

# Gas Chromatography and Gateway Sensors for On-Line State Estimation of Complex Fermentations (Butanol–Acetone Fermentation)

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A fermentation system has been designed to demonstrate the use of gas chromatography (GC) for on-line monitoring of the butanol–acetone and other complex saccharolytic fermentations. Tangential flow ultrafiltration was used to sterilely and continuously obtain a cell-free filtrate from the fermentation broth for on-line GC analysis of butanol, butyrate, acetate, acetone, ethanol, and acetoin. The liquid injection system consists of a phosphoric acid contactor, a slider-type injection valve, and a heater to address the difficulties (ghosting) encountered in the analysis of carboxylic acids. The fermentor headspace gas was also analyzed by on-line GC for nitrogen and carbon dioxide, while hydrogen was measured by difference. Raw chromatographic data were analyzed by a chromatography data system. Both raw and processed data were transmitted to a VAX 11/750 computer for further processing (using the fermentation equation) and archiving. The fermentation equation, which has recently been derived and tested on completed fermentation data, was also found to be valid during transient fermentations and thus useful as a gateway sensor for calculating various fermentation parameters on-line. Such parameters include glucose concentration and gas composition, as well as a number of unobservable parameters (such as  $Y_{ATP}$ , excess ATP, and NAD reduced by  $FdH_2$ ), which characterize the state of the fermentation.

## 1. INTRODUCTION

The renewed interest in complex multiproduct fermentations, such as the butanol–acetone<sup>1,2</sup> and butanediol<sup>3</sup> fermentations, combined with the realization that efficient and economical processes will require a more sophisticated reactor design and control than that of the simple batch reactor,<sup>1,4</sup> bring to light two of the major weak areas of biotechnology, process sensors and process control models.<sup>4,5</sup> Although no

reliable or practical dynamic models for process control are on the horizon for such multiproduct fermentations,<sup>1,2</sup> development of reliable technology to estimate the state of the fermentations on-line would indeed be a crucial advancement. Such instrumentation technology would expedite the study and understanding of the kinetics and biochemical control mechanisms of the fermentations and, perhaps, more importantly for the immediate future, would allow the employment of adaptive control strategies<sup>6</sup> for process optimization. Although a complete on-line characterization of the state of fermentation that would include such unobservables as the true intracellular rates of key enzyme production and reactions are beyond the realm of present reality, on-line measurement of the observable fermentation parameters would be a significant advancement. The observable parameters include the concentrations and/or rates of change of key nutrients (carbon and nitrogen sources, in most cases), biomass and extracellular products, in addition to the more conventional observables, pH, temperature, and oxygen concentration.

Mass spectroscopy (MS) has been explored in recent years<sup>7–9</sup> as a means of measuring concentrations of gases and volatile chemicals in both the headspace and the broth of the fermentations. Because of the many problems associated with the membrane inlet<sup>7,8</sup> (nonlinear calibration, membrane memory effects, slow response times), however, MS has yet to prove itself a useful tool in complex multiproduct fermentations.<sup>7–9</sup> Nevertheless, MS cannot be used for measuring non-volatile chemicals (such as substrate sugars), it is relatively expensive, and it requires sophisticated software for interpretation of results. Although inexpensive and easy to use, the recently proposed membrane gas sensor systems<sup>10</sup> share common difficulties with MS.

Chromatography remains as one of the most general and reliable methods of analysis of both volatile (GC)

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and nonvolatile (liquid chromatography, LC) chemicals. With the advances in hardware technology and computer automation (for both GC and LC), and the even more recent or forthcoming advents of ultrafast capillary GC and ultrafast HPLC (high-performance LC), chromatography may become the universal sensor in biotechnology. Recent work has shown that headspace GC can be employed successfully to monitor broth concentrations of ethanol<sup>11</sup> and the mixture of solvents (butanol, acetone, ethanol) in the butanol–acetone fermentation.<sup>12</sup>

The objective of this article is twofold. First, we want to demonstrate that on-line GC can be used to measure the composition of gases (N<sub>2</sub>, CO<sub>2</sub>, H<sub>2</sub>) in the headspace and the concentrations of all fermentation products (butanol, acetone, acetoin, ethanol, acetate, and butyrate) in the broth of the butanol–acetone fermentation. Second, we want to demonstrate that the fermentation equation we have recently derived<sup>1</sup> is valid under transient conditions and is therefore a useful tool (a “gateway” sensor<sup>1,4</sup>) for indirectly measuring observable and unobservable<sup>1,2</sup> parameters of the fermentation, as well as for checking the consistency of experimental data.

## 2. MATERIALS AND METHODS

### Organism and Growth conditions

The organism used was *Clostridium acetobutylicum* ATCC 824. The cultures were stored as spores at 2–4°C under nitrogen atmosphere in a 5% (w/v) corn mash, 0.5% (w/v) glucose medium.<sup>13</sup> Spore suspensions (10% v/v) were transferred to screw-cap tubes containing heat-sterilized soluble medium prepared by dissolving in 1 L distilled water: KH<sub>2</sub>PO<sub>4</sub>, 0.75 g; K<sub>2</sub>HPO<sub>4</sub>, 0.75 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.4 g; MnSO<sub>4</sub>·H<sub>2</sub>O, 0.01 g; FeSO<sub>4</sub>·7 H<sub>2</sub>O, 0.01 g; asparagine, 2.0 g; yeast extract (Sigma Chemical Co.), 5.0 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2.0

g; cysteine, 0.5 g; and glucose, 10 g. The culture pH was adjusted to 6.2–6.6 with 1N NaOH before the addition of spores. The cultures were sparged with sterile purified (O<sub>2</sub>-free) nitrogen, tightly capped, pasteurized at 70–80°C for 10 min, and then incubated at 37°C. Vegetative cultures prepared in this way were used as inocula for subsequent fermentations. The media used in all batch and continuous cultures were as above except for the cysteine, which was omitted, and the amounts of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and glucose, which were varied as is described below.

### Fermentor

A Biostat M fermentor (B. Braun Instruments) was used for growth of the microorganism. As is shown in Figure 1, the pH, temperature, agitation (rpm), and nitrogen flow were locally measured and controlled. For pH adjustment, 2N NaOH and 0.5N HCl solutions were used. The fermentor has a paddle wheel agitator, which was run at 200 rpm. Anaerobiosis was maintained by continuous flow of purified, oxygen-free, sterile nitrogen. For fed batch and continuous operation, a diaphragm dosing pump can easily be added to the fermentor. The Biostat M has a working volume of 0.5–1.4 L. Thus, the effect of fermentation broth sampling for off-line analysis on the reactor operation is small.

