0273-2289/90/2425—0591$02.40

Biomass Estimation Using On-Line Glucose Monitoring by Flow Injection Analysis

Application to *Candida Rugosa* Batch Growth

F. VALERO, J. LAFUENTE,* M. POCH, AND C. SOLÀ

Unitat d'Enginyeria Quimica, Universitat Autònoma de Barcelona, Spain

ABSTRACT

A new Flow Injection Analysis (FIA) system was developed that permits the on-line monitoring of glucose. This information may be used to identify the biomass concentration. The proposed manifold allows the automatic analysis of glucose from 0 to 90 g/L. The results are in agreement with the analyses provided by HPLC. The system was applied to on-line monitoring of substrate in several *Candida rugosa* batches grow without detecting mechanical or biochemical problems. This information was linked to a Extended Kalman Filter (EKF) recursive estimation scheme to identify the vector state of the system.

Index Entries: Flow Injection Analysis; on-line glucose analysis; biomass identification; *Candida rugosa*.

INTRODUCTION

Because of the characteristics of the bioprocesses, and in order to improve the quality of the products and to optimize productivity, it is important to have efficient control systems.

The control systems may be achieved (*1*) in the following ways:

1. Through a closed control loop by measuring the parameters to be controlled (biomass, products and so on);

*Author to whom all correspondence and reprint requests should be addressed.

2. By estimating these variables from measured process variables, using a mathematical model; and
3. By controlling the cell environment wthin the fermenter.

Although the first is the best option, it is not always applicable, because it is difficult to know all the variable values that define the state of the system. The third approach is to control one or more process variables, usually from oxygen uptake rate, pH, and temperature. In this case it is very difficult to be sure that a satisfactory control is achieved in terms of these process variables. For these reasons, important efforts are being made to develop methods of monitoring the characteristic process variables as well as to develop and apply identification methods.

Although the number of in-line sensors is increasing, they still present maintenance difficulties that other kinds of analytical devices (i.e., based on continuous sampling followed by on-line automatic analysis) do not exhibit. One of the most promising techniques for this type of analysis is the Flow Injection Analysis (FIA) system (*2*). FIA characteristics allow the use of this technique to determine the concentration of a given component in an extensive range of concentrations. This is one of the most important problems in the on-line analysis of glucose. Currently, when glucose concentration in the fermentation being studied is high, it is necessary to dilute the sample. Similar procedures should be followed when there are important variations in the concentration level, as in the batch fermentations (*3*).

Despite these efforts in developing new analysis methods, it is important to recognize that not all variables needed for the control of the process can be measured on-line. To overcome this problem, several digital filters have been applied. One of the most used in nonlinear systems is the Extended Kalman Filter (EKF), which has been used for identification of biomass growth and fermentation systems (*4–8*).

The utilization of the Kalman Filter assumes that the model and the initial values of its parameters are known. Also, a certain knowledge of the variability of the system expressed as a noise matrix is assumed. Because of the high complexity of biological systems, the strategy behind this approach is to work with as simple a model as possible and to rely on the filter, in order to adapt the model parameter values to changes in the behavior and so maintain the system controlled. One of the difficulties associated with this method is the definition of the noise matrices, poor estimations of which may produce the divergence of the method (*9*). These drawbacks have led to the proposal of other algorithms that use adaptive techniques to guarantee the stability of the method (*10*).

This paper shows the results obtained when identifying the biomass evolution by using only the glucose concentration measure. This study has used a *Candida rugosa* batch growth fermentation. The glucose concentration has been tracked using a FIA technique with stream splitting. The stream splitter allows the generation of different manifolds, one of them showing a linear interval of 0–90 g/L of glucose, without resorting to

sample dilutions. An EKF has been used for the identification stage. In spite of the drawbacks mentioned above, it allows the tracking of the evolution of all vector state parameters during the fermentation.

MATERIALS AND METHODS

Microorganisms and Medium

Candida rugosa (ATCC 14830) was maintained on malt extract, peptone, and agar plates. The fermentation medium had the following composition (*11*): glucose, 20 g/L; KH_2PO_4 6 g/L; urea, 1 g/L, $MgSO_4 \cdot 7H_2O$, 1 g/L; micronutrients: $FeCl_3 \cdot 6H_2O$, 10 mg/L; inositol, 0.004 mg/L; biotin, 0.008 mg/L; thiamine·HCl, 0.2 mg/L.

