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Use of Measurement Uncertainty Analysis to Assess Accuracy of Carbon Mass Balance Closure for a Cellulase Production Process

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Abstract

Closing carbon mass balances is a critical and necessary step for verifying the performance of any conversion process. We developed a methodology for calculating carbon mass balance closures for a cellulase production process and then applied measurement uncertainty analysis to calculate 95% confidence limits to assess the accuracy of the results. Cellulase production experiments were conducted in 7-L fermentors using *Trichoderma reesei* grown on pure cellulose (Solka-floc), glucose, or lactose. All input and output carbon-containing streams were measured and carbon dioxide in the exhaust gas was quantified using a mass spectrometer. On Solka-floc, carbon mass balances ranged from 90 to 100% closure for the first 48 h but increased to 101 to 135% closure from 72 h to the end of the cultivation at 168 h. Carbon mass balance closures for soluble sugar substrates ranged from 92 to 127% over the entire course of the cultivations. The 95% confidence intervals (CIs) for carbon mass balance closure were typically ±11 to 12 percentage points after 48 h of cultivation. Many of the carbon mass balance results did not bracket 100% closure within the 95% CIs. These results suggest that measurement problems with the experimental or analytical methods may exist. This work shows that uncertainty analysis can be a useful diagnostic tool for identifying measurement problems in complex biochemical systems.

Index Entries: Cellulase; carbon mass balance; cellulose; uncertainty analysis; *Trichoderma reesei*.

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Introduction

Carbon balancing a biobased production process is a useful technique for assessing the accuracy of performance measurements. Obtaining good carbon mass balance closure indicates internal data consisteny and provides reasonable confidence in the accuracy of the underlying data. This is extremely important when a technology is moving to commercialization because confidence in performance data is essential for engineering companies to commit to guaranteeing performance. If carbon mass balance closures near 100% cannot be obtained, then process stoichiometry or the accuracy of measuring one or more of the carbon-containing process streams is suspect. Typically, the output streams (e.g., carbon dioxide and soluble and insoluble products) are more difficult to measure because the input streams are better defined (e.g., sugar substrates) and are usually relatively easy to quantify, particularly for batch processing.

The conversion of lignocellulosic biomass to ethanol is one biobased process that is moving to commercialization *(1)*. As such, good carbon mass balance closure data and confidence in the accuracy of these performance results are essential. One proposed step in this process is the production of cellulase enzymes, which catalyze the hydrolysis of the cellulose component of the biomass to glucose. Cellulase enzymes could be produced using pretreated biomass since this provides a relatively inexpensive substrate for producing the enzyme. However, using insoluble substrates makes carbon mass balancing difficult because solid substrates are harder to measure and quantify than soluble substrates.

Wang and Stephanopoulos *(2)* developed a technique to determine gross measurement errors in biobased processing based on a set of equality constraints derived from material and energy balances. The technique has since been refined *(3–6)* and applied to a solid-substrate fermentation using *Monoscus purpureus (7)*. The major limitation with this technique is that the media have to be well defined and have only one carbon-containing substrate so that appropriate yield factors can be calculated. Additionally, redundant measurements must be made so that consistency of data can be checked.

We have used carbon mass balancing on the key processing steps involved in converting lignocellulosic materials to ethanol in order to assess the accuracy of product yields. Based on a methodology developed by Hatzis et al. *(8)*, McMillan et al. *(9)* reported an average carbon mass balance closure (carbon recovered as percentage of input carbon) of 106.3 ± 6.8% for a simultaneous saccharification and cofermentation process using *Zymomonas mobilis* as the fermentative microorganism.

There are only a few reported attempts to calculate carbon balances for cellulase production processes. Ross et al. *(10)* reported carbon balances ranging from 60 to 110% (estimated from a figure provided in the publication) for the first stage of a two-stage continuous cultivation of *Trichoderma reesei* QM 9414 on Avicel (a purified cellulose preparation). They accounted

Table 1
Carbon Mass Balance Runs

No. of experiment	No. of vessels	Substrate
SF1	4	Solka-floc
SF2	2	Solka-floc
SF3	2	Solka-floc
SF4	1	Solka-floc
G1	2	Glucose
G2	1	Glucose
L1[a]	1	Lactose

[a] Only run to use *T. reesei* Rut-C30.

for substrate, cell mass, enzyme, and CO_2 production. Carbon balances of 120 and 130% were reported for the second stage. Smits et al. *(11)* compared CO_2 production based on substrate weight loss and chemical composition with measured CO_2 production during a solid-state fermentation using *T. reesei* QM 9414 on wheat bran. They reported good agreement between the two values; however, there appears to be no attempt to account for other products (i.e., cell mass and enzyme).

