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Application of a Data Reconciliation Method to the Stoichiometric Analysis of *Fibrobacter succinogenes* Growth

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Abstract *Fibrobacter succinogenes* S85, a strictly anaerobic Gram-negative bacterium, was grown in continuous culture in a bioreactor at different dilution rates (0.02 to 0.092 h^{-1}) on a fully synthetic culture medium with glucose as carbon source. Glucose and ammonium sulfate consumption, as well as biomass, succinate, acetate, formate, and carbohydrate production were regularly measured. The relevant biomass elemental compositions were established for each dilution rate. Robustness of the experimental information was checked by C and N mass balances estimation, which were satisfactory. A detailed overall stoichiometry analysis of the process, including all substrates and products of the culture, was proposed. Online and off-line parameters measured during the culture brought a large number of data which were weighted by their respective variance associated to the measured value. The material balance resulted in an overdetermined linear system of equations made of weighted relationships including experimental data, elemental balances (C, H, O, N, S, Na), and an additional constraint. The mass balances involved in stoichiometric equations were solved using data reconciliation and linear algebra methods to take into account error measurements. This methodology allowed to establish the overall stoichiometric equation for each dilution rate studied.

Keywords *Fibrobacter succinogenes* · Stoichiometry · Data reconciliation · Continuous culture · Mass balance

Introduction

Fibrobacter succinogenes, a cellulolytic rumen bacterium, holds an enzymatic equipment well adapted to the anaerobic degradation of vegetable fibers and plants, especially when these are highly lignified. *F. succinogenes* has a full and complex enzymatic equipment, which includes endoglucanases, exoglucanases, and hemicellulases [1–3].

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The degradation steps lead to the production of cellobiose and glucose that are further metabolized by the bacterium. The fermentative metabolism of this bacterium has been studied and leads to the production of succinate, acetate, and formate [4–6]. *F. succinogenes* is also able to store intracellular glycogen, even in cells of young cultures [7, 8] and also to produce and release oligosaccharides [9, 10].

However, there is little information on the global stoichiometry of this metabolism. Such quantitative information is necessary for further understanding the growth of *F. succinogenes*, for example, through a global stoichiometric approach before metabolic flux modelling.

The aim of this work was to establish the overall stoichiometry of the growth of *F. succinogenes* S85 cultivated in a standardized continuous anaerobic culture process on a fully synthetic culture medium with glucose as carbon source for different dilution rates. Linear algebra and data reconciliation methods have been developed to solve the overdetermined system obtained from the large number of experimental data collected.

Materials and Methods

Bacterial Strain and Culture Conditions

The strain used was *F. succinogenes* S85 (ATCC 19169). The bacteria were grown anaerobically under 100% CO_2 in a synthetic medium [11] containing per liter: 0.45 g KH_2PO_4, 0.45 g K_2HPO_4, 0.9 g NaCl, 1.8 g $(NH_4)_2SO_4$, 0.09 g $MgSO_4$, 0.09 g $CaCl_2$, 3 mg $MnSO_4{\cdot}6H_2O$, 0.3 mg $CoCl_2{\cdot}6H_2O$, 8 mg $FeSO_4{\cdot}7H_2O$; volatile fatty acids: 1.64 mL acetic acid, 0.58 mL propionic acid, 0.39 mL butyric acid, 0.10 mL isobutyric acid, 0.10 mL valeric acid, 0.10 mL isovaleric acid, and 0.10 mL DL-α-methyl butyric acid; 0.025 mg biotin, 0.05 mg ρ-aminobenzoic acid, 0.7 mg hemin, 1 mg resazurine. The carbon source was glucose during continuous cultures.

The reactor was a modified B.BRAUN culture unit (Biostat ED, B.BRAUN Germany). The working volume was 5 L and the stirring speed 100 rpm. Temperature was controlled at 39°C, and the pH was maintained at 6.3 by automatic addition of Na_2CO_3 (70 g L^{-1}).

