On-line Growth Measurements in Bioreactors by Titrating Metabolic Proton Exchange

J. J. Lønsmann Iversen, Jens K. Thomsen and Raymond P. Cox

Institute of Biochemistry, Odense University, Campusvej 55, DK 5230 Odense M, Denmark.

Summary. Recording the amount of titrant required to maintain constant pH in a bioreactor where cell metabolism causes acidity changes allows on-line determinations of growth kinetics in computer-controlled batch cultures. A system for making such measurements is described and its performance is investigated. Transient bicarbonate accumulation occurs if the culture produces carbon dioxide at high pH values and low gas transfer rates. We have developed a mathematical model for the titrant requirement as a function of the cell growth rate, the gas transfer properties of the bioreactor and the culture pH. According to this model, bicarbonate accumulation affects the stoichiometry between titrant and biomass but does not prevent determination of growth rate constants. These predictions are confirmed using model experiments and measurements during batch growth of microbial cultures.

Introduction

Measurement of biomass is a perennial requirement during studies of microbial growth in bioreactors. During balanced growth there is a linear relationship between biomass concentration and the rate of change of the concentration of substrates or products, leading to the possibility of indirect growth measurements. The two metabolites most often monitored in bioreactors are O_2 and H^+. The difference between the measured dissolved O_2 and that in equilibrium with the gas phase can be used to measure rates of respiration as long as the k_La remains constant; unfortunately this is not always the case. The amount of acid or basic titrant required to maintain constant pH over a given time interval is a measure of the amount of active biomass, and recording titrant addition therefore provides a simple and obvious method for indirect growth measurements.

This approach has been known for many years. For example Kempe et al. (1956) monitored growth of *Lactobacillus delbrueckiae* by following the consumption of alkali required to neutralise the lactic acid formed. An automatic pH control system was used and titrant consumption was followed continuously by recording the level in the reservoir on a kymograph. Later applications of the same principle were reported by Veres et al. (1981) and by San and Stephanopolous (1984). The use of pH measurements for feedback regulation of medium addition in internally-limited continuous cultures is well established (Fraleigh et al. 1989); in this case the growth medium itself acts as the titrant in a "pH-auxostat" (Martin and Hempfling 1986). However, in spite of its simplicity and general applicability, the information obtainable from titrant measurements does not seem to be widely used to obtain information about growth kinetics in batch culture. We describe here the aproach which we have developed to allow accurate registration of titrant addition during computer-controlled batch cultures and present theoretical and experimental results about the effect of the conversion of CO_2 to bicarbonate on the process.

Materials and methods

Bioreactor. Growth and model experiments were made using a 3.0 litre BTS 0.5 bioreactor (Applikon, Schiedam, The Netherlands) with a working volume of 2.2 l. The bioreactor was equipped with a galvanometric oxygen electrode (Mackereth 1964) constructed in our workshops and a GK4103C autoclavable glass electrode (Radiometer, Copenhagen, Denmark) for measurements of pH.

Titration system. The titration system is shown schematically in Fig. 1. Acidic or basic titrant (typically 0.5 M HCl or 0.5 M NaOH) were added from burettes through silicon rubber tubing (2 mm i.d., 1 mm wall) via a type 101F peristaltic pump (Watson-Marlow, Falmouth, UK) controlled by the computer. A pulse lasting 1.0 s was given when the measured pH exceeded a preset value (acid addition) or

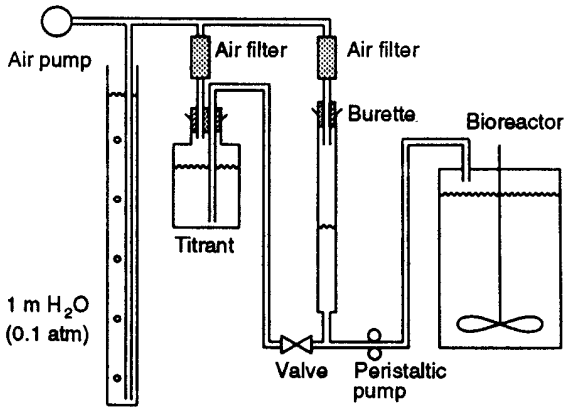

Fig. 1. Schematic diagram of the setup used for titration.

fell below a second preset value (base addition). The bandwidth between the two values was set to 0.15 pH units to prevent sequential additions of acid and base being triggered by noise. The titrant was held under a positive pressure of about 0.1 atm using an aquarium air pump and a water column; this prevented the formation of air bubbles in the tubing. The mean volume delivered per pulse was determined by reading the volumes in the burettes at the beginning and end of each experiment.