The objective of the present study was to develop a rigorous carbon mass balance methodology for cellulase production using a relatively pure cellulosic substrate (Solka-floc) and to assess the accuracy of the results using measurement uncertainty analysis. This technique is an alternative to the consistency analysis proposed by Wang and Stephanopoulos *(2)*.

Materials and Methods

Cellulase Production

Details of the method used for the cultivations are reported in another publication *(12)*. Briefly, the experimental work was conducted in New Brunswick (Edison, NJ) Bioflo 3000 fermentor systems in 7-L vessels utilizing a 4-L working volume. Experiments were performed with the cellulase-producing microorganism *T. reesei* strain L27 utilizing both insoluble (Solka-floc) and soluble (glucose) substrates; one cultivation was performed using *T. reesei* strain Rut-C30 grown on lactose. All cultivations were carried out in batch using an initial substrate loading of 5% (w/v). The soluble substrate cultivations were performed to check carbon balance closure on a simpler system (i.e., more readily measured sugar substrate and no enzyme production).

A total of 13 cultivations were performed at essentially the same operating conditions. Table 1 presents a summary of the runs and the substrate used. When performing the carbon balance calculation for the lactose run, it was assumed that the Rut-C30 cells had the same composition as the L27 cells used in all of the other runs.

Table 2
Sources of Carbon into and out of Cellulase Production Process and Carbon Content of Each Source

Carbon source	In	Out	Carbon content (%, [w/w])
Solka-floc	×	×	44.6
Glucose	×	×	40.0
CSL	×		36.2
Soluble protein	×	×	50.0
Adsorbed protein		×	50.0
Cells	×	×	50.5
CO_2		×	27.3

Carbon Balance Methodology

Carbon mass balance closures were calculated as output carbon mass divided by input carbon mass as shown in Eq. 1:

$$\text{Carbon Balance Closure (\%)} = \frac{\text{Carbon Out (g)}}{\text{Carbon In (g)}} \times 100\% \quad (1)$$

The methodology used for calculating the carbon mass balance is discussed in detail elsewhere *(13)*. Table 2 shows the carbon sources and how they were accounted for as inputs and outputs in the cellulase production process along with their carbon content. Cell mass was measured in the presence of Solka-floc (a purified cellulose) using near-infrared (NIR) spectrometry *(14)*. This technique determines the ratio of cell mass to cellulose, from which the amount of each component was calculated from a total solids measurement. Glucose was measured using a YSI Model 2700 Glucose Analyzer (Yellow Springs Instruments, Yellow Springs, OH). The amount of corn steep liquor (CSL) added to the cultivation is known. Protein was determined using the Pierce (Rockford, IL) bicinchoninic acid (BCA) protein assay kit, which is based on the colorimetric detection and quantitation of protein using BCA *(15)*. Adsorbed protein is estimated from the Solka-floc concentration assuming Langmuir adsorption *(13)*. A VG Prima 600 mass spectrometer (Fisons, Middlewich, UK) was used to measure the carbon dioxide mole fraction in the off gas. The inlet airflow rate was controlled at 5.0 L/min with MKS type 1159B mass flow controllers (MKS, Andover, MA), and the ratio of nitrogen in the inlet air and outlet gas was used to calculate the exhaust gas flow rate. We believe we have considered all of the major and even most of the minor sources of carbon, but it is possible that some minor sources of carbon are neglected.

Uncertainty Analysis

Confidence limits for mass balance closures were assessed using accepted uncertainty analysis procedures *(16,17)*. The approach was to

Table 3
Random and Bias Error Estimates for Carbon Source Parameters

Carbon source	Parameters	df[a]	Random error (S_i)	Bias error B_i	B_i^+	B_i^-
IN						
Solka-floc	Weight (g)	30	0.5		2.0	0.0
	Solids concentration (% [w/w])			0.5		
	Cellulose content (% [w/w])			2.0		
	Hemicellulose content (% [w/w])			0.05		
	Other content (% [w/w])				2.8	0.0
Glucose	Concentration (YSI) (g/L)	30	0.1	0.05		
	Initial vessel volume (L)			0.2		
CSL	CSL volume (mL)	30	1.0			
	Density (g/mL)			0.1		
	Solids concentration (% [w/w])			5.0		
	Carbon content (% [w/w])			2.0		
Soluble protein	Concentration (g/L)	2	0.02	0.1		
	Inoculum volume (mL)	30	3.0			
	Carbon content (% [w/w])			5.0		
Cells	Concentration (rel. %[b])	30	10.0			
	Initial vessel volume (L)			0.2		
	Carbon content (rel. %[b])			10.0		
OUT						
Solka-floc	Solids concentration (g/L)	2	0.35	0.5		
	Final vessel volume (L)			0.4		
	Fraction Solka-floc (via NIR) (rel.%[b])	30	10.0			
Glucose	Concentration (YSI) (g/L)	30	0.1	0.05		
	Final vessel volume (L)			0.4		
Soluble protein	Concentration (g/L)	2	0.036	0.1		
	Final vessel volume (L)			0.4		
	Carbon content (% [w/w])			0.5		
Adsorbed protein	Adsorbed protein (rel. %[b])	30	10.0			
	Final vessel volume (L)			0.4		
Cells	Solids concentration (g/L)	2	0.35	0.5		
	Final vessel volume (L)			0.4		
	Fraction Cells (via NIR) (% [w/w])	30	10.0			
	Carbon content (rel. %[b])			10.0		
CO_2	CO_2 production (rel. %[b])	10	See Fig.1	See Fig. 1		