The culture vessel was fed with the liquid medium completed with various glucose concentrations (from 8.2 to 19.1 g L^{-1}) at three volumetric flow rates (99, 255 and 464 mL h^{-1}) corresponding to three dilution rates (D=0.02, 0.051, and 0.092 h^{-1}). Culture vessel, effluent tank, medium tank, and carbonate bottle were interconnected by a gas system, and the pressure was maintained at 0.2 bars above atmospheric pressure with a 2-m bubble column filled with water (Fig. 1). The whole gas system was continuously flushed with 5 standard cubic centimeters per minute (sccm) of sterile, oxygen-free CO_2 during the culture to preserve anaerobic conditions. This flow rate was controlled using a mass flow controller (Tylan, FC 260, 0–5 sccm), while the gas flow at the exit of the reactor was measured with a mass flow meter (Brooks, 58605, 0–20 sccm).

Analysis

Samples were taken at regular time intervals during the experiment. Microscopic observations showed that the culture was always axenic. For each sample, the absorbance was measured at 600 nm (spectrophotometer, Safas mc^2). High-performance liquid chromatography apparatus (Agilent 1100 series, fitted with two Phenomenex Rezex ROA columns, 7.8 mm diameter and 300 mm length each) was used to determine glucose and organic acids concentrations.

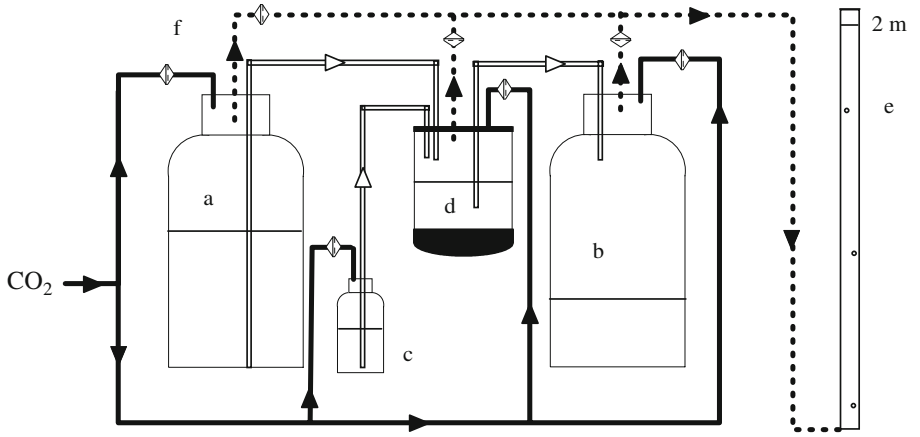

Fig. 1 Flow chart of the continuous culture system: **a** Medium tank, **b** Effluent tank, **c** Carbonate bottle, **d** Culture vessel, **e** Bubble column, **f** air filters; ═ liquid connection, ▬ Entry gas, ••• Exit gas

Culture supernatants were obtained after centrifugation of an aliquot (10,000×*g*, 5 min) and were used to perform the colorimetric assays of ammonium ions [12], proteins [13], and total carbohydrates [14, 15]. Soluble carbohydrate production was calculated by difference between total carbohydrates and glucose concentrations.

Biomass dry weight was determined after centrifugation (10,000×*g*, 5 min, 5°C) of 10 mL of crude sample. The resulting pellets were dried in an oven at 100°C for 24 h. A typical correlation OD-cell dry weight was established from data collected during the exponential growth phase of a batch experiment carried out in the same conditions of medium, pH, stirring, and temperature as:

$$\text{Cell dry weight} = 0.482(\pm 0.034)\ \text{OD}_{600\ \text{nm}}$$

where the cell dry weight is expressed as g L^{-1}(correlation coefficient of 0.989).

At steady-state, one sample was centrifuged (10,000×*g*, 15 min, 5°C), washed with 0.9% NaCl, and dried under vacuum at 65°C (48 h) to determine an average biomass formula (CHONSP) by elemental analysis (Service Central d'Analyses, CNRS); these data were used for elemental recovery calculations.

Gas at the exit of the bubble column was analyzed by gas chromatography (Hewlett Packard 5890 series II, fitted with a Thermal Conductivity Detector). Two 1.5 m length, 1/8-in. diameter stainless steel columns (Porapak Q and 5 Å molecular sieves) connected with a six-port commutation valve were used.