Computer system. The bioreactor was controlled using an IBM-compatible PC running a specially-written program. The concentration of dissolved O_2, the culture pH, and the number of pulses of acidic and basic titrant added since the beginning of the experiment, were stored on disc at 10 second intervals. To avoid uncertainty about the starting points of exponential curves, parameter values were usually estimated from Guggenheim plots of log $(x_t - x_{(t-\delta t)})$ versus t, where δt is a suitable time interval.

Other Methods. Escherichia coli strain B was grown on a mineral salts medium (Harrison and Pirt 1967). *Thiosphaera pantotropha* was cultivated as described previously (Thomsen et al. 1993) Biomass was routinely measured by culture turbidity at 600 nm after dilution to the linear range. Acetate (Thomsen et al. 1993) and nitrate (Thomsen and Cox 1990) were determined by HPLC.

Mathematica™ (Wolfram Software, Champaign IL) was used for the algebraic solution of the mathematical model.

Results

Mathematical models

Exponential growth without bicarbonate accumulation. During exponential balanced growth, the increase in biomass X is given by:

$$dX/dt = \mu X \tag{1}$$

The rate of proton production or uptake is given by:

$$dH/dt = Y_{H/X}\,\mu X \tag{2}$$

where $Y_{H/X}$ is a stoichiometric constant. The amount of proton production from time t_0 up to time t is given by:

$$H(t) = H_0 \exp(\mu t) \tag{3}$$

where $H_0 = Y_{H/X}\,X_0$.

Exponential growth with bicarbonate accumulation. There is a pH-dependent equilibrium between dissolved CO_2 and bicarbonate:

$$CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^- \Leftrightarrow 2\,H^+ + CO_3^{2-} \tag{4}$$

with pK_a values of 6.3 and 10.3 at 25°C. The number of protons (n) generated per molecule CO_2 converted to bicarbonate or carbonate depends on the pH:

$$n = \frac{2 \times 10^{2pH} + 10^{(pH + pK_{a2})}}{10^{2pH} + 10^{(pH + pK_{a2})} + 10^{(pK_{a1} + pK_{a2})}} \tag{5}$$

The rate of loss of inorganic carbon from a bioreactor depends on the gas transfer constant k_La and the fraction (f) of the total inorganic carbon present as CO_2:

$$f = \frac{10^{(pK_{a1} + pK_{a2})}}{10^{2pH} + 10^{(pH + pK_{a2})} + 10^{(pK_{a1} + pK_{a2})}} \tag{6}$$

The rate of change in acidity connected with growth, and hence the amount of titrant required to maintain constant pH within a given time interval, depends on the balance between production due directly to metabolism, production linked to the ionisation of metabolically produced CO_2, and the loss of inorganic carbon to the gas phase. The process can be described by a set of differential equations:

$$\frac{dH}{dt} = Y_{H/X}\frac{dX}{dt} + n\frac{dC}{dt} \tag{7}$$

$$\frac{dX}{dt} = \mu X \tag{8}$$

$$\frac{dC}{dt} = Y_{C/X}\frac{dX}{dt} - fk_La\,(C - C^*) \tag{9}$$

where $Y_{H/X}$ and $Y_{C/X}$ are stoichiometric constants, C is the concentration of all forms of inorganic carbon, and C^* is the concentration of inorganic carbon in equilibrium with the gas phase (equal to the concentration of CO_2 divided by f from Eqn. 6).

