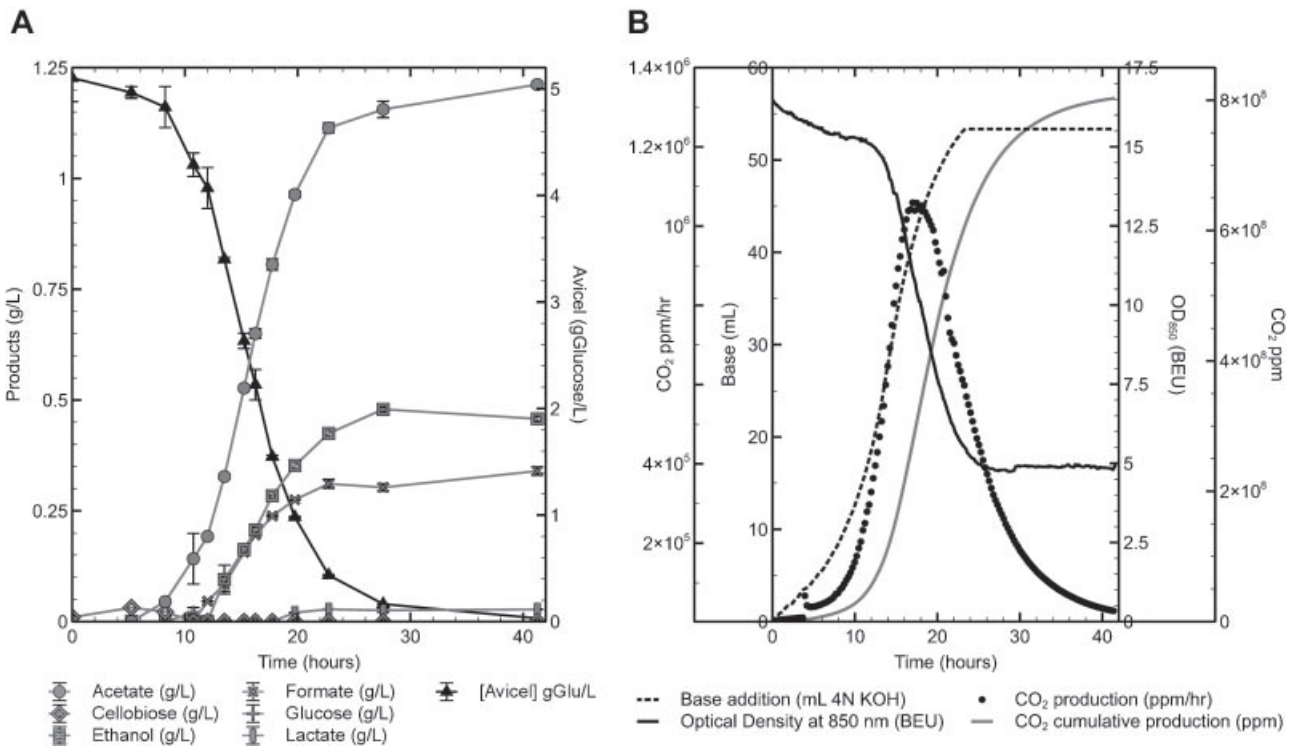

Figure 2. Fermentation time course for *C. thermocellum* grown on Avicel (PH105) in LC medium. **A**: Fermentation products and residual substrate concentration. **B**: On-line signals: base addition, CO_2 production rate, cumulative CO_2 production, and OD_{850}.

concentration exhibits a sigmoidal trend with the rate of substrate utilization accelerating but then decelerating well before substrate is exhausted (Fig. 2A). On-line signals again reflect trends observed based on off-line measurements of substrate and fermentation products (Fig. 2B). For example, the rate of CO_2 production from Avicel fermentation peaks when about 30% of the substrate is still remaining, whereas the maximum rate of CO_2 production from cellobiose coincides with substrate exhaustion.