Method for Stoichiometric Coefficients Estimation and Statistical Analysis

If a detailed analysis of a process leads to consider a set of c compounds, then an overall stoichiometric equation with $(c-1)$ unknown coefficients is established, knowing that one coefficient is arbitrarily fixed to a value of 1. To determine these $(c-1)$ stoichiometric coefficients, experimental data are needed, and it is necessary to keep in mind that calculations must be performed by meeting the constraint of Lavoisier (principle of elements conservation).

Let us introduce experimental data in a column vector $\hat{\mathbf{Y}}_{\mathbf{exp}}(n)$ of n mass yields, all calculated with the same compound as reference, glucose for example. Let us consider $\mathbf{C}(c)$

the column vector of the stoichiometric coefficients, with the relevant value for the reference compound, again glucose for example, being set to 1. Finally, $\mathbf{Y_r}(n)$ is the column vector of the yields values obtained after data reconciliation. The following matricial expression is written:

$$\mathbf{AC} = \mathbf{Y_r} \tag{1}$$

$\mathbf{A}(n, c)$ is the matrix enabling to build the values of the mass yields knowing the values of the stoichiometric coefficients.

The element conservation balances are written knowing the elemental formula of all compounds. This is a constraint for the identification and the need that one stoichiometric coefficient is fixed to 1. Let the resulting linear system of constraints be written as follows:

$$\mathbf{SC} = \mathbf{K} \tag{2}$$

$\mathbf{C}(c)$ is the aforementioned column vector of stoichiometric coefficients (negative for the substrates, positive for the products). $\mathbf{S}(p, c)$ contains the elemental formula of all compounds (six rows for six elements) and additional constraints (additional rows) such as the choice of the stoichiometric coefficient which is fixed to 1 and possibly other constraint equations between the coefficients. Therefore, p is the total number of equations. $\mathbf{K}(p)$ is the known column vector of resulting constraints, for example, 0 for corresponding conservation equations, 1 for the fixed stoichiometric coefficient, and 0 for the linear constraints equation between the coefficients.

The complete linear system for the calculation of the stoichiometric coefficients is obtained by concatenation of the previous expressions, leading to:

$$\mathbf{MC} = \mathbf{H} \tag{3}$$

where $\mathbf{M}$ is a $(n+p, c)$ matrix composed of matrix $\mathbf{A}$ and $\mathbf{S}$, and $\mathbf{H}$ is a $(n + p)$ column vector formed by concatenation of $\mathbf{Y_r}(n)$ and $\mathbf{K}$(p). Similarly, $\hat{\mathbf{H}}_{\mathbf{exp}}(n + p)$ is the column vector formed by concatenation of $\hat{\mathbf{Y}}_{\exp}(n)$ and $\mathbf{K}(p)$. A weighted diagonal matrix $\mathbf{W}$ $(n + p, n + p)$ is also be filled with the inverse of experimental variances for the n experimental data and with 1 for the constraint relationships. $\mathbf{W}$ enables to account for the difference in experimental errors of the measurement yields. The system $\mathbf{M}$ $\mathbf{C}$=$\mathbf{H}$ can be solved by direct inversion of the matrix $\mathbf{M}$, if both matrix rank and $(n+p)$ are equal to c (determined system). In such case:

$$\mathbf{C} = \mathbf{M}^{-1}\mathbf{H} \tag{4}$$

In the case of redundant information (more independent rows than columns, i.e., $(n+p)>$c), a data reconciliation method must be used to solve the overdetermined and constrained system. We propose to use a Lagrange method for solving this problem of optimization, with the assumption that error measurements (i.e., variances) follow a Gaussian law [16]. The method is developed as follows.

The variable to be minimized is the quadratic criterium (Φ) given by:

$$\Phi = \left(\hat{\mathbf{H}}_{\exp} - \mathbf{H}\right)^{\mathrm{t}}(\mathbf{W})\left(\hat{\mathbf{H}} - \mathbf{H}\right) \tag{5}$$

$$\text{i.e. } \Phi = \left(\hat{\mathbf{H}}_{\exp} - \mathbf{MC}\right)^{\mathrm{t}}(\mathbf{W})\left(\hat{\mathbf{H}}_{\exp} - \mathbf{MC}\right) \tag{6}$$

Φ is a dimensionless number if $\mathbf{W}$ is used.