### Analytical Procedures

Cell density was determined by measuring the optical density (OD) of the culture broth at 600 nm in a Gilford 250 spectrophotometer. Optical density readings were converted to biomass concentrations using a mass extinction coefficient of 5.0 ± 0.2 L/g biomass cm at 600 nm. The latter was established by dry-weight determinations of broth samples as is detailed in Roos.<sup>13</sup>

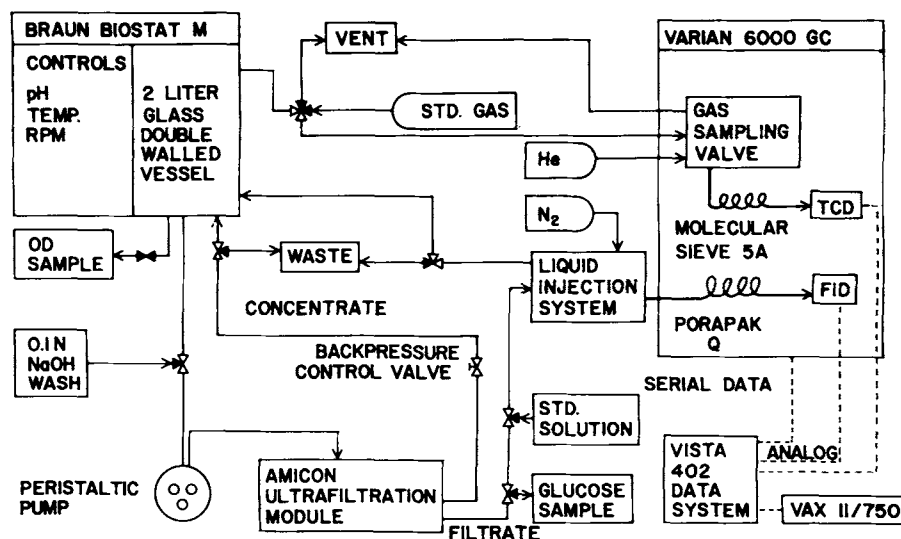


Figure 1. Fermentation monitoring system.

Glucose was determined by a peroxidase, glucose-oxidase enzymatic assay (Sigma Chemical Co.).

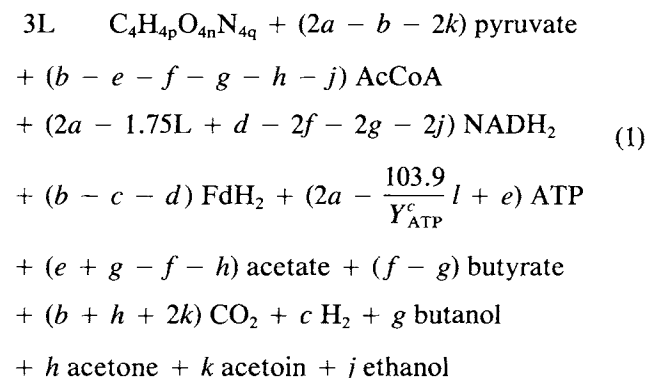
Concentrations of ethanol, acetone, acetate, butanol, acetoin, and butyrate were determined off-line using a Vista 6000 gas chromatograph and an 8000 autosampler (Varian Instrument Group). Clear filtrates were continuously produced from the fermentation broth as is described in the equipment design section. The clear filtrate samples were kept frozen until they could be analyzed. Chromatographic samples were prepared by mixing 0.1 mL 3*N* HCl with 1.0 mL filtrates in 1.4-mL screw-cap septum vials which were then loaded into the autosampler. The chromatographic analysis is described in the equipment design section.

### 3. CALCULATIONS

The data from the on-line gas chromatographic analyses can be used to calculate several important fermentation parameters, many of which are difficult to measure on-line (or at all in some cases). Of particular interest among these are the ATP yield ( $Y_{ATP}$ ), the amount of excess ATP, the amount of NADH<sub>2</sub> formed through the oxidation of FdH<sub>2</sub> (NfF) via the NADH-ferredoxin oxidoreductase,<sup>1,2</sup> and the glucose concentration in the fermentation broth. The data consistency can also be checked using the fermentation equation described below.

The calculations in this work are based on the following equation developed by Papoutsakis<sup>1</sup>:

(2L + a) glucose →



where  $C_4H_{4p}O_{4n}N_{4q}$  represents the dry microbial mass<sup>1</sup> and  $Y_{ATP}^c$  is a constant which represents the actual ATP yield of the particular fermentation. This stoichiometric equation describes the relationships between products, intermediates, biomass, and substrates for the acetone-butanol fermentation. Each unknown ( $a-h, j-l$ ) represents a specific reaction or pathway. Because there is no accumulation of the four intermediate species, pyruvate, AcCoA, NADH<sub>2</sub>, and FdH<sub>2</sub>, their coefficients in equation (1) are set equal to zero to yield

$$2a - b - 2k = 0 \quad (2)$$

$$b - e - f - g - h - j = 0 \quad (3)$$

$$2a - 1.75L + d - 2f - 2g - 2j = 0 \quad (4)$$

$$b - c - d = 0 \quad (5)$$

Equations (2)–(5) describe a system with 11 unknowns ( $a-h, j-l$ ) and four equations. Given seven more independent equations relating the various unknowns, one can then determine all 11 unknowns and use these to calculate other parameters of interest. Measured cumulative amounts of seven of the various products at a given point in time can be used to give the additional equations needed to determine the 11 unknowns.

Results from the liquid analysis provide the cumulative amounts of liquid products for a batch fermentation and are used directly in these calculations. The results from the gas analysis, however, provide only the effluent gas concentrations. Given the total gas flowrate, the gas concentrations are easily converted into the rates of production of the gaseous products. The cumulative amounts of the gaseous products must be calculated by integration of their rates of production.

The gas analysis provides the mole percent of nitrogen and carbon dioxide in the effluent gas. The mole percent of nitrogen in the effluent gas before the fermentation begins,  $x_{in}$ , is not 100%, but rather about 94%. The remaining 6% is water vapor in the effluent gas. The mole percent of hydrogen in the effluent gas is calculated by subtracting the percent carbon dioxide and nitrogen from  $x_{in}$ .

The total gas flowrate  $F$  is calculated using nitrogen as a tie component since the nitrogen flowrate is constant throughout the fermentation:

$$F = \frac{x_{in}}{x} F_0 \quad (6)$$

where  $F_0$  is the effluent molar gas flowrate before the fermentation begins and  $x$  is the mole percent of nitrogen in the effluent gas at time  $t$ . The CO<sub>2</sub> and H<sub>2</sub> flowrates are then calculated from the total gas flowrate and the gas composition.

The cumulative amounts of CO<sub>2</sub> and H<sub>2</sub> produced are calculated by integration of the CO<sub>2</sub> and H<sub>2</sub> flowrates. These amounts are then divided by the fermentation volume so that they are expressed on a liquid volume basis, as are the liquid products. For CO<sub>2</sub> we have

$$C_{CO_2}(t) = \frac{\int_0^t F(t)y(t) dt}{V(t)} \quad (7)$$

where  $C_{CO_2}$  is the total CO<sub>2</sub> produced up to time  $t$  per unit volume of fermentation broth,  $y$  is the mole fraction of CO<sub>2</sub> at time  $t$ , and  $V$  is the volume of fermentation broth at time  $t$ . If the integration is carried out using the trapezoidal rule, equation (7) becomes

$$C_{CO_2,i} = \frac{\sum_{k=1}^i (F_k y_k + F_{k-1} y_{k-1}) \Delta t}{2V_i} \quad (8)$$

where  $C_{CO_2,i}$  is the total CO<sub>2</sub> produced per unit volume

up to the  $i$ th datum point and  $F_k$  and  $y_k$  are the total molar flowrate and  $\text{CO}_2$  mole fraction at the  $k$ th datum point.

The  $\text{OD}_{600}$  reading provides a measure of the biomass. Since the  $\text{OD}_{600}$  measurements are made at different times than the gas and liquid analyses, linear interpolation was used to provide the  $\text{OD}_{600}$  reading at the same time as the gas and liquid analyses. This causes no significant error since the  $\text{OD}_{600}$  readings are taken frequently.