Inferring Biosynthate and Substrate Concentrations from Elemental Analysis

To evaluate pellet nitrogen as a proxy for the concentration of biosynthate, pellet nitrogen, pellet carbon, and dry weight were measured during batch cellobiose fermentation. As presented in Figure 3, data from replicate fermentations show strong correlations for pellet nitrogen and filtrate dry weight as well as pellet carbon and pellet nitrogen. In particular, the mass ratio of filtrate biosynthate dry weight to pellet biosynthate nitrogen ($R_{\mathrm{Bbdw/Npb}}$) was 8.85 ($r^2 = 0.99$) and the mass ratio of pellet biosynthate carbon to pellet biosynthate nitrogen ($R_{\mathrm{Cpb/Npb}}$) was 3.82 ($r^2 = 0.99$). Pellet biosynthate carbon and cell dry weight were also strongly correlated with $R_{\mathrm{Bp/Cpb}} = 2.32$ ($r^2 = 0.99$; data not shown).

The data in Figure 3 are consistent with the following equations for biosynthate dry weight and pellet biosynthate nitrogen and pellet biosynthate carbon:

$$N_{\mathrm{Tp}} = N_{\mathrm{Bp}} + N_{\mathrm{Sp}} = B_{\mathrm{p}} R_{\mathrm{Npb/Bp}} + S_{\mathrm{p}} R_{\mathrm{Ns/S}} \tag{7}$$

$$C_{\mathrm{Tp}} = C_{\mathrm{Bp}} + C_{\mathrm{Sp}} = B_{\mathrm{p}} R_{\mathrm{Cpb/Bp}} + S_{\mathrm{p}} R_{\mathrm{Cs/S}} \tag{8}$$

where N_{Tp}, total nitrogen in the pellet (g/L); N_{Bp}, biosynthate nitrogen in the pellet (g/L); N_{Sp}, substrate nitrogen in the pellet (g/L); B_{p}, pellet biosynthate concentration (g/L); $R_{\mathrm{Bbdw/Npb}}$, unitless ratio of pellet biosynthate nitrogen:pellet biosynthate dry weight; S_{p}, pellet substrate concentration (g/L); $R_{\mathrm{Ns/S}}$, unitless mass fraction of nitrogen in the substrate; C_{Tp}, total carbon in the pellet (g/L); C_{Bp}, biosynthate carbon in the pellet (g/L); C_{Sp}, substrate carbon in the pellet (g/L); $R_{\mathrm{Cpb/Bp}}$, unitless ratio of pellet biosynthate carbon:pellet biosynthate dry weight; $R_{\mathrm{Cs/S}}$, unitless mass fraction of carbon in the substrate.

If the substrate contains negligible nitrogen ($R_{\mathrm{Ns/S}} = 0$), valid for Avicel, Equation (7) becomes

$$N_{\mathrm{Tp}} = R_{\mathrm{Npb/Bp}} B_{\mathrm{p}} \tag{9}$$

which can be used to calculate the pellet biomass concentration using Equation (10)