[a]Degrees of freedom.

[b]Percentage relative error in parameter value; actual error = % relative error/100 × parameter value.

identify random and bias errors associated with each of the measured sources identified in Table 2 and then combine errors using propagation-of-error techniques. The carbon content of each of these source terms is calculated from other parameters as identified in Table 3. Random errors

are the scatter in data associated with repeat measurements of the same thing and are characterized by a mean and SD. The number of measurements used to determine the mean minus one is the degrees of freedom (df). Degrees of freedom are used to estimate the *t*-statistic for the uncertainty calculation, which for df values >30 is set equal to 2. Bias or systematic error is an error that remains constant throughout a measurement process. Bias errors are typically produced during the calibration process and introduce a fixed error into the measurement. Since each of the terms in Table 2 was calculated from other measured parameters (e.g. concentrations, volumes), it was necessary to estimate random and bias errors associated with each parameter that were also combined using propagation-of-error techniques to produce the final error estimates for the source terms.

Estimates of random (S_i) and bias errors (both symmetrical, B_i, and non-symmetrical, B_i^+, B_i^-) and df for each of the parameters are presented in Table 3. In some cases, there are redundant entries (i.e., the same measurement was used for multiple sources), but they are included for completeness. In general, if the parameter was measured, then a random error and possibly a bias error were assigned. If the parameter was not measured—if it was known from a previous measurement or previously had been assigned a value (e.g., Solka-floc composition)—then a bias error was assigned. The assumption of a known value introduces a fixed difference between the true value and the assumed value, which is assumed large enough to cover the variation in random error.

In general, the following additional guidelines were also used when assigning values for random or bias errors. If a series of repeated measurements was used to determine errors, then the random error was the standard error and the df was one less than the number of samples. When random error values were based on judgment, the value was assumed to represent a standard error for a large sample size and the df was assigned a value of 30. Except as noted, bias errors were based on judgment.

The following sections document the random and bias error estimates for all parameters. As previously noted, many of the values are assumptions and are based on judgment. We believe they are good values based on our knowledge of the measurement systems and reasonable estimates of measurement accuracies, but they are still assumptions.

Solka-floc

1. Weight (in): The random error on the weight measurement was assumed to be 0.5 g (df = 30). Nonsymmetrical bias limits were used assuming that up to 2.0 g of Solka-floc could remain in the measuring container used to transfer the Solka-floc to the vessel.
2. Solids concentration (in), cellulose content (in), hemicellulose content (in), other content (in): The solids concentration (97.1% [w/w], cellulose (88.6% [w/w] dry basis), hemicellulose (7.7% [w/w] dry basis), and ash (0.9% [w/w dry basis] content of Solka-floc were

based on an analysis of Solka-floc using methods previously discussed *(18)*. The remaining material (2.5% [w/w] dry basis) is identified as "Other" and is primarily, but not exclusively, lignin. A symmetrical bias limit of 0.5% was assumed for solids concentration and hemicellulose content and 2.0% for cellulose content. Ash was assumed to be inert. The carbon contents of these materials are known from stoichiometry and no errors were assigned. A nonsymmetrical bias limit was assigned to the "Other" content assuming that none of this material was converted to measured products.

3. Solids concentration (out): The random error for solids concentration was based on three independent measurements, and the bias error was assumed to be 0.5 g/L.
4. Final vessel volume (out): A bias error of 0.4 L was assumed for the final vessel volume (final volume of approx 4 L) for all calculations that required this parameter.
5. Fraction Solka-floc (out): A relative random error of 10% (df = 30) for the accuracy of the NIR correlation was assumed based on results described previously *(14)*. This material was assumed to be 100% cellulose, and there was no error assigned to the cellulose carbon content.

Glucose

1. Concentration (in, out): The input and output glucose concentration measurements were assumed to have a random error of 0.1 g/L (df = 30) and a bias error of 0.05 g/L.
2. Initial vessel volume (in): Initial volume was assumed to be more accurately known than the final volume and was assumed to have a bias error of 0.2 L.