Growth Conditions

The yeast was grown in a 6-L Braun fermenter Biostat E. The standard operating conditions were stirring rate, 500 rpm; temperature, 30°C; non-controlled pH; and air flow rate between 0 and 15 L/min in order to ensure that the value of the dissolved oxygen inside the culture broth was not less than 20% of the saturation value.

Sampling Device

The broth was extracted through a peristaltic pump, followed by microfiltration unit (Millipore, Minitan™, Barcelona, Spain) with two membranes of 0.45μm pore size (PK). This permitted a biomass free flow. The retentate and the filtrate not used for the glucose analysis were returned to the fermenter.

Sterilization Conditions

The medium was sterilized in the fermenter, except vitamins and $FeCl_3 \cdot 6H_2O$, which were microfiltered. The sampling device and the recirculation circuit (filter and membranes inclusive) were chemically sterilized by the following sequence: 10 min, 0.1*M* NaOH; 5 min, 30% ethanol; 10 min 0.1*M* HCl followed by cleaning with distilled water until neutral pH was obtained. All the recirculation and sampling connections of the system were sterilized in an autoclave.

Glucose Analyzers

Glucose was analyzed by FIA and HPLC techniques. The FIA tubes were 0.8-mm-diameter PTFE tubes (Tekator AB, Höganäs, Sweden) joined by PVC fittings (home made). Solution pumping was done by peristaltic pump (Gilson Minipuls-3). An Eyela peristaltic pump was used to aspire the solution through the loop of the injection valve (microtube pump

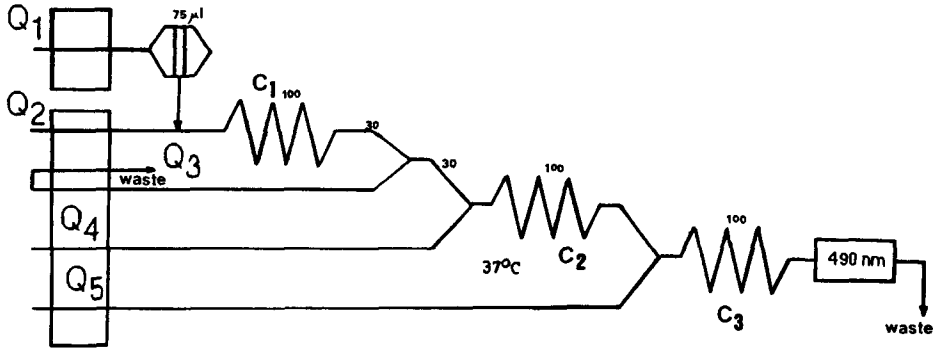

Fig. 1. Set-up of the FIA system with stream splitter: Tube dimensions: length in cm, diameter = 0.8 mm. Q_1: sample stream, Q_4: enzymes stream, Q_5: phenol stream, $Q_1 = Q_4 = Q_5 = 1$ mL/min. Q_2: carrier stream. Q_3: stream splitter (*12*).

MP-3). Samples were introduced by a four-way rotating injection valve (Hamilton) coupled with a remote control motor (Sirtek, Barcelona). The absorbance measurements were made at 490 nm with a Hellma flux cell of 18 µL at a controlled temperature of 37°C.

The glucose determination is based on the following enzymatic reactions:

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 + 2O_2$$

$$2H_2O_2 + \text{4-Aminophenazone} + \text{phenol} \xrightarrow{\text{POD}}$$

$$\text{Monoimino-}p\text{-benzoquinone-4-phenazone} + 4H_2O$$

The concentration of reagents were: GOD (glucoseoxidase), 1200 U/L, equivalent to 0.052 g/L (Boehringer, Barcelona, Spain); POD (peroxidase), 1250 U/L, equivalent to 0.0043 g/L (Boehringer); 4-aminophenazone, 0.3049 g/L (Fluka, Barcelona, Spain); phenol, 0.0093*M* (Merck, Barcelona, Spain); and KH_2PO_4/Na_2HPO_4 buffer, pH 7.