$$B_{\mathrm{p}} = \frac{N_{\mathrm{Tp}}}{R_{\mathrm{Npb/Bp}}} \tag{10}$$

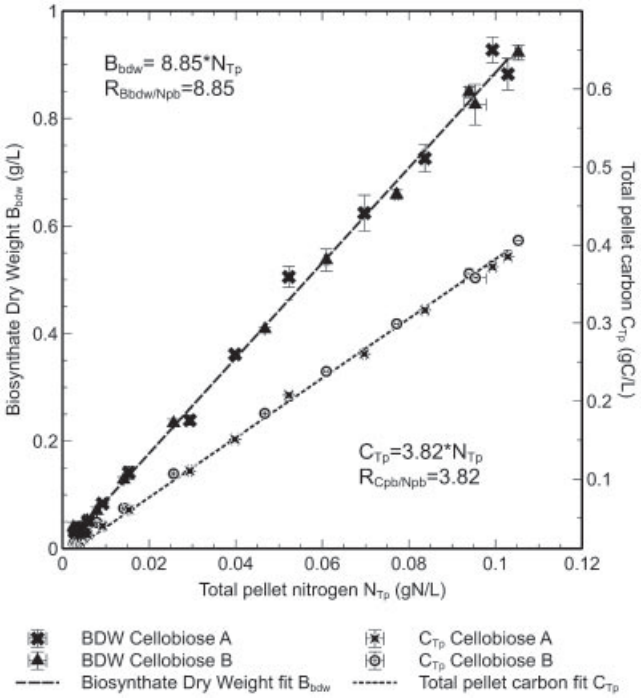

Figure 3. Pellet nitrogen versus biosynthate dry weight (BDW) and total organic carbon in the pellet from two batch fermentations (fermentations A and B) of 5 g/L cellobiose by *C. thermocellum*.

For a cellobiose-grown culture, Equation (8) becomes

$$C_{Tp} = B_p R_{Cpb/Bp} \tag{11}$$

Combining Equations (10) and (11) and defining $R_{Cpb/Npb}$ as the carbon to nitrogen ratio of biosynthate ($=R_{Cpb/Bp}/R_{Npb/Bp}$) gives

$$C_{Tp} = N_{Tp} R_{Cpb/Npb} \tag{12}$$

Equation (8) can be rewritten using Equation (12) assuming that the carbon and nitrogen content for cells does not change when grown on soluble or insoluble substrate. This yields an equation that can be used to calculate substrate carbon

$$C_{Ts} = C_{Tp} - C_{Tb} = C_{Tp} - N_{Tp} R_{Cpb/Npb} \tag{13}$$

where C_{Ts}, total pellet substrate carbon ($S_p R_{Cs/Sp}$); C_{Tb}, total pellet biosynthate carbon ($B_p R_{Cpb/Bp}$).

When biosynthate as well as substrate are present in the solid phase (pellet), it is generally impractical to measure their concentration directly and their concentration must instead be inferred. Compositional analysis is one approach to such inference. Because nitrogen is the most abundant element present in cells that is often (but not always) not abundant in cellulosic substrates, and since it is a major component of most polymeric cell components (protein, nucleic acids, and peptidoglycans) typically comprising about 3/4 of the dry weight of microorganisms (Stouthamer, 1979), it is a logical basis for inference of biosynthate concentration using relationships similar to Equation (7) or Equation (10). Substrate concentration may also be inferred if an element in addition to nitrogen present in the substrate is also measured. Carbon is convenient for inference of the concentration of insoluble carbohydrates using a relationship of the form of Equation (10) or Equation (13).

In Figure 4, total pellet carbon concentration determined by elemental analysis and the inferred cellulose carbon concentration calculated based on carbon and nitrogen analysis using Equation (13) are plotted as a function of the cellulose carbon concentration based on direct measurement using quantitative saccharification. When the calculated biosynthate carbon is subtracted from total pellet carbon, excellent agreement is obtained ($r^2 = 0.99$) between inferred ($r^2 = 0.99$ with $y = x$, the regression equation is $y = 1.026 + 5.52\text{E}{-}03$) and direct measurements of residual cellulose concentration.

Figure 5A and B shows experimental results for residual substrate and biosynthate, as well as curves fit to experimental data for the biosynthate concentration using an exponential equation for cellobiose fermentation (see Eq. 1), and a 5-parameter Richards curve (Richards, 1959)