Corn Steep Liquor

1. CSL volume (in): A random error of 1.0 mL (df = 30) was assumed for the measured CSL volume (40 mL) added to each vessel.
2. Density (in), solids concentration (in), carbon content (in): Bias errors of 0.1 g/mL, 5.0% (w/w), and 2.0% (w/w) were assumed for the density, solids concentration, and carbon content of CSL, respectively.

Soluble Protein

1. Concentration (in): The random error for protein concentration was based on three independent measurements (df = 2) of a sample containing a low amount of protein (<5.0 g/L). A bias error of 0.1 g/L was assumed.
2. Inoculum volume (in): A random error of 3 mL (df = 30) was assumed for the inoculum volume of 200 mL.
3. Carbon content (in, out): A bias error of 5.0% (w/w) was assumed for the carbon content of protein for both the input and output streams.

Table 4
Random and Bias Errors Associated with Measurement of CO_2 Production

Parameter	S_i	df	B_i
CO_2 concentration (mol %)	0.01	30	0.002
N_2 concentration (mol %)	0.05	30	0.15
Exhaust gas flow rate (L/min)	0.016	9	0.032

4. Concentration (out): The random error for protein concentration was based on three independent measurements (df = 2) of a sample containing a high amount of protein (>5.0 g/L). A bias error of 0.1 g/L was assumed.

Adsorbed Protein

1. Adsorbed protein (out): Adsorbed protein concentration was assumed to have a 10% relative random error.

Cells

1. Concentration (in): A relative random error of 10% (df = 30) was assumed for time zero cell mass concentration.
2. Carbon content (in, out): Both the input and output cell carbon contents were assumed to have a relative bias error of 10%.
3. Solids concentration (out), fraction cells (out): Solids concentration and fraction cells errors were the same as used for the output Solkafloc calculation.

Carbon Dioxide

The equations used to calculate CO_2 production were previously described *(13)*. Briefly, CO_2 production is calculated by integrating instantaneous CO_2 production rate, which is calculated from the exhaust gas CO_2 concentration as measured by the mass spectrometer and the exhaust gas flow rate. The exhaust gas flow rate is determined from the measured inlet airflow rate using a nitrogen mass balance.

Table 4 presents the errors associated with each of the parameters used to calculate CO_2 production. The random errors for the CO_2 and N_2 measurements (df = 30) were assumed to be the tolerance values set in the mass spectrometer software for each gas. When the concentration measurement of a component in the check gas deviates from the known value by more than the tolerance, the mass spectrometer is recalibrated. (A check gas is a gas of known composition that is used to assess the instrument's accuracy.) Thus, these values provide a conservative estimate of the SD observed in repeated measurements of the check gas composition. The bias errors for the gas concentration measurements are assumed to be the accuracy specifications (0.2%) for the calibration gases.

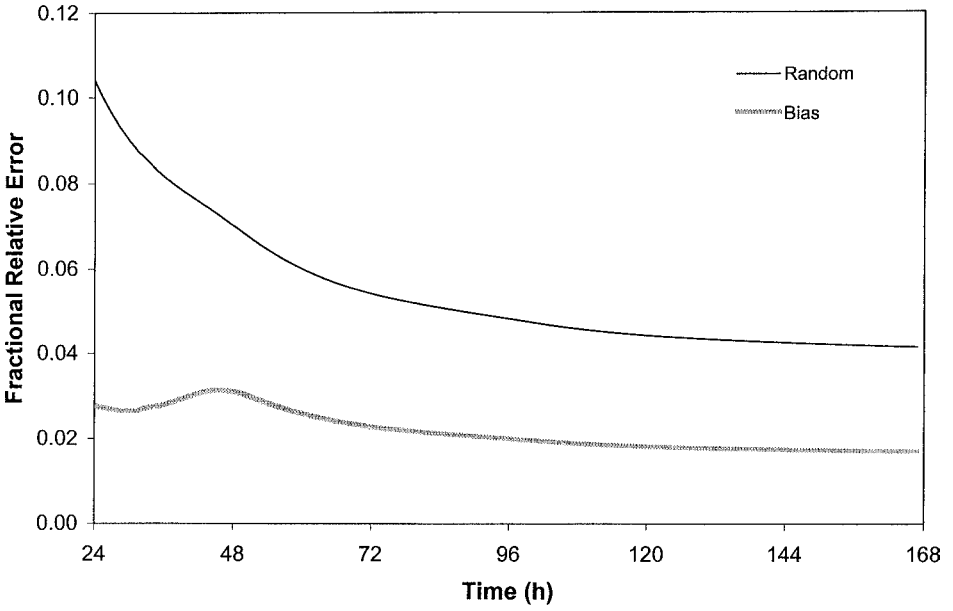

Fig. 1. Relative random and bias error estimates for CO_2 production as a function of cultivation time (culture aerated at 5.0 L/min).