The set of equations 7-9 can be integrated to give

$$H(t) = a_0 + a_1 e^{\mu t} + a_2 e^{-fk_L a t} \tag{10}$$

where

$$a_0 = H_0 - n\,(C_0 - C^*) - Y_{H/X}\,X_0$$

$$a_1 = Y_{H/X}\,X_0 + \left(\frac{\mu}{fk_La + \mu}\right) n\,Y_{C/X}\,X_0$$

$$a_2 = n\,(C_0 - C^*) - \left(\frac{\mu}{fk_La + \mu}\right) n\,Y_{C/X}\,X_0$$

The solution shows that the kinetics of titrant addition depend on the sum of two terms; one increasing exponentially at a rate depending on the growth rate constant μ and one decreasing exponentially at a rate depending on the product of k_La and the fraction of inorganic carbon present as CO_2. In cases where the second term decreases rapidly or remains constant, the expression for $H(t)$ can be simplified to a constant plus a term depending on the exponential growth rate of the cells.

Fig. 2 shows the effect of the ratio between k_La at pH 7.0 and μ on the ratio between the integrated proton production $H(t)$ and biomass increase $(X-X_0)$ as a function of time. A ratio of 0 corresponds to a closed system and 1000 corresponds to very rapid gas exchange compared to growth, as for a well-aerated bioreactor. The family of curves varies between 2 extremes with different stoichiometries between titrant and biomass. One extreme corresponds to the situation where all the CO_2 remains in the system (predominantly as bicarbonate), while in the other case bicarbonate does not accumulate. With high or low k_La values, the ratio between titrant and biomass increase

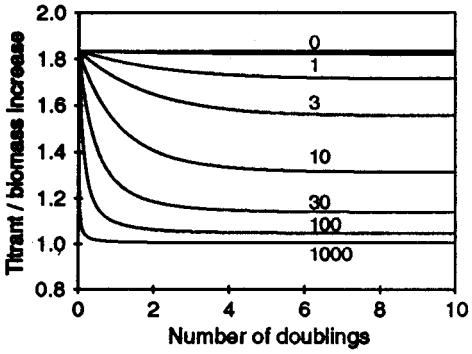

Fig. 2. Time-dependence of the calculated ratios between total amount of titrant required according to Eqn. 10 and the increase in biomass ($X(t)-X_0$). The curves correspond to different k_La/μ ratios at pH=7.0. The parameter values used were $C^*=0$ and $Y_{HX} = Y_{OX} = 1$. The initial values were $X_0 = 1$ and $H_0 = C_0 = 0$,

remains constant throughout the growth period. At intermediate k_La values the ratio attains a stable value after a transient lasting a few generations, introducing a potential source of error in the use of titration data to monitor growth under these conditions. Aiba and Furuse (1990) reached analogous conclusions from a theoretical analysis of the effect of the pH-dependent accumulation of inorganic carbon on the accuracy of measurements of the ratio of O_2 uptake to CO_2 production from analysis of the compositions of the exit gases. The errors involved can in principle be corrected for by using Eqn. 10 to fit the whole time course, possibly after making an independent estimate of fk_La.

Non-exponential growth. Under conditions such as oxygen limitation, growth will not be exponential. If biomass increase is linear the stoichiometry between the amount of biomass and the rate of proton exchange involves both growth-dependent and growth-independent components. The expected change in titrant consumption with time has the form $H(t) = a + bt + ct^2 + d\exp(-kt)$. Under these conditions there is no straightforward relationship between titrant consumption and biomass.

Model experiments

In order to test the reliability of the titration system we carried out model experiments in which metabolic proton production was simulated by the addition of concentrated acid to a bioreactor containing a buffered medium. Using a computer to control a peristaltic pump it was possible to obtain an exponential increase in the amount of acid added. Fig. 3 shows that under good aeration conditions and at neutral pH, the system maintained the medium pH within narrow limits and that the addition of basic titrant followed the kinetics of the programmed acidification. The time course of titrant addition was fitted to an exponential function using the Simplex algorithm (Nelder and Mead 1965). The best fit value for the time constant was 1.37 h^{-1} compared to the expected value of 1.33 h^{-1}. The residuals between the experimental and best fit curves are shown in Fig. 3 on an expanded scale.