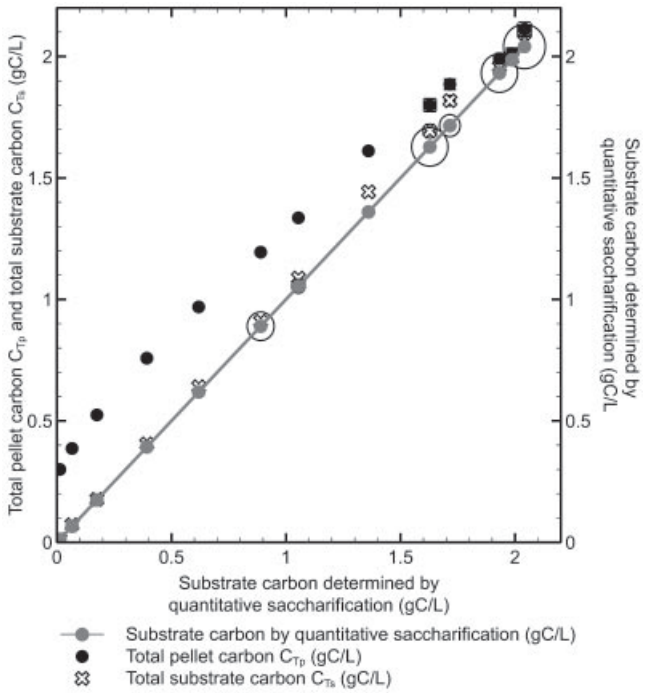

Figure 4. Total pellet carbon and total pellet carbon minus biosynthate carbon compared to residual substrate as determined by quantitative saccharification. The solid grey line with solid grey circles represents quantitative saccharification data, the amount of total pellet carbon (C_{Tp}) is shown as black solid circles and substrate carbon ($C_{Ts} = C_{Tp} - C_{Tb}$) is shown as open crosses. The course of the fermentation is from right to left.

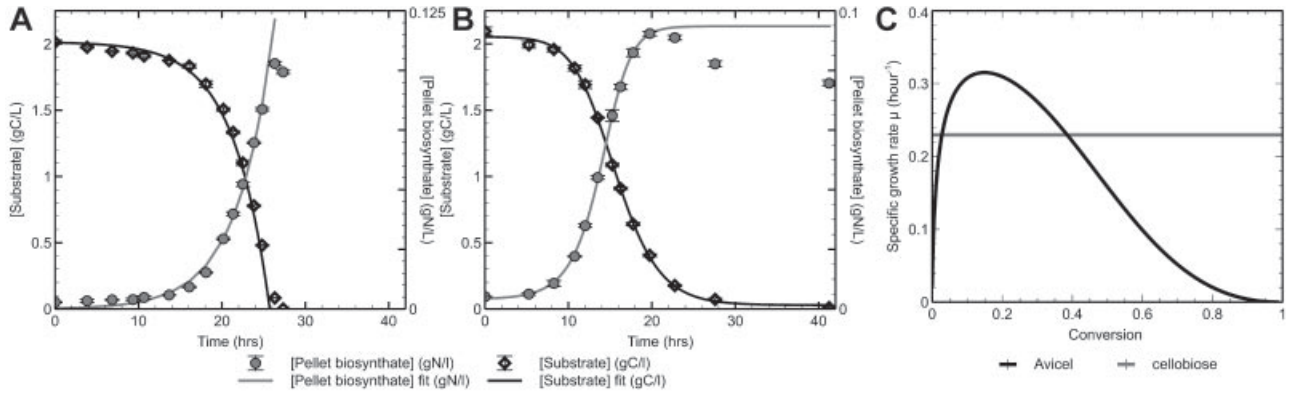

Figure 5. Experimental data and fit curves for residual substrate and biosynthate for cellobiose (**A**) and Avicel (**B**), and the specific growth rates for both substrates as a function of fractional substrate conversion (**C**). Biosynthate and residual substrate (Avicel) curves are fitted based on elemental analysis data (see text). Residual cellobiose concentration is determined by HPLC and fitted according to Equation (3).

for the Avicel fermentation (see Eq. 4). A similar 5-parameter Richards curve was applied to residual cellulose data (determined by biosynthate carbon inferred using Eqs. 12 and 13). The fit curve for residual cellobiose was obtained using a constant yield for biosynthate and substrate (see Eq. 3). As shown in Figure 5, there is excellent agreement between the fitted and actual values (for cellobiose grown biosynthate $r^2 = 0.99$, for residual cellobiose $r^2 = 0.99$, for Avicel/cellulose grown biosynthate $r^2 = 0.99$ and residual Avicel/cellulose concentration $r^2 = 0.99$). Figure 5C shows the specific growth rates calculated using the sigmoidal and exponential fit curves (Eq. 2 for cellobiose and Eqs. 4 and 5 for Avicel) versus the fraction of substrate utilized (fractional conversion χ). The growth rate on Avicel transiently exceeds the growth rate on cellobiose, with a maximum at about 0.18 fractional conversion.