The gas mass flow rate measurement errors were based on 10 measurements of airflow rate made by a bubble meter with the mass flow controllers set at 3.0 L/min. This was the largest flow that could be accurately measured with the bubble meter. The errors were similar for all four mass flow controllers associated with each of the four vessels. The SD of the 10 measurements was used to calculate a relative random error that was then applied to the normal flow rate of 5.0 L/min used in the cultivations.

Since the calculation of the combined df for the CO_2 production measurement was unwieldy, and since the relative errors associated with the gas flow rate measurements were greater than the errors associated with the gas composition measurement, the combined df was assumed to be dominated by the gas flow rate measurement and was set equal to 9. The total bias error was determined by combining the offset error (using sum of squares addition) between the controller set point and the measured flow rate (0.03 L/min) with an assumed bias error in the bubble meter measurement of 0.01 L/min.

The relative random and bias errors for CO_2 production were calculated as a function of time and are shown in Fig. 1. The errors were calculated at each time point when data were collected, and then the total error at the time point of interest was determined by combining errors from all previous time points (i.e., summing errors from time zero through the time of interest) using root sum of squares. The plot was generated using data from one cultivation, which was assumed to apply to all other cultivations.

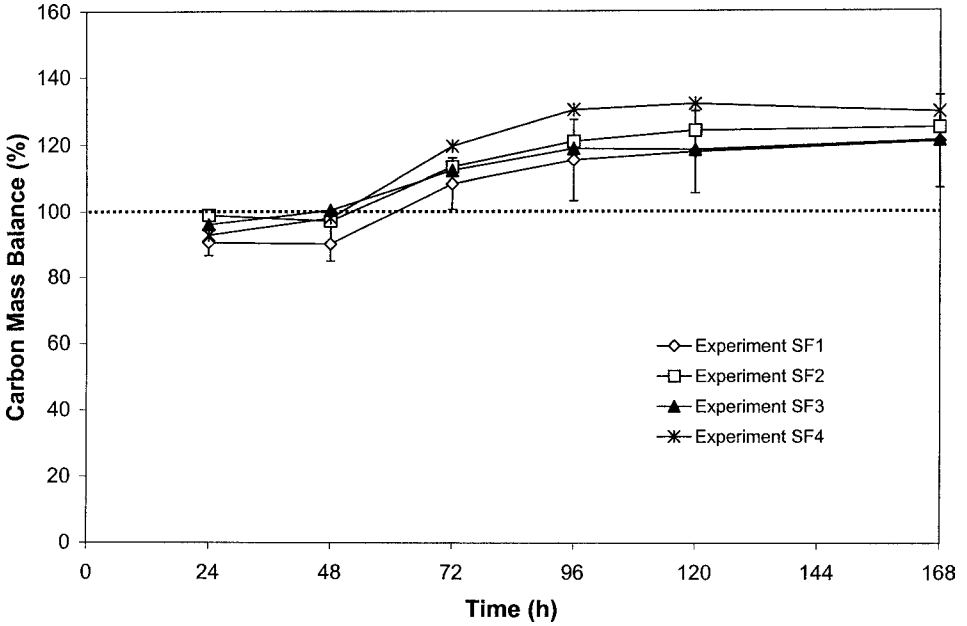

Fig. 2. Average carbon mass balance closure for 5% (w/v) Solka-floc runs. One SD error bars are shown for the combined experiment SF1 results.

Results

Carbon Balance Results on Solka-floc

Average carbon mass balance closures for all Solka-floc runs are shown as a function of run time in Fig. 2. One SD error bars are shown for the combined experiment SF1. The error bars for the other experiments that had multiple runs are similar to the results of experiment SF1 but are not shown to avoid cluttering the plot. Consistent trends are evident in all runs. The carbon mass balance closure is typically between 90 and 100% during the early part of the run (at times <72 h), and then increases during the latter part of the run to values >100%. The values at the final time point (168 h) for all vessels range from 104 to 133%. The biggest contributor to the carbon balance at 168 h is CO_2 production, which accounts for >60% of the total carbon in most of the runs (data not shown). Thus, an accurate measurement of CO_2 production is critical to obtaining accurate carbon mass balance closure.