In this study, the errors on experimental values are known, and the confidence interval (i_y), the standard deviation (σ_y) of the model are given:

$$\begin{aligned} y &= \hat{y} \pm i_y \\ \Delta_y &= 2i_y \\ \Delta_y &= 2t_{1-\alpha/2}\sigma_y \\ \sigma_y &= i_y / t_{1-\alpha/2} \end{aligned} \tag{7}$$

If the number of points for the estimation is sufficiently large, $t_{0,975} \approx 2$ (α=0.05), and the interval is a confidence interval of 95%. The weights for all measured yields are given by:

$$\mathbf{W}_{y_j} = \frac{1}{\sigma_{y_j}^2} = \frac{4}{i_{y_j}^2} \tag{8}$$

The constraints to fulfill are given by:

$$\mathbf{SC} = \mathbf{K} \tag{9}$$

The Lagrangian function to be minimized is:

$$\mathbf{L} = \Phi - \Lambda(\mathbf{SC} - \mathbf{K}) \tag{10}$$

where $\mathbf{\Lambda}$ is the row vector (p) of the Lagrange coefficients.

The p Lagrange coefficients and the c stoichiometric coefficients are obtained by solving the (p+c) system of equations formed by:

$$\begin{aligned} \frac{\partial \mathbf{L}}{\partial \mathbf{C}} &= 0 \quad (c \text{ equations}) \\ \mathbf{SC} &= \mathbf{K} \quad (p \text{ equations}) \end{aligned} \tag{11}$$

$\frac{\partial \mathbf{L}}{\partial \mathbf{C}} = 0$ is a matrix derivation which leads to the c following equations:

$$\frac{\partial \mathbf{L}}{\partial \mathbf{C}} = -2\mathbf{M}^t\mathbf{W}\left(\hat{\mathbf{H}}_{\text{exp}} - \mathbf{MC}\right) - \mathbf{S}^t\,\Lambda^t = 0 \tag{12}$$

Let us write:

$$2(\mathbf{M}^t\mathbf{WM})\mathbf{C} = 2\mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\text{exp}} + \mathbf{S}^t\,\Lambda^t \tag{13}$$

then:

$$\mathbf{C} = (\mathbf{M}^t\mathbf{WM})^{-1}\mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\text{exp}} + 1/2(\mathbf{M}^t\mathbf{WM})^{-1}\mathbf{S}^t\,\Lambda^t \tag{14}$$

and

$$\mathbf{S}(\mathbf{M}^t\mathbf{WM})^{-1}\mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\text{exp}} + 1/2\mathbf{S}(\mathbf{M}^t\mathbf{WM})^{-1}\mathbf{S}^t\,\Lambda^t = \mathbf{K} \tag{15}$$

Let us consider:

$$\Lambda^{\text{t}} = 2\left[\mathbf{S}(\mathbf{M}^t\mathbf{WM})^{-1}\mathbf{S}^t\right]^{-1}\left[\mathbf{K} - \mathbf{S}(\mathbf{M}^t\mathbf{WM})^{-1}\mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\text{exp}}\right] \tag{16}$$

if $\boldsymbol{\psi}$=$\mathbf{M}^t\ \mathbf{W}\ \mathbf{M}$, $\mathbf{C}$ is:

$$\mathbf{C} = \boldsymbol{\psi}^{-1}\mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\text{exp}} + \boldsymbol{\psi}^{-1}\mathbf{S}^t\left[\mathbf{S}\boldsymbol{\psi}^{-1}\mathbf{S}^t\right]^{-1}\left[\mathbf{K} - \mathbf{S}\boldsymbol{\psi}^{-1}\mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\text{exp}}\right] \tag{17}$$

Summarizing and combining computed matrices, the calculation leads to:

$$\boldsymbol{\psi} = \mathbf{M}^t\mathbf{W}\mathbf{M} \tag{18}$$

$$\Omega = \mathbf{M}^t\mathbf{W}\,\hat{\mathbf{H}}_{\exp} \tag{19}$$

The solution is given by:

$$\mathbf{C} = \boldsymbol{\psi}^{-1}\left[\Omega + \left(\mathbf{S}\boldsymbol{\psi}^{-1}\mathbf{S}t\right)^{-1}\left(\mathbf{K} - \mathbf{S}\boldsymbol{\psi}^{-1}\Omega\right)\right] \tag{20}$$