On-Line Methods

Inferred substrate concentration versus time is plotted in Figure 6 based on three normalized on-line measurements: base consumption, cumulative CO_2 production, and OD_{850} with or without a correction for final OD (see methods). The residual substrate curve fit based on elemental analysis (Fig. 5A and B) is shown for comparison. The normalized optical density for the Avicel fermentation in Figure 6 is shown in two ways: one with zero set as the minimum value ("OD normalized A"), and one with the minimal data point

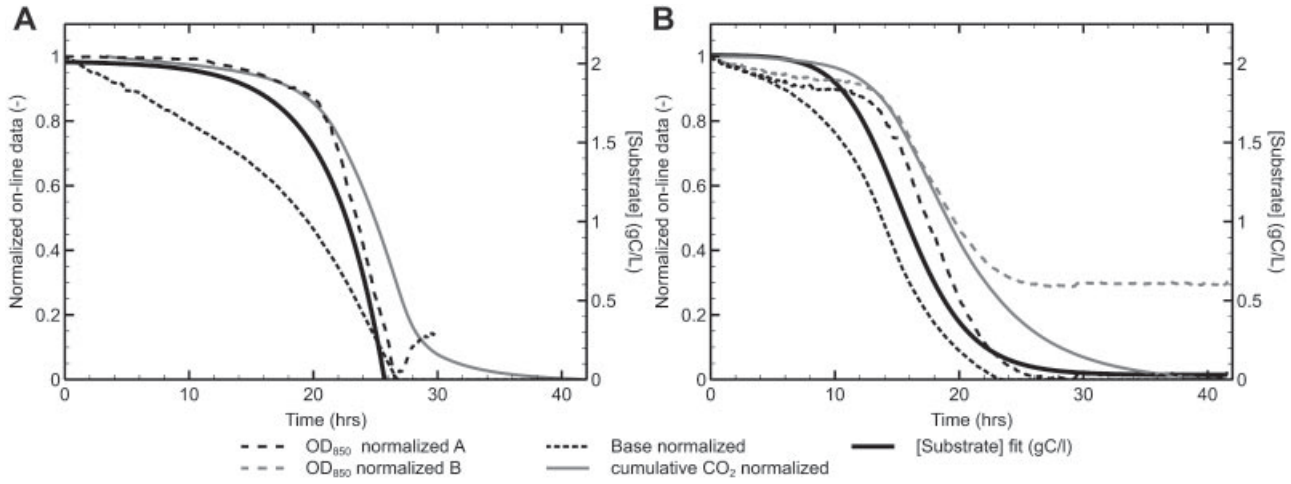

Figure 6. Time course of fit curves for total substrate carbon ($C_{Ts} = C_{Tp} - C_{Tb}$) and residual cellobiose concentration versus time for 5 g/L Cellobiose (**A**) and Avicel PH105 (**B**) fermentations with normalized on-line data for base addition, cumulative CO_2 production and OD_{850}. Normalized data for base addition, cumulative CO_2 production and OD_{850} in the cellobiose figure and base addition and cumulative CO_2 production data in the Avicel figure is plotted as $1 - N(t)$ using Equation (6).

set as the minimum value ("OD normalized B"). All three on-line measurements represent the overall substrate depletion trend reasonably well. However, base addition underestimated substrate carbon concentration, and CO_2 and OD_{850} measurements overestimated substrate carbon concentration. In general, results from on-line measurements were not as accurate as results from elemental analysis.

Discussion

The central issue impeding physiological studies of cellulolytic microbes is that microbial cell mass and insoluble substrates are both present in the solid phase and are thus difficult to distinguish from each other. The situation is further complicated because microbes are often present both adhered to solid substrates and free in the medium, and because pellets from cultures of microbes growing on solid substrates contain substrate-adhered extracellular enzymes as well as cells and cell-associated enzymes and exopolysaccharide. Although relevant data are not widely available, it is not in general reasonable to assume that cellulase synthesis is a negligible fraction of cell synthesis on a mass basis (Zhang and Lynd, 2005).