The  $\text{OD}_{600}$  readings are converted to molar concentrations ( $C_b$ ) of biomass ( $\text{C}_4\text{H}_{4n}\text{O}_{4p}\text{N}_{4q}$ ) according to the equation

$$C_b = \frac{\text{OD}_{600}}{\varepsilon L(\text{MW})_b} \quad (9)$$

where  $\varepsilon$  is the mass extinction coefficient (5.0 L/g cm) for *C. acetobutylicum* as determined earlier,<sup>13</sup>  $L$  is the length of the cell (1 cm), and  $(\text{MW})_b$  is the molecular weight of biomass based on four carbon atoms per mole and a carbon mass fraction ( $\sigma_b$ ) of 0.462,<sup>1</sup> so that  $(\text{MW})_b = (12)(4)/(0.462) = 103.9$  g/mol.

Two different sets of values of seven of the products were used in addition to equations (2)–(5) to complete the set of 11 equations. This first set consists of the biomass, ethanol, acetone, acetate, butanol, acetoin, and butyrate molar concentrations,  $C_b$ ,  $C_{\text{Et}}$ ,  $C_{\text{Aco}}$ ,  $C_{\text{Aca}}$ ,  $C_{\text{Bl}}$ ,  $C_{\text{Aci}}$ , and  $C_{\text{Bt}}$ , respectively. The seven additional equations thus are

$$3l = C_b \quad (10)$$

$$j = C_{\text{Et}} \quad (11)$$

$$h = C_{\text{Aco}} \quad (12)$$

$$e + g - f - h = C_{\text{Aca}} \quad (13)$$

$$g = C_{\text{Bl}} \quad (14)$$

$$k = C_{\text{Aci}} \quad (15)$$

$$f - g = C_{\text{Bt}} \quad (16)$$

Equations (2)–(5) and (10)–(16) provide 11 independent equations with 11 unknowns. These are solved using a standard Gauss elimination<sup>14</sup> routine. Once the coefficients  $a$ – $h$  and  $j$ – $l$  are known, other fermentation variables are determined according to the equations

$$C_{\text{Glu}} = C_{\text{Glu},\text{in}} - (2l + a) \quad (17)$$

$$\text{ATP}_{\text{tot}}^* = 2a + e \quad (18)$$

$$\text{ATP}_{\text{ex}}^* = 2a + e - \frac{103.9}{Y_{\text{ATP}}^c} l \quad (19)$$

$$Y_{\text{ATP}} = \frac{C_b(\text{MW})_b}{\text{ATP}_{\text{tot}}^*} \quad (20)$$

$$Y_s = \frac{C_b}{2l + a} (\text{MW})_b \quad (21)$$

$$\text{NfF}^* = d \quad (22)$$

$$C_{\text{Hy}} = c \quad (23)$$

$$C_{\text{CO}_2} = b + h + 2k \quad (24)$$

where  $C_{\text{Glu},\text{in}}$  and  $C_{\text{Glu}}$  are the initial and at time  $t$  molar concentrations of glucose,  $\text{ATP}_{\text{tot}}^*$  is the molar concentration of the total amount of ATP produced,  $\text{ATP}_{\text{ex}}^*$  is the molar concentration of the amount of ATP produced in excess of what is required for biosynthesis,  $\text{NfF}^*$  is the molar concentration of NAD reduced by  $\text{FdH}_2$ , and  $C_{\text{Hy}}$  is the moles of  $\text{H}_2$  produced per unit volume of fermentation broth.

The second set of equations is obtained by replacing equation (10) by

$$c = C_{\text{Hy}} \quad (25)$$

After solving this second set of equations, the coefficients are used as before to calculate  $C_{\text{Glu}}$ ,  $\text{ATP}_{\text{tot}}^*$ ,  $\text{ATP}_{\text{ex}}^*$ ,  $Y_{\text{ATP}}$ ,  $Y_s$ ,  $\text{NfF}^*$ , and  $C_{\text{CO}_2}$ . In addition, the biomass concentration and hence the  $\text{OD}_{600}$  are estimated. Many of the calculated or measured values are converted into yields based on the amount of glucose fermented by using the amount of glucose consumed,  $2l + a$ . Obviously, any set of values of seven of the products and/or glucose consumed can be used to calculate the values of the remaining two products or consumed glucose. This is a matter of choice. It is also possible to calculate the “best” values of certain desired fermentation parameters, such as  $\text{ATP}_{\text{tot}}^*$  or  $\text{NfF}^*$ , or the extent of a key intracellular reaction represented by 1 or a combination of the 11 coefficients, by employing more experimental data values than the minimum number required for the solution of the system. This is a least-squares problem,<sup>14</sup> the solution of which was discussed in detail in Papoutsakis and Meyer.<sup>15</sup> Equation (1) can be used to check the consistency of experimental data by comparing calculated to experimental data as has been discussed in detail elsewhere.<sup>1</sup>

In the derivation of equation (1) two well-accepted biological regularities were used<sup>1</sup> — the values of  $\sigma_b$ , the weight fraction of carbon in biomass, and  $\gamma_b$ , the degree of reduction of biomass. The value of  $\sigma_b$  was taken to be 0.462<sup>1</sup>; measured values of  $\sigma_b$  for *C. beijerinckii* were found to be 0.48, 0.45, and 0.462 for growth on minimal, complex, and nitrogen-limited media, respectively.<sup>16</sup> The value of  $\gamma_b$  was taken to be 4.291,<sup>1</sup> while measured values of  $\gamma_b$  for *C. beijerinckii* were found to be 4.68, 3.69, and 4.01 for growth on minimal, complex, and nitrogen-limited media, respectively.<sup>16</sup> Among all the calculated quantities, only the value of  $\text{ATP}_{\text{ex}}^*$  [eq. (19)] is affected by the value of  $\sigma_b$ , and only in a very weak way because of the small percentage of carbon incorporated into biomass, which results in a small relative  $l$  value. The value of  $\gamma_b$  also has only a very weak effect on the calculations based on equation (1) because of the small coefficient

(1.75) of  $l$  in equation (4) and the small relative value of  $l$  as was just discussed. Thus, 10–20% variations in the values of  $\sigma_b$  and  $\gamma_b$  have an insignificant effect on the calculations. In the original derivation<sup>1</sup> of equation (1), a value of 10.5 was assumed for  $Y_{ATP}^c$ . This value is indeed a well-accepted<sup>1</sup> mean value for the ATP yield from many measurements with various microorganisms. As has been discussed already<sup>2</sup> and as will be discussed presently, the value of  $Y_{ATP}^c$  changes not only from batch to batch in fermentations of *C. acetobutylicum* but also quite dramatically within the same batch, achieving a maximum in the early exponential phase and dropping thereafter. Since the  $Y_{ATP}^c$  value affects only the value of  $ATP_e^*$ , we take it to be the maximum value achieved early in the fermentation as calculated according to equation (20). This results in the maximum estimate for  $ATP_{ex}^*$ . Note also that the values of  $ATP_{tot}^*$  and  $Y_{ATP}$  do not depend on either  $\sigma_b$  or  $\gamma_b$  but only on the concentrations of the biomass, acetate, butyrate, acetone, and carbon recovered in products (i.e.,  $a$ ), as can be seen by solving for  $e$  in equations (12), (13), (16), and (18).

Finally, the gamma ratio is the ratio of the degree of reductance of biomass and other fermentation products divided by the degree of reductance of carbon substrate(s), assuming that the degree of reductance of the nitrogen source is the same as that of  $NH_3$ , namely zero.<sup>1</sup> For totally synthetic media a gamma ratio value close to the ideal value of 1.0 is an indication of good data consistency. For fermentations on complex media, if only a small percentage of the biomass and product carbon is derived from the complex carbon source, a gamma ratio value close to 1.0 is expected again.