The SDs of the calculated coefficients are estimated by diagonal elements of matrices of covariances:

$$\begin{aligned}
&\mathrm{Covar}\left(\hat{\mathbf{C}}\right) = \left(\mathbf{M}^t\mathbf{W}\mathbf{M}\right)^{-1} = \boldsymbol{\psi}^{-1} \text{ if } \mathbf{W} \text{ is known from variances}\\
&\mathrm{Covar}\left(\hat{\mathbf{C}}\right) = \left(\mathbf{M}^t\mathbf{M}\right)\frac{\Phi}{n+p-c} \text{ if } \mathbf{W} \text{ is not known}
\end{aligned} \tag{21}$$

Covariance on estimates of the model is:

$$\mathrm{Covar}\left(\hat{\mathbf{H}}_{\exp}\right) = \mathbf{M}\,\mathrm{Covar}\left(\hat{\mathbf{C}}\right)\mathbf{M}^t \tag{22}$$

This method is already used in the laboratory for metabolic flux modeling [17] and is applied here to the characterization of continuous cultures of *F. succinogenes* at different dilution rates.

Results and Discussion

Molar Biomass Formulae and Element Recoveries

The average molar biomass formulae established after elemental analysis during steady-state are presented in Table 1. The average formula changed weakly with the dilution rate, the most important modification concerning the nitrogen mass fraction that increases with the dilution rate. This modification could be explained by the variation in glycogen to protein ratio that has been evidenced when the growth rate is increased [11].

To calculate C-recovery, consumptions of sodium carbonate and glucose were both taken into account as carbon sources as well as production of biomass, soluble proteins, succinate, acetate, formate, and soluble carbohydrates. As reported in Table 2, C-balance was between 96.6% and 101.3%. Data considered in N-recovery were the nitrogen source consumption (ion ammonium), the nitrogen content in cell dry weight, and the soluble proteins measured in the culture supernatant. At low dilution rate (0.02 h^{-1}), N-balance was satisfactory with a value of 98.2%, but at high dilution rate (0.051 and 0.092 h^{-1}), N-balances were low with, respectively, 62.1% and 81.7%.

N-recovery was dilution rate dependent and thus growth rate dependent. This dependence has already been shown in *F. succinogenes* by Wells and Russell [18]. Growing cultures of *F. succinogenes* were reported to assimilate more ammonia than could be accounted for cellular protein, RNA, or DNA, and released large amounts of

Table 1 Average biomass formulae for different dilution rates.

Dilution rate (h^{-1})	Average biomass formula				
	C	H	O	N	S
0.020	3.688	6.760	2.665	0.247	0.010
0.051	3.656	6.821	2.791	0.255	0.009
0.092	3.799	6.981	2.545	0.380	0.008

nonammonia nitrogen that were not identified and quantified. Moreover, low sensitivity of the analytic method may also be another reason of the mismatch in the N-balance.

All these results showed a rather good consistency of the experimental results obtained during the course of the culture, indicating that the data reconciliation technique can be valuably applied.

Stoichiometric Equations Analysis and Data Reconciliation

The following detailed stoichiometric equation was proposed to describe the culture. The chemical dissociation imposed by the pH value (6.3) was taken into account in the elementary formulae of the organic acids. Nevertheless, one could assume that in the range of pH tolerated by this bacterium (from 6.0 to 7.0), the relevant formulae remained rather identical. The stoichiometric equation included 13 compounds and, therefore, 12 stoichiometric coefficients had to be determined, the coefficient for glucose being, as already pointed out, set to 1:

$$\begin{aligned} C_6H_{12}O_6 + \alpha_1(NH_4)_2SO_4 + \alpha_2Na_2CO_3 \rightarrow & \alpha_3C_{3.69}H_{6.76}O_{2.66}N_{0.25}S_{0.010}\ \text{(biomass, see Table 1)} \\ & + \alpha_4C_4H_{4.1864}O_4Na_{1.8136}\ \text{(sodium succinate)} \\ & + \alpha_5C_2H_3O_2Na_{\text{(sodium acetate)}} \\ & + \alpha_6CHO_2Na_{\text{(sodium formate)}} \\ & + \alpha_7C_6H_{10}O_5\ \text{(carbohydrates)} \\ & + \alpha_8C_{4.43}H_{7.09}O_{1.29}N_{1.27}S_{0.042}\ \text{(proteins)} \\ & + \alpha_9H_2O + \alpha_{10}NaHCO_3 \\ & + \alpha_{11}CO_2 + \alpha_{12}Na_2SO_4 \end{aligned} \tag{23}$$