In approaching the physiology of cellulolytic microbes and related measurements, it is desirable where possible to make use of conceptual frameworks established based on studies involving soluble substrates. At the same time, some accommodation for the methodological challenges associated with insoluble substrates is likely to be practical and in some cases required. For many purposes involving cellulolytic microbes, reporting the sum of cell and protein synthesis—regardless of whether or not proteins are physically associated with cells—seems to us preferable in lieu of reporting cell mass only. Reporting of "biosynthate" in this manner is consistent conceptually with the objective of an aggregate measure of biosynthesis, and avoids methodological complications associated with distinguishing cells from enzymes present in various forms for situations where this is appropriate.

Growth on cellulose appears to have an advantage over growth on cellobiose, as the maximum specific growth rate is higher for Avicel grown cultures compared to cellobiose grown cultures. We speculate that early in the fermentation when the substrate to cell ratio is high, *C. thermocellum* would appear to gain access to soluble carbohydrate molecules more easily when growing on cellulose than when growing on cellobiose. More detailed explanation of this phenomenon is of great interest, but can only be speculated on at this time. It may be relevant to note that cellobiose is not thought to be the dominant carbohydrate species taken up by C. thermocellum both in natural environments and when growing on model cellulosic substrates such as Avicel. Transporting higher cellodextrins is energetically more efficient than transporting cellobiose as shown by Zhang and Lynd (2005). However, this advantage is only temporarily as for most of the fermentation the specific growth rate on cellulose is lower compared to that of cellobiose. Limitations in the available surface area of the Avicel particles could be involved in this rapid decline of the specific growth rate for Avicel grown cells.

The most appropriate form of measurement and any related approximations of course depend on the question being asked. As summarized in Table I, on-line measurements such as optical density, CO_2 production, and base addition are convenient and amenable to high-throughput data collection, but at least in our hands, provide a semi-quantitative indication of substrate consumption, are not of sufficient quality for many types of kinetic analysis, and are insensitive to changes in the biosynthate yield. Of these three methods, optical density was the most practical and gave the best agreement for residual substrate compared to more precise measurements based on elemental analysis. The accuracy of these methods, however, depends on system configuration (e.g., with respect to dead volume for CO_2 analysis) and it is thus possible that studies carried out in a different apparatus could rank the accuracy of the techniques differently.

Using off-line analysis of C and N, we obtained far more accurate analysis of both substrate and cells as compared to any of the off-line methods tested. In particular, the data set obtained using this method appears to be of sufficient quality to allow evaluation of the instantaneous rate of cellulose utilization on both an absolute and biosynthate-specific basis. We are not aware that such a data set has been reported previously. Two-element compositional analysis has the added advantage over on-line methods that it allows direct inference of biosynthate and it thus can detect, and is sensitive to, changes in the biosynthate yield. However, two-element compositional analysis does not allow measurement of changing allocation between cells and cellulase. If independent analysis of cells and cellulase is desired, an additional measurement specific to cellulase is required. We did not undertake such measurements in the work reported here, but this approach has been explored using both ELISA (Zhang and Lynd, 2003) and proteomic measurements based on tandem mass spectrometry (B. Hettich ORNL, personal communication).

In presenting results from two-element analysis, there is a choice between presenting biosynthate in terms of an element largely or entirely specific to cells (nitrogen in the work presented here) or in terms of inferred biosynthate concentrations based on the mass fraction of the element present in microbial biomass and protein (which fortunately are not very different in the case of nitrogen). Reporting elemental data involves fewer assumptions but reporting inferred biosynthate concentrations facilitates interpretation. Recognizing that the data will be used differently by different investigators, we report here both pellet biosynthate nitrogen/carbon, and measured and inferred biosynthate dry weight. We suggest that others may want to do likewise.

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