In 12 experiments of this type the ratio between the total amount of added acid and the total amount of basic titrant

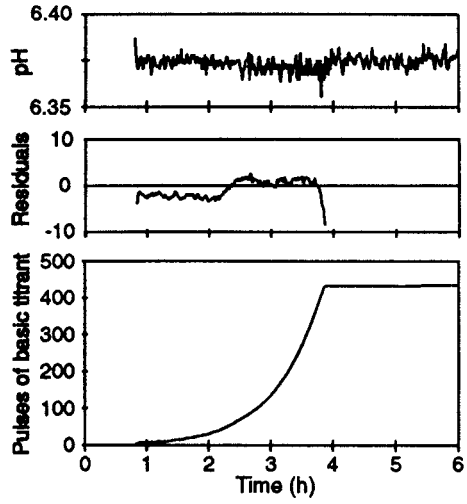

Fig. 3. Titration response of the pH control system with set pH of 6.3 in response to an exponentially increasing concentration of acid. The bioreactor contained growth medium without carbon source, containing 56 mM phosphate buffer, and was aerated through the sparger. The gradient was started by adding an initial volume of acid (18 pulses) and the remaining 982 pulses were added over 3 hours with a rate constant of 1.33 h^{-1}. The center panel shows the residuals between the number of pulses of basic titrant and the best fit of an exponential function to the time-course.

required was 1.00 ± 0.01 (S.D). The mean volume delivered per pulse for the 12 experiments was 108.3 µl; the standard deviation of the mean value for each experiment was 0.9 µl.

Fig. 4 shows the results of a similar model experiment under conditions designed to promote the accumulation of bicarbonate. Only headspace aeration was used and the

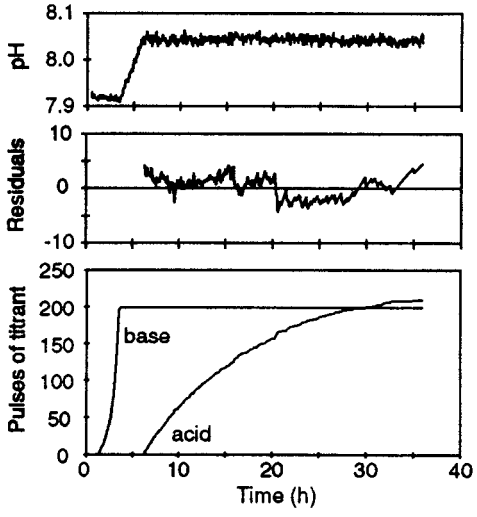

Fig. 4. Titration response of the pH control system with set pH of 8.0 in response to exponentially increasing concentrations of HCl and $NaHCO_3$ added through separate inlets. Headspace aeration was used. 0.75 M $NaHCO_3$ and 0.5 M HCl were added as described in the legend to Fig. 3. Titrant acid was 0.24 M HCl and titrant base was 0.25M NaOH. The center panel shows the residuals between the amount of acid titrant added and the best fit of an exponential function to the time-course.

titration system was set to maintain the pH at 8.0 where the equilibrium ratio of HCO_3^- to CO_2 is about 50. Solutions of acid and bicarbonate were pumped into the bioreactor through separate inlets at an exponentially increasing rate. The figure shows the expected consumption of basic titrant to neutralise the acid and the subsequent slow alkalinisation as CO_2 is lost.

Growth experiments

Fig. 5 shows the results of an experiment in which titrant addition was recorded during batch growth of *E. coli* on a medium containing succinate, ammonium and mineral salts. The utilisation of the acid substrate caused alkalisation of the medium which was compensated by the addition of acidic titrant. The kinetics of titrant addition were exponential with the same kinetics as the oxygen uptake rate determined from the dissolved oxygen concentration.