The manifold shown in Fig. 1 has been used (*12*). The sample, Q_1 (75 µ L), is introduced into the system through the injection valve into the carrier stream (Q_2) flowing through a 1-m coil, C_1. At the end of the coil, there is a splitter. The value of the Q_3 depends on the desired linear range of glucose. The other flow reacts with the corresponding reagents in coils C_2 and C_3. The analyses of glucose by HPLC were made using a Bio Rad Aminex HPX 87-H column and water as eluent at 0.6 mL/min flow rate.

Biomass Analysis

Biomass was determined by dry weight: 5 mL of a grown culture was passed through a glass fiber filter (pore size 0.65–0.45 µm) that was dried overnight and weighted.

Mathematical Method (EKF)

The EKF is an extension to nonlinear ordinary differential equations of the Kalman linear approach (*13*). The EKF method tries to estimate optimally the process state of the system by assuming that the behavior of the system is described by the model, and that the mean and the covariances of the measurement errors, inputs, and outputs are known. The nonlinear model may be expressed in a general form by

$$dx/dt = f(x,t) + e(t) \tag{1}$$

where x is an augmented state vector (set of dependent variables that adequately describes the situation in the reactor at any time-point that includes the parameters of the model. The function $e(t)$ represents the random disturbances of the system. The observations are related to the system by

$$y = h(x) + E(t) \tag{2}$$

where $E(t)$ represents the noise of the measurements and $h(x)$ is the measurement matrix.

It was assumed that the system and the measurement noises were independent random gaussian white noise with zero mean and covariance matrices **Q** and **S**. The EKF method works in two steps:

1. A prediction stage between observations in which the uncertainty in the estimates increases with time; the state vector and the variance are calculated by

$$\hat{x}(t/t_{k-1}) = f(\hat{x},t) \tag{3}$$

$$\mathbf{P}(t/t_{k-1}) = f_x(\hat{x})\mathbf{P} + \mathbf{P}f_x^T(\hat{x}) + \mathbf{Q} \tag{4}$$

   where **P** is the variance matrix.

2. A correction step that takes place when observations occur. The estimate is corrected by a term that is proportional to the difference between the actual measurement and the prediction.

$$\hat{x}(t_k/t_k) = \hat{x}(t_k/t_{k-1}) + K(t_k)\,[y(t_k) - h(\hat{x}(t_k/t_{k-1}),t_k)] \tag{5}$$

$$\mathbf{P}(t_k/t_k) = \mathbf{P}(t_k/t_{k-1}) - \mathrm{K}(t_k)\,\hat{h}_x\,\mathbf{P}(t_k/t_{k-1}) \tag{6}$$

$$\mathrm{K}(t_k) = \mathrm{P}(t_k/t_{k-1})\,\hat{h}_x^T[\hat{h}_x\,\mathrm{P}(t_k/t_{k-1})\,\hat{h}_x^T + \mathrm{S}]^{-1} \tag{7}$$

   where k is the gain matrix.

RESULTS

Analysis System Description

In order to have an efficient system for automatic analysis, it is necessary to consider three critical issues: 1) The sampling device should be

Table 1
FIA Manifold Specifications for Different Stream-Splitters

Q_2 mL/min	Q_3 mL/min	Linear range, g/L	Regression coefficient	P. height = A(glucose) + B		Detection limit, g/L	Standard deviation, %
				A	B		
4.1	2.93	30	0.9996	0.450	0.227	0.3	3.1
4.1	3.15	50	0.9993	0.384	0.247	0.4	3.5
4.1	3.45	70	0.9997	0.303	0.149	0.6	3.5
4.1	3.70	90	0.9992	0.172	0.004	0.8	3.8

able to get a sample flow without biomass. 2) An analysis method should work independently and provide results in short time periods. 3) A management system should be able to automate the process.

1. To tackle this problem, equipment with membranes is normally used. This type of equipment has several problems: membrane plugging, dead volumes, sample volume required, and sterile conditions at the sample outlet. The system worked properly in several fermentations (*12*). The microfiltration equipment did not present pressure increases. Later observations have confirmed the absence of plugging. Microscope observations and Petri plate inoculations have not shown contamination. Because of recirculation through the filtration equipment, the microorganism behavior has not changed.
2. In order to work in different conditions with the manifold, it is only necessary to change the flow rate leaving the system (*see* Table 1). The system works properly in a linear range from 0 to 90 g/L. The detection limit is 0.8 g/L with a standard deviation of 3.8%.
3. Management system. An efficient system should work in an autonomous control fashion during the whole fermentation. This has been achieved with the incorporation of an automatic valve that, depending on the value of the electric signal it receives, fills up the injection loop or recirculates the flow. The other components were moved through relays connected to an analog-digital converter (HP-3497-A). The microfiltration equipment worked continuously during the experiments to keep the filter unplugged. The analytic equipment was connected following the algorithm shown in Fig. 2. The maximum sampling frequency achieved with this system was 30 samples/h, although for this particular fermentation, 10 samples/h was considered enough to monitor the glucose concentration.