#### 4. EQUIPMENT DESIGN

In addition to the requirements discussed in the introduction, the system we set out to design and construct had to be durable, for reliable operation over many and long fermentation runs, as well as versatile, to accommodate various modes and conditions of fermentation. Also, it had to be designed with the constraint of having one gas chromatograph available with a single oven and the ability to simultaneously process the data from up to two detectors.

The system shown in Figure 1 fulfills the objectives, requirements, and constraints discussed above. Its major components are the fermentor, the ultrafiltration module, the liquid and gas injection valves, the gas chromatograph, and the chromatography data system (CDS). The fermentor has been described in the materials and methods section.

##### Ultrafiltration Module

An ultrafiltration system was chosen to provide cell-free liquid samples to the liquid sampling valve. Similar

systems have previously been used for various fermentation applications. A cell-recycle reactor for lactic acid production that utilizes ultrafiltration has been described by Vick Roy et al.<sup>17</sup> An ultrafiltration membrane has also been used to provide cell-free samples for a glucose enzyme thermistor in a system developed by Chotani and Constantinides.<sup>18</sup> A hollow-fiber system by Amicon Co. was first tested. Growth of cells inside the fibers, however, resulted in plugging and unsteady operation. Those problems probably resulted from operating the hollow-fiber module significantly below its design capacity. The unit finally chosen is a thin-channel, spiral flow system (Amicon CEC1), which utilizes 90-mm disc membranes. This unit has several advantages, such as stable operation over long time periods, membrane visibility, and minimal retention volume. Continuous operation is accomplished by diafiltration, where the sample is fed tangentially across the membrane. Thus, the cells do not concentrate on the membrane but are gently removed by the fluid flow. Three membranes were tested, with 50,000, 100,000, and 300,000 MW (molecular weight) cutoff. The 100,000-MW cutoff membrane was found to provide the best results. The 100,000- and 300,000-MW membranes both provided adequate filtrate flow, while that at MW 50,000 did not. Glass tubing for the feed and concentrate return lines was used to minimize and monitor wall growth. Other tubing was either stainless steel, latex, or Cole Palmer C-flex to minimize oxygen diffusion. The system layout was designed to minimize the residence time of the sample. The residence time for the cells is about 1.7 min and for the cell-free filtrate about 3.2 min. The total volume of the sample system is approximately 35 mL. The extensive amount of tubing and the ultrafiltration module requirements prevent the use of autoclaving for sterilization. A 5% formalin solution was used for sterilization as recommended by the manufacturer of the ultrafiltration module. For washing, 0.1N NaOH was chosen to clean the membrane and neutralize the electrostatic charges that build on the membrane and reduce the separation efficiency. Membrane and cells (which have a net negative charge) must repel each other to minimize plugging and growth on the membrane. NaOH neutralizes any positively charged sites and gives the membrane a net negative charge. The high pH also denatures most proteins. Both the concentrated-cell and cell-free streams return to the fermentor except for the small portions required for analyses. This sampling system gives excellent results with minimal impact on the fermentor operation.

##### Liquid Injection System

The liquid injection system, shown in Figure 2, consists of a phosphoric acid contactor, a liquid injection valve, and an auxiliary vaporization heater. Analysis of volatile organic acids is complicated by their tendency to adsorb to most porous metal surfaces. This problem

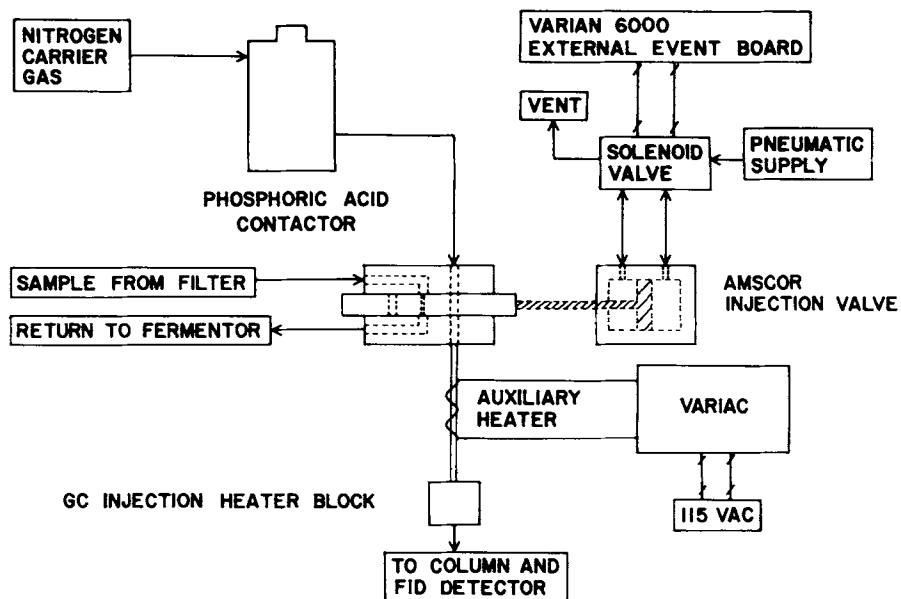


Figure 2. Liquid injection system on gas chromatograph.

leads to the phenomenon known as ghosting, which has been described by Drucker<sup>19</sup> as the appearance of "mysterious phantom" peaks after a later injection. To correct this problem, an auxiliary heater was installed on the stainless steel tubing leading from the injection valve to the GC column. Use of stainless steel was also minimized where possible. In addition, the carrier gas was originally passed over a 97% formic acid solution, as recommended by Drucker,<sup>19</sup> to minimize adsorption. Most commercially available formic acid preparations contain small amounts of acetic acid, however, which interferes with the analysis. As a result, phosphoric acid was ultimately used instead of formic acid. The phosphoric acid also stabilizes the pH of the liquid sample at vaporization, as well as the pH of the GC column, so that the sensitivity of the flame ionization detector (FID) to the two acids (butyric and acetic) is virtually independent of the culture pH in the pH range (3.5–7.0) of interest. Passing the carrier gas over the phosphoric acid made a significant improvement in the liquid analysis reproducibility.

The liquid injection valve is a slider-type valve (Amcor), which provides a direct path from the valve to the column, unlike a rotary valve. The valve is actuated by a Humphrey Products four-port, three-way pneumatic solenoid valve activated by a 115-V relay controlled by the gas chromatograph through the external-events board.

### Gas Chromatograph

The gas chromatograph used in this system is a Vista 6000 (Varian Instrument Group). Its ability to handle multiple columns and detectors makes possible the simultaneous analyses of both the fermentation broth and the effluent gas. Its temperature programming capabilities include multiple-ramp functions that allow

for optimal temperature settings for both analyses. Continuous operation is greatly facilitated by its time-programmed control of injection valves and its automation capabilities.

For the liquid analysis the column is constructed from glass tubing with a 6.25 mm o.d. and 2 mm i.d. The column is 2 m long and is packed with 80–100 mesh Porapak Q (Alltech). A FID was used for peak detection with the ionization oven set at 245°C. Temperature programming is used to minimize analysis time. The column temperature is held at 115°C for 10 min after injection and then increased to 170°C at a rate of 2°C min<sup>-1</sup>. This temperature is held until the last peak elutes. Finally, to ensure that all contaminants are removed from the column, the temperature is increased to 210°C at 15°C min<sup>-1</sup> where it is held constant for 5 min. Ultrapure nitrogen is used as a carrier gas at 30 mL/min.