Neutral carbon sources such as carbohydrates or alcohols are metabolised to CO_2 without permanent changes in pH. However, the uptake of either nitrate or ammonium as nitrogen sources will be followed by uptake or loss of protons from the medium. Fig. 6 shows the results obtained during a batch growth experiment in which *E. coli* was grown on a medium containing a mixture of glucose and xylose at pH 7.0. This is a substrate mixture known to give diauxie in batch culture (Monod 1942; Standing et al. 1972). The acidification observed during growth results mainly from the uptake and incorporation of ammonium. Comparison of the traces for titrant addition and that for dissolved O_2 allows 4 phases to be distinguished. The first phase involves exponential growth at the expense of the preferred substrate, glucose. In the second phase respiration stops briefly (shown as a transient return of the O_2 trace towards the equilibrium value) and then restarts in a process which does not cause titrant addition, although transient pH changes are observed within the bandwidth of the titration system. The third phase involves growth at the expense of xylose with ammonium incorporation and proton release. A final phase involves proton uptake corresponding to the consumption of some accumulated acidic metabolite. These results demonstrate the complementary nature of O_2 uptake and titrant consumption measurements.

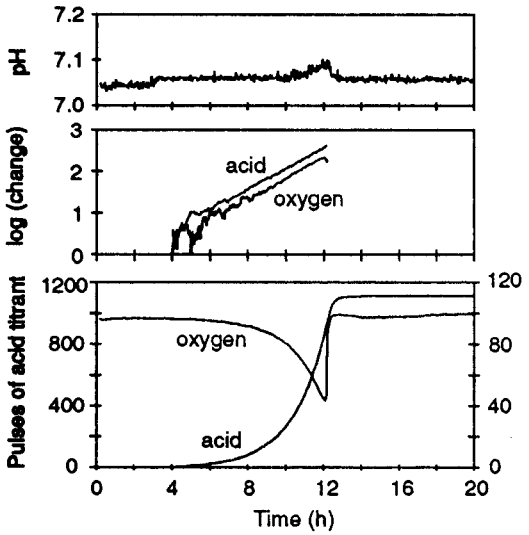

Fig. 5. Titrant consumption and changes in dissolved oxygen during batch culture of *E. coli* on a succinate-mineral salts medium with a set pH of 7.0. Sparger aeration was used. The center panel shows Guggenheim Plots of the changes in titrant and dissolved O_2 over the preceding 1.0 h.

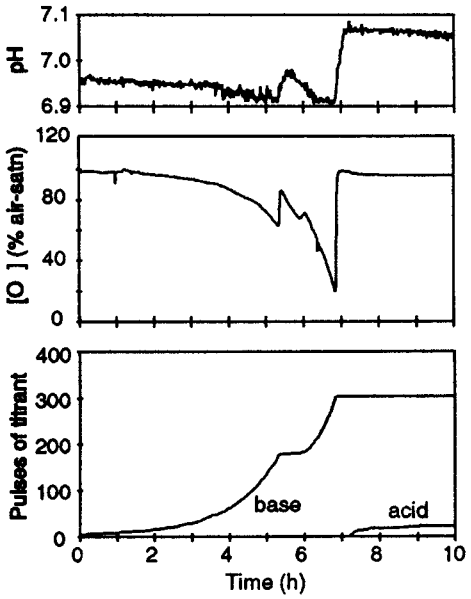

Fig. 6. Titrant consumption and changes in dissolved oxygen during batch culture of *E. coli* on a growth medium containing glucose (2.5 g l^{-1}), xylose (2.5 g l^{-1}), and mineral salts. Sparger aeration was used and the pH was set to 7.0.

Fig. 7 shows the relationship between biomass measured as culture turbidity and titrant consumption for *E. coli* during batch growth on a variety of substrates. The linear relationship observed conforms the validity of using titration data to follow growth. Growth on acetate or lactate gives, as expected, a higher titrant demand than growth with sugars plus ammonium where the acid production is solely dependent on the amount of nitrogen incorporated.

Fig. 8 shows the results of a growth experiment under conditions which cause transient bicarbonate accumulation. The denitrifying bacterium *Thiosphaera pantotropha* was cultivated at pH 8.0 on acetate with nitrate as oxidant under a gas phase of N_2. The uptake of the acidic carbon source