Figure 3 presents carbon mass balance results as well as the corresponding 95% confidence interval (CI) for the Solka-floc cultivation performed in experiment SF4. The results show the same trend as in all the other cultivations (as seen in Fig. 2). The 95% confidence limits bracket 100% closure during the early part of the run (<72 h). At 72 h and beyond, the carbon mass balance values clearly lie outside the 95% CI (approx ±11 to 12 percentage points), suggesting that there is a problem quantifying one or more of the carbon sources.

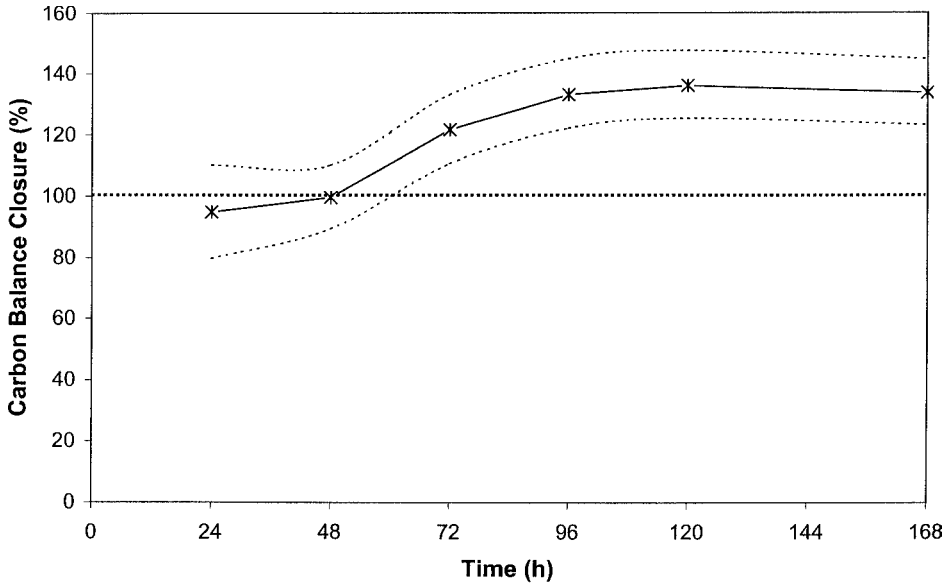

Fig. 3. Carbon mass balance closure for a Solka-floc run (from experiment SF4) showing 95% confidence limits (dashed lines).

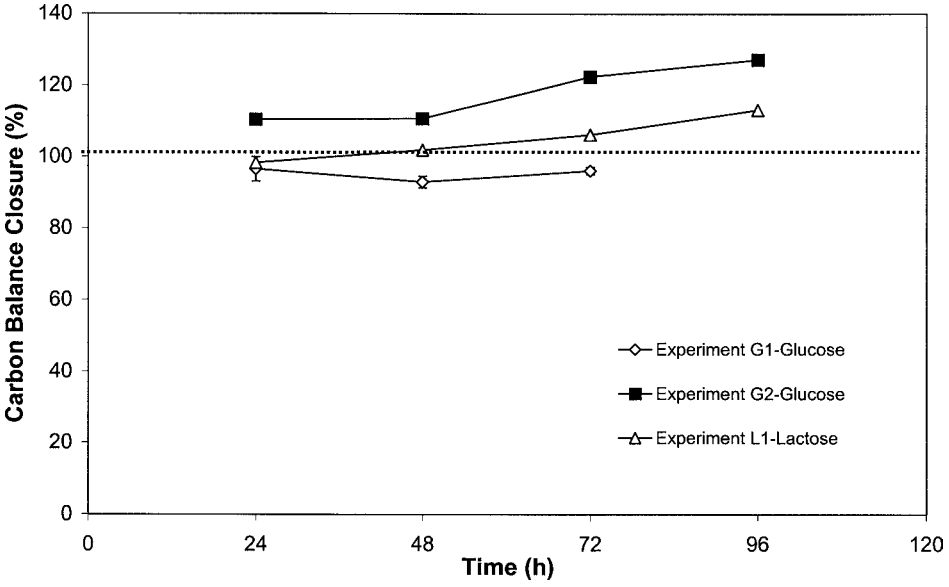

Fig. 4. Carbon mass balance closure for soluble sugar cultivations. One SD error bars are shown for the two glucose runs from experiment G1.