Injection of the gas sample was controlled in a manner similar to that used for the liquid analysis. The injection valve is a rotary type (Valco six-port) and is located in the GC oven. The gas was analyzed with 2-m, 3.13-mm ID stainless steel column packed with washed molecular sieve 5A (Alltech). A thermal conductivity detector (TCD) was used with helium as the carrier gas at 30 mL/min. The TCD oven and filament temperatures were 210 and 230°C, respectively.

### Chromatography Data System (CDS)

The data system consists of a Vista 402 CDS (Varian Instrument Group) and a serial data interface to a VAX 11/750 computer. The 402 CDS performs the integration, peak identification, and result calculation based on peak area and calibration factors. Baseline subtraction is used in the integration to account for baseline drift due to temperature programming. The

results from the 402 CDS calculations are in units of millimole per liter for the liquid and mole percent for the gas. After each analysis the results are transmitted to the VAX 11/750 computer for data storage and higher-level calculations as was previously described.

## 5. SYSTEM OPERATION

### Monitoring

During normal operation of the system, all valves are set as shown in Figure 1. Samples for determination of biomass concentration via the OD<sub>600</sub> readings are taken manually directly from a sample port on the fermentor.

For the liquid analysis, a stream of fermentation broth is continuously pumped from the fermentor to the ultrafiltration module where it is split into a concentrate stream and a cell-free filtrate stream. The concentrate is returned directly to the fermentor. A valve on the concentrate line is used to control the relative flowrates of the filtrate and concentrate streams. The pump and the backpressure control valve are set so that the filtrate flowrate is approximately 5–10 mL/min. The filtrate continues on through the liquid sampling valve on the gas chromatograph and then back to the fermentor. Samples of the filtrate are taken periodically through the sample valve located immediately after the filtration module. These samples are used for off-line analysis of glucose and the fermentation products.

For the gas analysis, the off-gas from the fermentor flows through the gas sampling valve and then to a vent. Gas and liquid samples are injected into the gas chromatograph via the sampling valves about once every hour. The liquid injection volume is 1  $\mu$ L and the gas is 0.25 mL. The samples are analyzed by the gas chromatograph and CDS and the results of the analyses are transferred to the VAX computer for further processing.

### Calibration

Calibration of the gas chromatograph for liquid and gas analyses generally precedes inoculation of the fermentor. For long fermentations, however, it may be necessary to calibrate during the fermentation.

For calibration of the liquid analysis, flow through the filtration system is stopped and a standard solution containing all liquid products of the fermentation is circulated through the sampling valve. Initially, the standard solution flows through the liquid sampling valve and then to the waste vessel. After the sample loop has been thoroughly purged, the flow is diverted from the waste vessel back to the standard solution vessel to allow circulation of the standard solution. Five to 10 injections of the standard solution are made. The 402 data system calculates and stores calibration

factors for the calculations to be performed during the actual analyses. When calibration is complete, the filtration module and sample loop are washed with a 0.1N NaOH wash solution. This procedure prevents any of the standard solution from entering the fermentor.

Gas analysis calibration is accomplished using a standard gas mixture prepared and analyzed by Airco Industrial Gases. The fermentor off-gas is diverted directly to the vent via a four-port valve that allows the standard gas to flow through the sample valve and to the vent. The flowrate is adjusted to be approximately equal to the fermentor off-gas flow. The calibration injections are done along with the liquid calibration injections.

### Sterilization

Sterilization is accomplished in several steps by a combination of autoclaving, washing with a 5% formalin solution, and filtration. The pH electrode is first calibrated and placed in the fermentor vessel, which is filled with distilled water. The fermentor and all other vessels with vapor space that would not be directly exposed to the formalin solution are sterilized by autoclaving. Jumper lines of flexible tubing are attached to the openings to allow flow of the formalin solution through all parts of the sample system. A vessel with approximately 0.8 L of 5% formalin solution is attached to the input line of the ultrafiltration module. The flexible lines are connected to a sterile receiving vessel. Formalin solution is then circulated through the system with periodic valve switching and pump reversal to ensure that all portions of the system are adequately sterilized. To remove formaldehyde from the system, 2.5 L sterile distilled water is passed through the system in a similar manner. The jumper and feed lines are then clamped off and the receiving and solution feed vessels removed. Next, the fermentor connections are made after flaming to ensure sterility. Calibration fluid, which has been sterilized using a 0.2- $\mu$ m filter (Syborn Nalge 120-0020), is placed in the standard solution vessel.

**Table 1.** Covariances of liquid and gas analyses using the system of Figure 1.

Species	Covariance		Percent different
	Off-line	On-line	
Butanol	0.0204	0.0365	79
Acetone	0.0153	0.0351	129
Butyrate	0.0255	0.0538	111
Acetate	0.0245	0.0473	93
Ethanol	0.0193	0.0415	115
Acetoin	0.0257	0.0676	163
N <sub>2</sub>		0.0024	
CO <sub>2</sub>		0.0060	
H <sub>2</sub>		0.0060	

**Table II.** Fermentation balances for batch fermentations with the same initial ammonium–glucose ratio (0.136) but different controlled pH values.

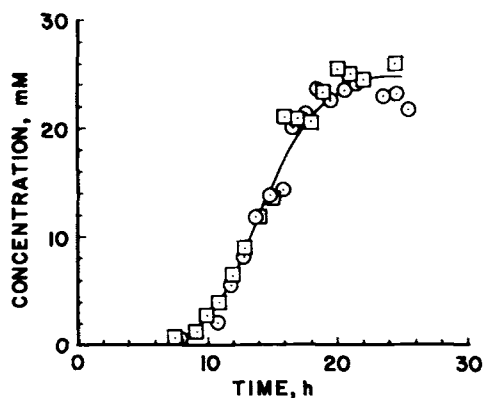
	Fermentation 1 pH 3.7	Fermentation 2 pH 4.0
Biomass (g/L)	0.95	1.21
Initial glucose (mM)	54.4	51.6
Residual glucose (mM)	0.0	0.0
$\mu$ (h <sup>-1</sup> )	0.37	0.62
$Y_x$	0.097	0.130
$Y_{ATP}^a$	5.5	9.00
Gamma ratio <sup>a</sup>	1.002	1.013
	mol/100 mol glucose fermented	
Butyrate	36.4	61.3
Acetate	43.7	33.8
CO <sub>2</sub>	180	208
H <sub>2</sub>	183	194
Ethanol	2.8	1.6
Butanol	18.8	2.2
Acetone	9.0	1.3
Acetoin	1.8	2.2
Carbon recovered in products (as glucose)	90.0	93.0
Nff <sup>a</sup>	-15.4	-21.4
H <sub>2</sub> /CO <sub>2</sub>	1.02	0.93

<sup>a</sup> Calculated quantities according to the fermentation equation.