At least 12 theoretical and/or experimental data were necessary. Online and off-line parameters measured during the culture provided nine experimental mass yields ($\hat{\mathbf{Y}}_{\text{exp}}$) related to ammonium sulfate, sodium carbonate, biomass, succinate, acetate, formate,

Table 2 C and N element recoveries, redox potential and dissolved CO_2 partial pressure for different dilution rates.

Dilution rate D (h^{-1})	C recovery (%)	N recovery (%)	Redox potential (mV)	Dissolved CO_2 partial pressure (mbar)
0.020	101.3	98.2	−358	1,167
0.051	97.6	62.1	−347	1,191
0.092	96.6	81.7	−345	1,193

carbohydrates, carbon dioxide, and protein, each value being weighted by a SD associated to the measured value (Table 3). For these data, a **A**(9, 13) matrix and $\hat{\mathbf{Y}}_{exp}$ (9) column vector could be built with molar mass of compounds and nine experimental mass yields. Elemental balances on C, H, O, N, S, and Na provided six linear equations of constraints. Coefficient of glucose was here again fixed to 1, allowing to build a **S**(7, 13) matrix filled with chemical formulae of compounds and coefficient of glucose, and a **K**(7) column vector filled with 0 for conservation equations and 1 for fixed stoichiometric coefficient.

The resulting system of 16 linear equations was made of 9 relationships obtained from experimental measurements and 7 constraints relationships resulting from elemental balances (C, H, O, N, S, Na) and glucose coefficient fixed to 1. It was overdetermined, as there were only 12 unknown coefficients to calculate. The advantage of data reconciliation was that it allowed the use of all available information to reduce inaccurate data due to experimental errors. Reconciled molar yields ($\mathbf{Y_r}$) were thus calculated from the calculated stoichiometric coefficients.

In a first time, this linear system composed of nine weighted relations from experimental measurements and seven constraints from elemental balances was used to reconcile molar yields obtained at three different dilution rates (Table 3).

Table 3 Experimental mass yield values ($\hat{\mathbf{Y}}_{exp\ mass}$) [g substrate or product. (g glucose)$^{-1}$] and comparative values of experimental ($\mathbf{Y}_{exp\ molar}$) and reconciled ($\mathbf{Y_r}$) average molar yields [mol substrate or product. (mol glucose)$^{-1}$] with their associated variances for different dilution rates.