Carbon Balance Results for Soluble Substrates

Figure 4 gives the carbon balance closures for soluble substrate runs. The results of experiment G1 also include 1 SD error bars; however, the deviations were small because there was excellent reproducibility between

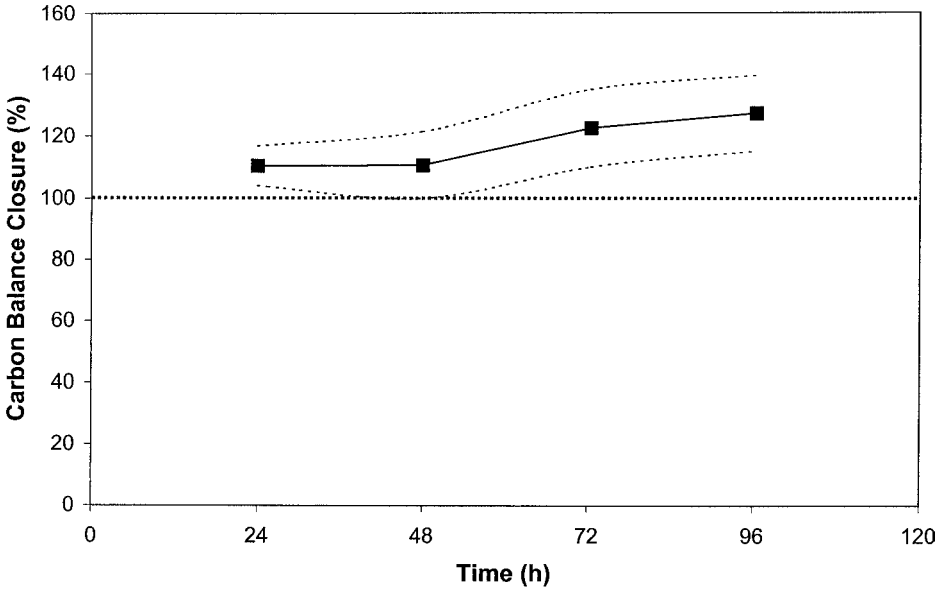

Fig. 5. Carbon mass closure for glucose run (from experiment G2) showing 95% confidence limits (dashed lines).

the two cultivations. Nevertheless, there were significant differences between the two experiments utilizing glucose; the carbon mass balance closure level for experiment G2 is about 20 to 30 percentage points higher than for experiment G1. The lactose results from experiment 58 are also included for completeness. By the end of these runs, all of the output carbon was either cell mass or CO_2, since no enzyme is produced under these conditions.

Figure 5 shows the 95% confidence limits for the glucose cultivation performed in experiment G2. For this run, 100% closure is outside the 95% confidence limits (again approx ±11 to 12 percentage points after 24 h) for the entire run, although the confidence limits for experiment G1 data shown in Fig. 5 would bracket 100% closure (not shown).

Figure 6 shows how the initial and final carbon mass measurements differed for the glucose runs of experiments G1 and G2. The initial amount of glucose was only slightly different in the two experiments, but the amounts of cell mass produced and CO_2 evolved at approx 72 h were significantly different. Clearly, the much greater amount of CO_2 measured in experiment G2 contributed to its higher carbon mass balance closure. The amount of cell mass produced in experiment G2 was also higher, however, it is much easier to quantify accurately this component because total insoluble solids are relatively easy to measure when using soluble substrates.

Discussion

Carbon mass balancing was performed on 13 separate cellulase production runs in a series of four experiments performed using Solka-floc,

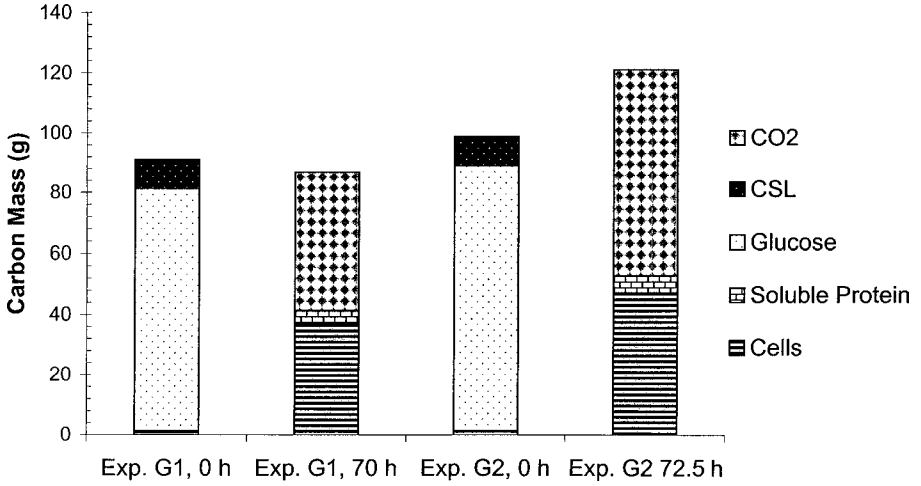

Fig. 6. Comparison of initial and approx 72-h carbon mass measurements for glucose runs performed in experiments G1 and G2.

glucose, or lactose as substrates. For all runs on Solka-floc, the carbon mass balance closures were higher than expected for run times of 72 h or longer. Carbon mass balance closures were usually less than but near 100% for times ≤48 h. These results are superior or comparable to the 120–130% closures reported by Ross et al. *(10)*. Although more limited, the soluble sugar results basically show the same general trends.