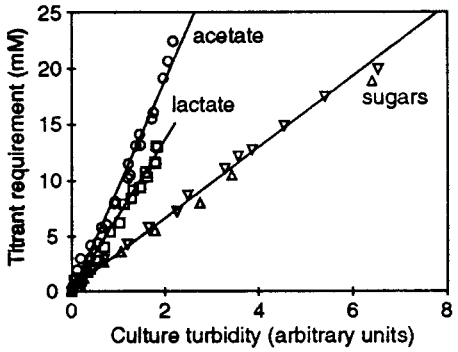

Fig. 7. Relation between biomass increase and titrant consumption for *E. coli* grown on a variety of substrates. Biomass was measured by turbidimetry using samples removed from the bioreactor. O, 2.5 g l^{-1} sodium acetate (acidic titrant). □, 2.5 g l^{-1} sodium lactate (acidic titrant), Δ, 2.5 g l^{-1} glucose + 2.5 g l^{-1} xylose (basic titrant). ∇, 2.5 g l^{-1} glucose + 2.5 g l^{-1} galactose (basic titrant).

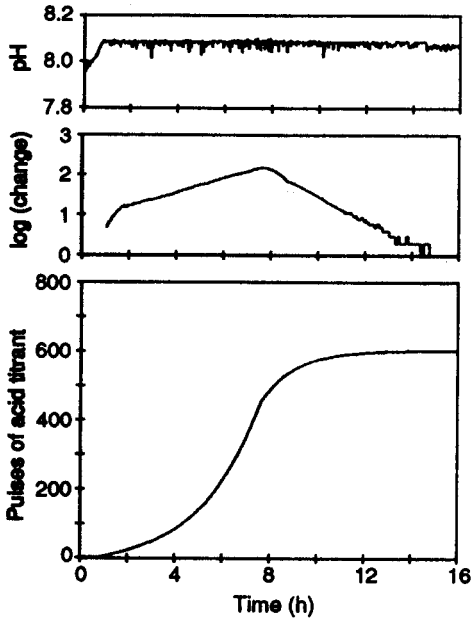

Fig. 8. Titrant consumption during denitrifying batch culture of *Thiosphaera pantotropha* at pH 8.0. The bioreactor was configured with a single impeller and no baffles. Impeller speed was 1000 rev min^{-1} with sparger aeration. The center panel shows a Guggenheim Plot of the amount of titrant added during the the preceding 1.0 h.

and nitrate are both expected to cause medium alkalisation and acid addition to maintain constant pH. In this experiment ment where there was a low rate of exchange between the growth medium and the gas phase, the transient accumulation of bicarbonate decreases the extent of the expected alkalisation during the growth period. Titrant addition shows two phases; an increasing rate corresponding to the period of growth and a decreasing rate which depends on the rate of gas exchange over the liquid-gas interface. The end of the first phase was shown to correspond to the exhaustion of acetate (results not shown).

Fig. 9 shows the correlation between titrant consumption, biomass and the consumption of acetate and nitrate, confirming the conclusion from our mathematical model that the kinetics of titrant addition follow growth and metabolism even during bicarbonate accumulation.

Discussion

Proton exchange as a result of metabolism can be the consequence of several types of process. The consumption of acidic carbon sources like acetate or succinate, the production of acidic fermentation products like lactate, or the utilisation of proteins or amino acids as carbon and energy sources following deaminination, involve major changes in culture pH. Even if the carbon source is neutral, as is the case with carbohydrates, uptake of inorganic nitrogen sources like ammonium or nitrate will cause a smaller growth dependent proton exchange which is perfectly adequate for following growth kinetics. Thus the only type of growth mode which cannot be readily monitored by titration involves the use of nitrogen sources such as dinitrogen or neutral amino acids together with a neutral carbon source; even in this case it may be possible to obtain pH changes due to transient bicarbonate accumulation.

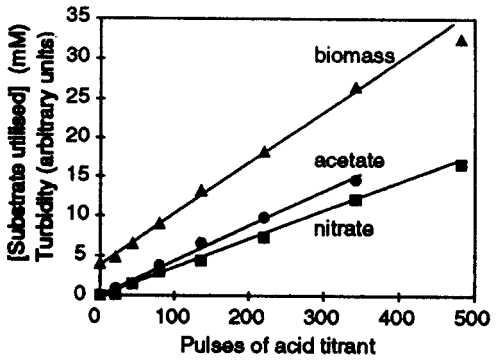

Fig. 9. Relationship between titrant consumption and substrate utilisation or culture turbidity, for the experiment shown in Fig. 8.