Dilution rate (h^{-1})	Substrate and product	$\hat{\mathbf{Y}}_{exp\ mass}$	$Y_{exp\ molar}$	SD of $Y_{exp\ molar}$	Y_r	SD of Y_r	Confidence interval after data reconciliation	Y_r/Y_{exp}
0.020	$(NH_4)_2SO_4$	−0.044	−0.060	0.008	−0.071	0.004	0.008	1.19
	Na_2CO_3	−0.603	−1.024	0.038	−1.025	0.019	0.038	1.00
	Biomass	0.207	0.387	0.047	0.343	0.024	0.048	0.89
	Succinate	0.553	0.630	0.030	0.630	0.015	0.030	1.00
	Acetate	0.147	0.322	0.028	0.322	0.014	0.028	1.00
	Formate	0.004	0.108	0.012	0.108	0.006	0.012	1.00
	Carbohydrates	0.237	0.235	0.139	0.260	0.067	0.134	1.11
	CO_2	0.000	0.000	0.939	0.389	0.423	0.846	–
	Proteins	0.026	0.048	0.004	0.047	0.002	0.004	0.97
0.051	$(NH_4)_2SO_4$	−0.140	−0.191	0.017	−0.124	0.009	0.018	0.65
	Na_2CO_3	−0.553	−0.939	0.048	−0.940	0.024	0.048	1.00
	Biomass	0.448	0.814	0.041	0.844	0.020	0.040	1.04
	Succinate	0.502	0.572	0.017	0.569	0.009	0.018	0.99
	Acetate	0.141	0.310	0.021	0.308	0.010	0.020	0.99
	Formate	0.055	0.147	0.014	0.146	0.007	0.014	1.00
	Carbohydrates	0.157	0.173	0.131	0.000	0.063	0.126	–
	CO_2	0.007	0.027	1.297	0.537	0.541	1.082	20.00
	Proteins	0.014	0.025	0.003	0.026	0.001	0.002	1.03
0.092	$(NH_4)_2SO_4$	−0.165	−0.225	0.018	−0.159	0.009	0.018	0.71
	Na_2CO_3	−0.589	−0.999	0.027	−1.000	0.024	0.048	1.00
	Biomass	0.496	0.906	0.038	0.751	0.020	0.040	0.83
	Succinate	0.562	0.641	0.018	0.606	0.009	0.018	0.95
	Acetate	0.168	0.369	0.036	0.340	0.010	0.020	0.92
	Formate	0.058	0.152	0.005	0.149	0.007	0.014	0.98
	Carbohydrates	0.038	0.042	0.347	0.000	0.063	0.126	–
	CO_2	0.055	0.227	1.144	0.670	0.494	0.988	2.95
	Proteins	0.014	0.026	0.005	0.025	0.003	0.006	0.98

At a dilution rate of 0.02 h^{-1}, reconciled molar yields of acid production and sodium carbonate consumption were equal to experimental yields (Table 3). Biomass and protein yields were slightly decreased which led to an increase of ammonium sulfate yield from −0.060 (σ=0.008) to −0.071 (σ=0.004). These variations were of the same order of magnitude than the SD. There was also a slight increase in soluble carbohydrate yield from 0.234 (σ=0.139) to 0.260 (σ=0.067) at this dilution rate (0.02 h^{-1}) although the SD associated to this value was high. So, this linear system was sufficient to obtain satisfactory results at a dilution rate of 0.02 h^{-1}.

At the other dilution rates of 0.051 and 0.092 h^{-1}, this linear system did not give satisfactory results for the soluble carbohydrates yield, as a negative value was calculated. This result would lead to consider that under these conditions, soluble carbohydrates were a substrate. This idea could not be considered as realistic, as glucose was the sole carbon source in the fresh medium. It was thus necessary to modify the linear system by adding a new constraint on soluble carbohydrates for dilution rates of 0.051 and 0.092 h^{-1}. This supplementary constraint was to fix the coefficient of soluble carbohydrates to zero.

The new system also resulted in an overdetermined linear system of equations, then made of eight relationships from experimental measurements, six constrained equations from elemental balances (C, H, O, N, S, Na), and two reference coefficients (glucose set to 1 and carbohydrate set to 0). **A**(8, 13) was the matrix of known constant coefficients and $\hat{\mathbf{Y}}_{\mathbf{exp}}$(8) was the column vector of experimental yields. These data allowed to build a **S**(8, 13) matrix filled with chemical formulae of compounds, and a **K**(8) column vector.

The average reconciled yields and the relevant variances were calculated from this second linear system using data reconciliation. Experimental and reconciled values were compared in Table 3.

This modified linear system gave rather satisfactory results for dilution rates of 0.051 and 0.092 h^{-1} with very close experimental and identified yield values. Particularly $\mathbf{Y_r}/\mathbf{Y}_{exp}$ ratios were often close to 1 except for carbohydrates that had been set to zero by the supplementary constraint. The same major reconciliations were observed on carbon dioxide and ammonium sulfate yields. The discrepancy between $\mathbf{Y_r}$ and $\mathbf{Y}_{exp}$ on ammonium sulfate was explained by N-balances that were not satisfactorily assessed at these dilution rates. $\mathbf{Y_r}$ took into account only ammonium sulfate used for cellular growth and protein production. During continuous culture, no significant carbon dioxide gas production or consumption had been measured, and there was an important SD on these measurements. Carbon dioxide gas production or consumption was calculated by difference between inlet and outlet gas. The whole gas system was flushed with a regular gas flow of 5 sccm of carbon dioxide minimum to preserve correct anaerobic conditions during the continuous culture. However, effluent and medium tank volumes were about ten times higher than the reactor volume and were not regulated in temperature. Therefore, carbon dioxide solubility was permanently modified by ambient temperature variations that consequently led to unreliable carbon dioxide flow rate at the exit of the culture vessel. As carbon dioxide yield, carbohydrate yield was obtained indirectly by difference between total carbohydrate and glucose concentrations.