Uncertainty analysis yielded 95% CIs of ±11 to 12 percentage points for results obtained after 48 h. Although some of the carbon mass balance results for the Solka-floc cultivations were close to 100% within the 95% CI for times ≤48 h, the results for later time points were significantly greater than 100%. Thus, uncertainty analysis results suggest that a measurement problem may exist that is causing consistently high carbon mass balance closures to be obtained at the later stages of the cultivation.

Assuming that the input carbon is well characterized and not a major source of error, these results suggest that problems quantifying one or more of the output carbon-containing process streams are causing high carbon closures. As already discussed and as shown by the results presented in Fig. 6, CO_2 is the dominant contributor to the increasing amounts of carbon observed during the latter stages of these cultivations. Errors in quantifying CO_2 are more likely to influence carbon balances than any other source. Smaller and nearly equal contributions of carbon are derived from protein and cell mass; however, results from the glucose cultivation suggest that protein measurement is not likely to be the problem because protein is not produced in these cultivations and the carbon closure was still high in one of the glucose runs. In addition, cell mass is not likely to be a significant source of error in the glucose substrate system because it is relatively easy to quantify using a total solids measurements. Again, this reasoning leads to the hypothesis that errors in quantifying CO_2 are most

likely the major underlying factors responsible for high carbon mass balance closures.

Difficulties with closing carbon balances around chemical processes are well known, although perhaps not well documented. Biomass conversion processes pose even more challenges because measurements are usually required on three different material phases (solid, liquid, and gaseous) and often on multiple solid phases (e.g., cells, cellulose) as well. Furthermore, it is difficult to assess the accuracy of carbon balancing unless redundant measurements are made to enable the use of consistency analysis methods of the type proposed by Wang and Stephanopoulos *(2)*. This work has shown that measurement uncertainty analysis can also be used to detect measurement errors in biobased conversion processes.

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References

1. Wyman, C. E. (1999), *Annu. Rev. Energy Environ.* **24,** 189–226.
2. Wang, N. and Stephanopoulos, G. (1983), *Biotechnol. Bioeng.* **25,** 2177–2208.
3. Stephanopoulos, G., Aristidou, A., and Nielsen, J. (1998), in *Metabolic Engineering: Principles and Methodologies,* Academic, San Diego, CA, pp. 115–146.
4. van der Heijden, R., Heignen, J., Hellinga, C., Romein, B., and Luyben, K. (1994), *Biotechnol. Bioeng.* **43,** 3–10.
5. van der Heijden, R., Heignen, J., Hellinga, C., Romein, B., and Luyben, K. (1994), *Biotechnol. Bioeng.* **43,** 11–20.
6. van der Heijden, R., Heignen, J., Hellinga, C., Romein, B., and Luyben, K. (1994), *Biotechnol. Bioeng.* **44,** 781–791.
7. Rosenblitt, A., Agosin, E., Delgado, J., and Perez-Correa, R. (2000), *Biotechnol. Prog.* **16,** 152–162.
8. Hatzis, C., Riley, C., and Philippidis, G. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 443–459.
9. McMillan, J., Newman, M., Templeton, D., and Mohagheghi, A. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 649–665.
10. Ross, A., Schugerl, K., and Scheiding, W. (1983), *Eur. J. Appl. Microbiol. Biotechnol.* **18,** 29–37.
11. Smits, J., Rinsema, A., Tramper, J., Van Sonabeek, H., and Knol, W. (1996), *Appl. Microbiol. Biotechnol.* **46,** 489–496.
12. Hayward, T. K., Hamilton, J., Tholudur, A., and McMillan, J. (2000), *Appl. Biochem. Biotechnol.* **84–86,** 859–874.
13. Sáez, J. C., Colucci, J. A., McMillan, J. D., Schell, D. J., Hamilton, J., Tholudur, A., and Hames, B., (2001), *Biotechnol. Bioeng.*, submitted.
14. Tholudur, A., Hames, B., Meglen, R., Hayward, T. K., Sáez, J. C., Hamilton, J., and McMillan, J. D. (2001), submitted.
15. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985), *Anal. Biochem.* **150,** 76–85.

16. Abernethy, R. and Benedict, R. (1985), *ISA Trans.* **24 (1),** 75–79.
17. American Society of Mechanical Engineers (1986), ANSI/ASME PTC 19.1–1985, ASME, NY.
18. Vinzant, T. B., Ponfick, L., Nagle, N. J., Ehrman, C. I., Reynolds, J. B., and Himmel, M. E. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 611–626.