A major advantage of this approach to biomass determination is that no extra apparatus is usually necessary since most bioreactors are provided with equipment for pH control. The measurements depend on the amount of active biomass in the whole bioreactor, and the method is thus equally suitable for homogenous bacterial suspensions, for mycelial pellets or biofilms. A limitation is that the measurements are indirect and a conversion factor is needed to relate titrant to a measure such as cell dry weight or cell carbon; however, this information is easily obtained.

The question arises as to why such a generally applicable approach to bioreactor monitoring is not more commonly employed. One possible reason is uncertainty about the validity of assuming a correlation between biomass and titrant; our results show that although this question will need to be investigated for each new set of experimental conditions, there is likely to be a satisfactory result. Another factor limiting wider adoption may be the requirement for the use of a suitable computer-controlled system to allow titrant additions to be registered; even if a computer is already used the control program must either already be able to make the necessary calculations or be capable of modification; the latter is easy with programs written by the user but may be more of a problem with commercial software.

Our results demonstrate the possibility of making accurate measurements of titrant addition and using these to monitor microbial growth. We have demonstrated that transient accumulation of bicarbonate is not usually a major source of error. Our conclusion is that this approach to indirect biomass monitoring deserves to be more widely used.

Nomenclature

C_t	concentration of all forms of inorganic carbon
C^*	concentration of all forms of inorganic carbon in a liquid in equilibrium with the gas phase
f	fraction of total inorganic carbon as dissolved CO_2
H	proton or titrant concentration
k_La	gas liquid transfer coefficient for CO_2
n	number of protons produced for each CO_2 generated
X	biomass
Y_{CX}	stoichiometric coefficient: CO_2 produced / biomass formed
Y_{HX}	stoichiometric coefficient: protons produced / biomass formed
μ	growth rate constant

Acknowledgments.
We acknowledge the invaluable contribution of Lars Teil Nielsen in writing the computer program used to control the bioreactor.

References

Aiba S, Furuse H (1990) Some comments on respiratory quotient (RQ) determination from the analysis of exit gas from a fermentor. Biotechnol. Bioeng. 36: 534-538

Fraleigh SP, Bungay HR, Clesceri LS (1989) Continuous culture, feedback control and auxostats. Trends Biotechnol. 7: 159-164

Harrison DEF, Pirt SJ (1967) The influence of dissolved oxygen concentrations on the respiration and glucose metabolism of *Klebsiella aerogenes* during growth. J. Gen. Microbiol. 46: 193-211

Kempe LL, Gillies RA, West RE (1956) Acid production by homofermentative bacteria at controlled pH as a tool for studying the unit process of fermentation. Appl. Microbiol. 4:175-178

Mackereth FJH (1964) An improved galvanic cell for determination of oxygen concentrations in fluids. J. Sci. Instrum. 41:38-41

Martin GA, Hempfling WP (1976) A method for the regulation of microbial population density during continuous culture at high growth rates. Arch. Microbiol 107: 41-47

Monod J (1942) Recherches sur la croissance des cultures bacteriènnes. Hermann et Cie, Paris.

Nelder JA, Mead R (1965). A simplex method for function minimalization. Computer. J. 7: 308-313

San K-Y, Stephanopolous G (1984) Studies on on-line bioreactor identification. IV. Utilization of pH measurements for product identification. Biotechnol. Bioeng. 26: 1209-1218

Standing CN, Frederickson AG, Tsuchiya HM (1972) Batch and continuous culture transients for two-substrate systems. Appl. Microbiol. 23:354-359

Thomsen JK, Cox RP (1990) Alkanesulphonates as eluents for the determination of nitrate and nitrite by ion chromatography with direct UV detection. J. Chromatograph. 521: 53-61.

Thomsen JK, Iversen JJL, Cox, RP (1993) Interactions between respiration and denitrification during growth of *Thiosphaera pantotropha* in continuous culture. FEMS Microbiol. Lett. 110: 319-324

Veres A, Nyeste L, Kurucz I, Kirchknopf L, Szigeti L, Holló J (1981) Automated fermentation equipment. I. Program-controlled fermentor. Biotechnol. Bioeng. 23: 391-404