These results showed that the stoichiometric equation was dilution rate dependent. When the dilution rate increased from 0.020 to 0.092 h^{-1}, biomass and ammonium sulfate yields also significantly increased. The biomass yield was improved from 0.343 (σ=0.024) to 0.751 (σ=0.020) mol biomass (mol glucose)$^{-1}$ [from 0.183 to 0.412 g biomass (g glucose)$^{-1}$]. An important result was to notice that no significant variations were observed on succinate, acetate, formate, and sodium carbonate yields. In the range of dilution rates studied, the rates of succinate, acetate, and formate production were proportional to the

rate of glucose consumed into the system. This tended to demonstrate that the metabolism of *F. succinogenes* was not limited by the production of these acids which were directly linked to energy metabolism.

These results showed that assays used to track products of fermentation, consumption of nitrogen and carbon source were efficient as well as analysis of the biomass. This information was reliable to establish a stoichiometric equation for each dilution rate. It should also be pointed out that soluble carbohydrate production should be measured using a more specific technique. This information must be accurately available to go further in the analysis of *F. succinogenes* growth by the use of a metabolic flux model.

References

1. McGavin, M., & Forsberg, C. W. (1988). *Journal of Bacteriology*, *170*, 2914–2922.
2. Gong, J., Lo, R. Y. C., & Forsberg, C. W. (1989). *Applied and Environmental Microbiology*, *55*, 132–136.
3. Smith, D. C., & Forsberg, C. W. (1991). *Applied and Environmental Microbiology*, *57*, 3552–3557.
4. Bryant, M. P., & Burkey, L. A. (1953). *Journal of Dairy Science*, *36*, 205–217.
5. Stewart, C. S., & Flint, H. J. (1989). *Applied Microbiology and Biotechnology*, *30*, 433–439.
6. Weimer, P. J. (1993). *Archives of Microbiology*, *160*, 288–294.
7. Stewart, C. S., Paniagua, C., Dinsdale, D., Cheng, K. J., & Garrow, S. (1981). *Applied and Environmental Microbiology*, *41*, 504–510.
8. Gaudet, G., & Gaillard, B. (1987). *Archives of Microbiology*, *148*, 150–154.
9. Wells, J. E., Russel, J. B., Shi, J. B., & Weimer, P. J. (1995). *Applied and Environmental Microbiology*, *61*, 1757–1762.
10. Nouaille, R., Matulova, M., Delort, A. M., & Forano, E. (2005). *FEBS Journal*, *272*, 2416–2427.
11. Gaudet, G., Forano, E., Dauphin, G., & Delort, A. M. (1992). *European Journal of Biochemistry*, *207*, 155–162.
12. Patton, C. J., & Crouch, S. R. (1977). *Analytical Chemistry*, *49*, 464–469.
13. Bradford, M. (1976). *Analytical Biochemistry*, *72*, 248–254.
14. Dubois, M., Gilles, K., Hamilton, J. K., Rebers, P. A., & Smith, F. (1951). *Nature*, *168*(4265), 167.
15. Herbert, O., Phipps, P. I., & Strange, R. E. (1971). *Methods in Microbiology* (vol. 5B, pp. 244–249). London and New York.
16. Himmelblau, D. M. (1970). *Process analysis by statistical methods*. NY: John Wiley Ed.
17. Urrieta-Saltijeral, J. M., Dussap, C. G., Pons, A., Creuly, C., & Gros, J. B. (2001). *Engineering and Manufacturing for Biotechnology*, 143–156.
18. Wells, J. E., & Russel, J. B. (1996). *Environmental Microbiology*, *62*, 1342–1346.