## 6. RESULTS

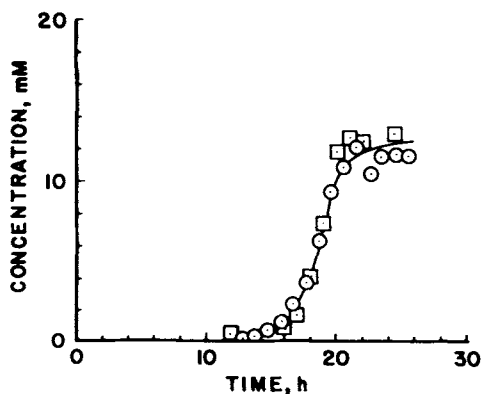
### System performance

The performance of the on-line liquid analysis system can best be evaluated by comparison with the off-line measurements. Table I shows the covariances for each component in the two measurements. The covariance is a dimensionless standard deviation and indicates the repeatability of a measurement. If the covariance is minimized and the system is properly calibrated, the analyses performed will be accurate. The values for the covariance were estimated using a standard solution and five injections. While the repeatability of the on-line measurements is not as good as the off-

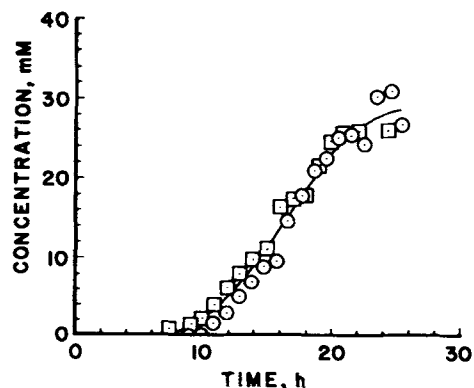


**Figure 4.** Comparison of off-line (□) to on-line (○) GC analyses of butyrate in fermentation broth of batch fermentation 1 (Table II).

line ones, it is quite acceptable. The capabilities of the system for on-line monitoring of complex fermentations and the validity and usefulness of the fermentation equation [eq. (1)] will be demonstrated by employing two butyric acid fermentations, which have been discussed in more detail in Roos et al.<sup>2</sup> The balances of the two fermentations are shown in Table II. Both fermentations were performed with an ammonium–glucose ratio of 0.136. The fermentation pH affects the availability of complex nitrogen (yeast extract) in the medium,<sup>2</sup> and thus, fermentation 1 produces solvents while 2 produces exclusively acids. The on- and off-line time profiles of butanol, butyrate, and acetate concentrations for fermentation 1 are shown in Figures 3, 4, and 5, respectively. These profiles are typical of the agreement between the on- and off-line measurements for all fermentations we have carried out and also of the remaining components (ethanol, acetone, and acetoin). The agreement of the two measurements is indeed quite good, although further improvement can be achieved, as is discussed next. The larger percentage of error comes from two sources. The first is the adsorption of acids on the stainless steel tubing between the liquid injection valve and the GC column. The second source of error is the variability



**Figure 3.** Comparison of off-line (□) to on-line (○) GC analyses of butanol in fermentation broth of batch fermentation 1 (Table II).



**Figure 5.** Comparison of off-line (□) to on-line (○) GC analyses of acetate in fermentation broth of batch fermentation 1 (Table II).

of the liquid sample volume ( $1 \mu\text{L}$ ), which is a hardware problem. The experimental error can be reduced (i) by reducing the sample volume to  $0.5 \mu\text{L}$ , (ii) by reducing the total analysis time and thus increasing the injection frequency, and (iii) by using glass-lined stainless steel tubing. The reduced sample volume and glass-lined tubing will significantly reduce the acid adsorption problem. The increased sampling frequency will reduce the impact of the sample volume variability because of better averaging and possible application of data filtering. For example, on the basis of a slow-changing component (such as ethanol or acetoin), a (bad) injection can be rejected if it is judged to produce an unreasonable value for that component. More general filtering techniques (such as Kalman filtering) can also be implemented. The sample volume variability can also be improved by better hardware design and construction. We are currently implementing remedies (i) and (ii) of the above, and preliminary results indicate that significant improvements over the figures of Table I can be achieved. By employing a shorter glass column and different temperature programming, we have already reduced the total analysis time by almost 50%. Further improvements appear almost certain.

Evaluation of the system performance for gas analysis cannot be made directly since we had no alternative method of analysis for comparison, and an off-line analysis is certainly less accurate. However, the gas analysis has few sources of error, and in view of the excellent analysis repeatability of both standard and unknown samples (Table I), it appears very accurate for the direct measurement of  $\text{N}_2$ ,  $\text{CO}_2$ , and gases like  $\text{O}_2$  and  $\text{CO}$  that can be performed with the same column and TCD. We have also improved the (already very good, Table I)  $\text{H}_2$  measurement by switching from the presently reported indirect measurement to a direct GC measurement employing a slightly different column configuration (but the same packing) and a different temperature programming. As we shall show below, the comparison between the measured and the calculated gas values is very good.

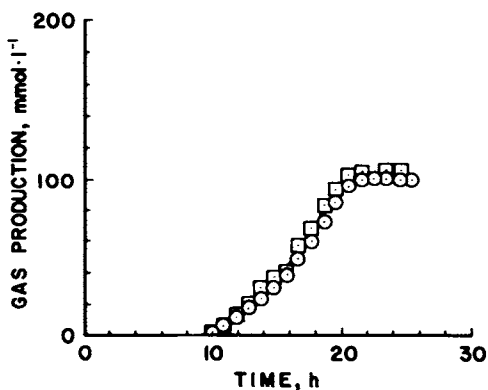


Figure 6. Comparison of calculated ( $\square$ ) to measured ( $\odot$ )  $\text{CO}_2$  amounts ( $\text{mmol/Liter}$  fermentation broth) produced in batch fermentation 1 (Table II).

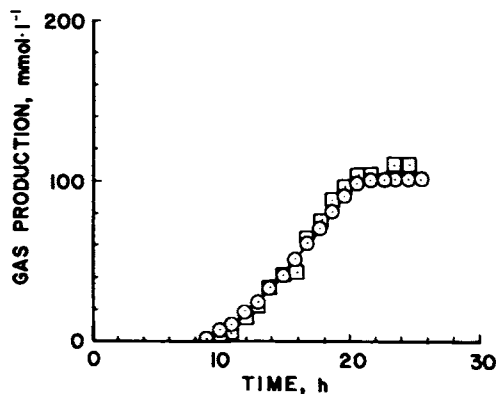


Figure 7. Comparison of calculated ( $\square$ ) to measured ( $\odot$ )  $\text{H}_2$  amounts ( $\text{mmol/L}$  fermentation broth) produced in batch fermentation 1 (Table II).

### Validity of Fermentation Equation (Gateway Sensors)

We had argued<sup>1</sup> that the pseudo-steady-state hypothesis can be applied to the four species corresponding to equations (2)–(5), which would thus be valid under transient conditions, in addition to being valid for steady-state or completed fermentations. Comparisons between the measured and calculated values for  $\text{CO}_2$ ,  $\text{H}_2$ , and glucose are shown in Figures 6, 7, and 8, respectively. The calculations employ the experimental values corresponding to equations (10)–(16). The comparison between the measured and calculated values is very good to excellent, and thus the fermentation equation proves valid also under transient conditions. The agreement is surprisingly good in view of the fact that incorporation of components of the yeast extract into biomass and products renders the balances of carbon, nitrogen, and available electrons less applicable. In conclusion, the fermentation equation is a useful tool for monitoring fermentations on complex media, which are typically employed in industrial fermentations, and not only on totally synthetic media, which are less practical from an industrial standpoint. These calculations of the concentrations or amounts

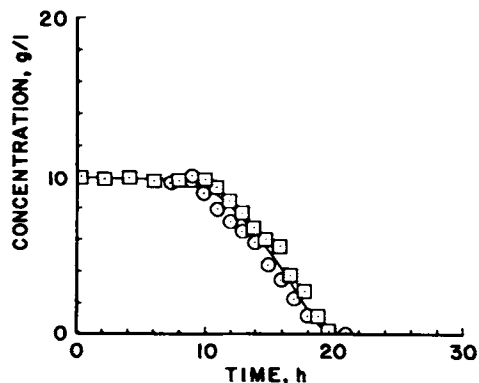
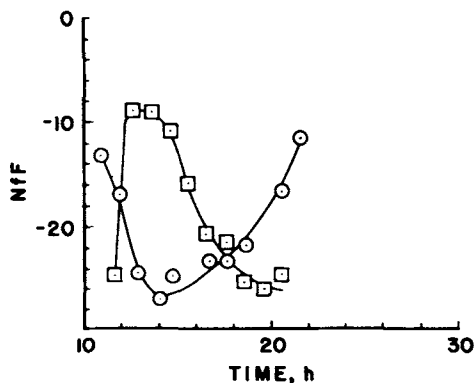


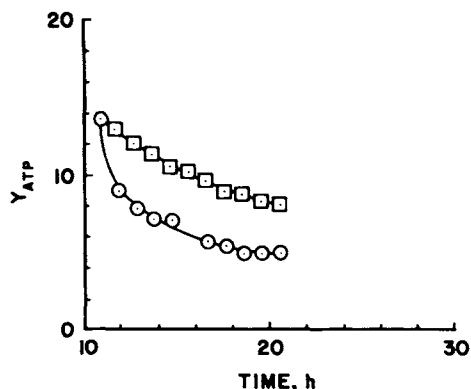
Figure 8. Comparison of calculated ( $\square$ ) to measured ( $\odot$ ) glucose concentrations in batch fermentation 1 (Table II). Equations (10)–(16) employed in calculations.

of  $\text{CO}_2$ ,  $\text{H}_2$ , and glucose can be viewed as gateway sensors<sup>1,4</sup> for the three fermentation parameters. For the calculation of the gas molar rates or amounts, it was necessary to correct the calculated gas production for the effect of liquid sampling (for off-line GC analysis and  $\text{OD}_{600}$  measurement) on the reactor volume. If this correction is not made, significant variation between calculated and measured gas production is observed.

The fermentation equation can also be used to calculate unobservable fermentation parameters, such as the values of  $\text{NfF}$ ,  $Y_{\text{ATP}}$ ,  $\text{ATP}_{\text{tot}}$ , and  $\text{ATP}_{\text{ex}}$ , and of the various extents or rates of key intracellular reactions.<sup>1</sup> For example,  $a$  and  $da/dt$  represent the extent and rate, respectively, of the reaction from pyruvate to  $\text{AcCoA}$ ;  $2f - g$  and  $d/dt(2f - g)$  represent the extent and rate, respectively, of the reaction from  $\text{AcCoA}$  to butyryl-CoA via crotonyl-CoA.<sup>1</sup> Here we present the use of the fermentation equation as a gateway sensor for  $\text{NfF}$ ,  $Y_{\text{ATP}}$ , and  $\text{ATP}_{\text{ex}}$  as shown in Figures 9, 10, and 11, respectively. The experimental values corresponding to equation (10)–(16) were also used for these calculations. Note that  $\text{NfF}$  represents cumulative, normalized values, that is, it represents the total amount of  $\text{NADH}_2$  produced from the oxidation of  $\text{FdH}_2/100$  mol glucose fermented up to a given fermentation time. The instantaneous  $\text{NfF}$  values can be measured from the slopes of the curves in Figure 9. For the pH 3.7 fermentation (Figures 3–8, Table II),  $\text{NfF}$  decreases in the early part of the fermentation, where only acids are produced, and increases thereafter when solvents are produced. In this latter phase the instantaneous  $\text{NfF}$  values are positive, but the cumulative  $\text{NfF}$  values remain negative. For the pH 4.0 fermentation (where only acids are produced, Table II),  $\text{NfF}$  shows an early increase and then it continuously decreases, remaining negative at all times. Fermentations with significant solvent production show a totally distinct  $\text{NfF}$  behavior, which leads to positive cumulative  $\text{NfF}$  values.<sup>2</sup> The values and slopes of the  $\text{NfF}$  curve, we found, not only adequately characterize the nature of the fermentation (acid versus solvent) but also predict the direction of the fermentation toward



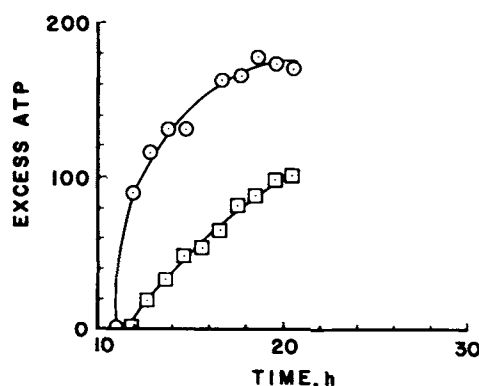
**Figure 9.** Time profiles of  $\text{NfF}$  (mol  $\text{NAD}$  reduced by  $\text{FdH}_2/100$  mol glucose fermented) for batch fermentations 1 (○) and 2 (□) (Table II).



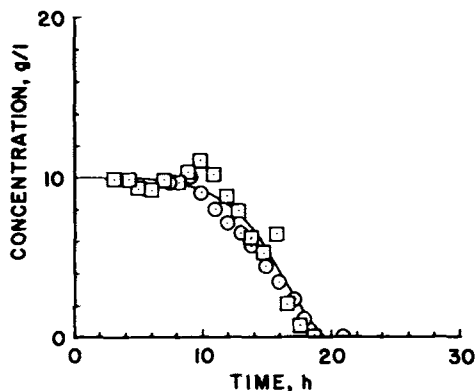
**Figure 10.** Time profiles of  $Y_{\text{ATP}}$  for batch fermentations 1 (○) and 2 (□) (Table II).

acid or solvent production. We are currently exploring its potential as a criterion for bioreactor control. The  $Y_{\text{ATP}}$  and the (normalized)  $\text{ATP}_{\text{ex}}$  shown in Figures 10 and 11 are also unobservable cumulative values that characterize the state of the fermentation in a different way. One observes, for example, that the steepest decrease in  $Y_{\text{ATP}}$  or increase in  $\text{ATP}_{\text{ex}}$  came just before solvent production commenced, which proved valid for all solvent-producing fermentations in our laboratory.<sup>2</sup> As is shown in Figure 10, where the  $Y_{\text{ATP}}$  drops even during the constant  $\mu$  exponential growth phase, and as we have discussed in more detail earlier,<sup>2</sup> the  $Y_{\text{ATP}}$  in all batch cultures we have carried out does not appear to obey the usual dependence on  $\mu$  and a maintenance coefficient.<sup>2</sup> It appears justified then to ignore the maintenance energy requirements in the derivation and use of the fermentation equation<sup>1,2</sup> in batch cultures.

Although various combinations of experimental values can be used to predict three observable parameters and the unobservable ones, as was discussed above, not all of these combinations give equally good results. This is because the errors in experimental values propagate differently in the calculated values, depending on the combination of the experimental values used. For example, if the values of equations (11)–(16) and (25) are used, the predicted glucose values (Fig. 12),

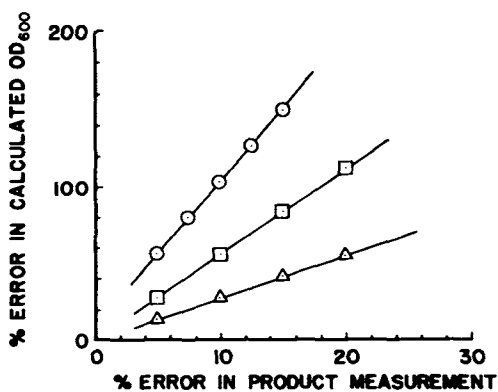


**Figure 11.** Time profiles of  $\text{ATP}_{\text{ex}}$  (mol/100 mol glucose fermented) for batch fermentations 1 (○) and 2 (□) (Table II).



**Figure 12.** Comparison of calculated (□) to measured (○) glucose concentration in batch fermentation 1 (Table II). Equations (11)–(16) and (25) employed in calculations.

although still reasonable, are not as good as those of Figure 8. If the same combination is used to predict the biomass concentration, the results are very poor. This is because small errors in the employed values amplify enormously in the calculation of  $C_b$ , as is shown in Figure 13, for errors in the acetate and  $H_2$  values and their combination. Better results are obtained if equations (11), (12), and (14)–(16), the glucose value, and either equation (23) or (24) are used to predict the biomass concentration. The results are still not acceptable for process monitoring. We believe that the main reason for the poor results in the prediction of biomass concentration is the incorporation of yeast extract into both products and biomass. Even if the percentage of yeast extract carbon fermented is only 5% of that of glucose, the glucose carbon may be approximately equal to the yeast extract carbon incorporated into biomass since typically only 1–10% of the glucose is going into biomass. Although this apparently does not affect the use of experimental data for predicting the concentrations of products and glucose, it has an almost catastrophic effect on the prediction of biomass concentration. We are currently working to develop a semiempirical method for cal-



**Figure 13.** Effect of experimental product concentration error on calculated  $OD_{600}$  value. (△) Positive error in acetate measurement, (□) negative error in  $H_2$  measurement, (○) positive error in acetate and negative error in  $H_2$  measurements.

culating biomass concentration from the glucose,  $CO_2$ , and liquid product data. Finally, for the combination of equations (11)–(16) and the value of  $C_{CO_2}$ , the problem has no solution (a singular matrix is obtained<sup>15</sup>), and thus this combination cannot be used for calculations.

## 7. DISCUSSION AND CONCLUSIONS

We have shown that the system of Figure 1 that we have designed and employed gives a good performance for on-line monitoring of complex fermentations. This system represents only a first attempt, and significant improvements on its performance can be achieved as was discussed in the results section. The system provides a complete analysis of the gas and liquid phases for fermentation products. If it is combined with either an on-line glucose analyzer<sup>18</sup> or an on-line  $OD_{600}$  measurement, and by employing the fermentation equation (1), a complete description of the observable parameters of the fermentation can be achieved. Because of the slowly evolving fermentation processes, the currently presented frequency of one sample per hour was acceptable but perhaps barely adequate. We have discussed that the present system can achieve frequencies of up to 2 samples per hour. In an industrial environment, if two chromatographs are used, one for gas analysis and the other for liquid analysis, and each with two ovens and two columns and detectors, a frequency of up to 24 gas samples and 6 liquid samples per hour can be easily achieved. If the two analyses are uncoupled, the gas analysis can be carried out in less than 5 min and the liquid analysis in less than 20 min. Such sampling frequencies will be higher than necessary for all practical fermentations. Further reduction in analysis time and equipment cost may yet be achieved in the near future with the wider use of the ultrafast capillary GC combined with the fast-evolving miniaturization technology. There already exist commercially available miniature GC modules based on the so-called silicon micromachining technology<sup>20</sup> (Microsensor Technology, Inc.), which are cost-effective and can perform single-component analyses in less than 1 min.

The system of Figure 1 can be immediately expanded to include HPLC analysis of the liquid sample for sugar substrates or nonvolatile products (amino acids, peptides, high-MW acids, alcohols, etc.). The liquid filtrate stream simply passes through the liquid sampling valve of the high-performance liquid chromatograph. The currently available (e.g., Varian Instrument Group) ultrafast HPLC technology can complete the analysis of up to five sugars in less than 5 min. The HPLC data output can be handled by the same CDS. The combination of GC and HPLC provides the most general and most versatile analytical tool for fermentation technology. It may also become the most widely used one in the near future.

Fermentation equation (1) is a very useful tool for the estimation of the state of the fermentation. It can be used to check the data consistency and to calculate both observable and unobservable fermentation parameters. Generalization of the procedure for its derivation and derivation of equations for other fermentations have been discussed by Papoutsakis and Meyer.<sup>15,21</sup>

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## NOMENCLATURE

$a, b, c, d, e, f, g,$ $h, j, k, l$	stoichiometric coefficients or unknowns, in eqs. (1)–(5) and (10)–(16)
AcCoA	acetyl-CoA
ATP <sub>ex</sub>	mol excess ATP/100 mol glucose fermented
ATP <sub>ex</sub> *	mol excess ATP/unit volume fermentation broth, in eq. (19)
ATP <sub>tot</sub>	mol total ATP produced/100 mol glucose fermented
ATP <sub>tot</sub> *	mol total ATP produced/unit volume fermentation broth, in eq. (18)
CDS	chromatography data system
$C_{\text{Ac}}, C_{\text{Ac}}, C_{\text{Ac}}, C_{\text{Ac}},$ $C_b$	molar concentrations of acetate, acetoin, acetone, and biomass in eqs. (13), (15), (12), and (10), respectively
$C_{\text{Bt}}, C_{\text{Bt}}, C_{\text{Glu}}$	molar concentrations of butanol, butyrate, and glucose in eqs. (14), (16), and (17), respectively
$C_{\text{Glu, in}}$	initial $C_{\text{Glu}}$ , in eq. (17)
$C_{\text{CO}_2}, C_{\text{H}_2}$	mol $\text{CO}_2$ and $\text{H}_2$ produced/unit volume fermentation broth in eqs. (24) and (23), respectively
$C_{\text{CO}_2, i}$	$C_{\text{CO}_2}$ at datum point $i$
$F$	total molar gas flowrate in eq. (6)
$F_0$	initial $F$ , in eq. (6)
$F_k$	$F$ at the $k$ th datum point, in eq. (8)
FdH <sub>2</sub>	reduced form of ferredoxin
$L$	length of spectrophotometric cell (cm), in eq. (9)
(MW) <sub>b</sub>	molecular weight of biomass ( $\text{C}_4\text{H}_9\text{O}_4\text{N}_{4q}$ ), in eq. (9)
NfF	mol NAD reduced by FdH <sub>2</sub> (via the NADH–ferredoxin oxidoreductase) per 100 mol glucose fermented
NfF*	mol NAD reduced by FdH <sub>2</sub> (via the NADH–ferredoxin oxidoreductase) per unit volume fermentation broth

OD <sub>600</sub>	optical density of biomass at 600 nm
$V$	volume of fermentation broth (L or mL)
$V_i$	$V$ at datum point $i$ , in eq. (8)
$x$	mole percent of nitrogen in effluent gas, in eq. (6)
$x_{\text{in}}$	initial $x$
$y$	mole fraction of $\text{CO}_2$ in effluent gas, in eq. (7)
$y_k$	$y$ at datum point $k$ , in eq. (8)
$Y_{\text{ATP}}$	ATP yield (g biomass/mol ATP utilized in biomass synthesis)
$Y_{\text{ATP}}^c$	actual constant $Y_{\text{ATP}}$ of a fermentation, in eq. (1)
$Y_s$	biomass yield (g biomass/g glucose fermented)
$\epsilon$	extinction coefficient (L/g cm), in eq. (9)
$\mu$	specific growth rate ( $\text{h}^{-1}$ )

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