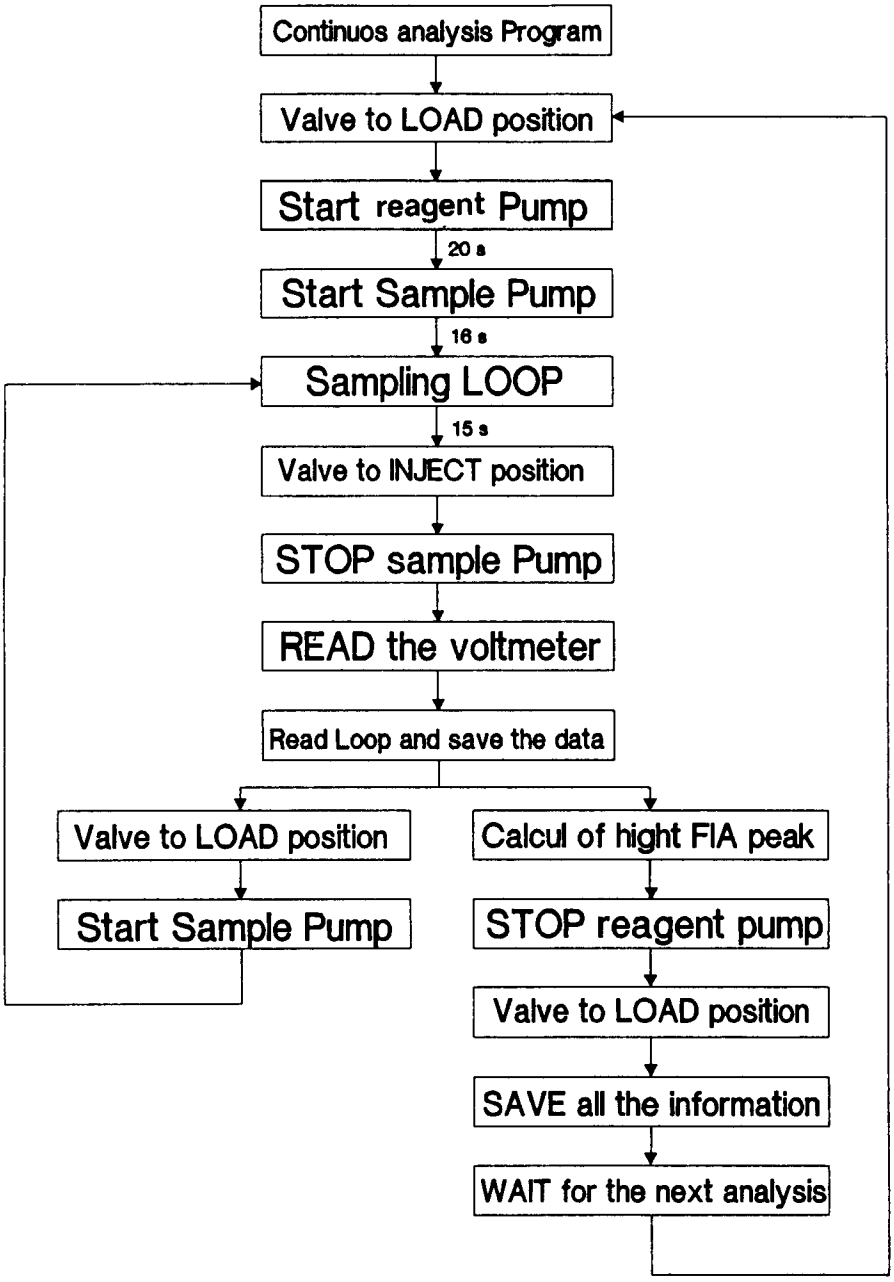

Fig. 2. Actuation and control algorithm for the automatic glucose analysis.

Fermentation Model

The fermentation selected for the present work is a system that was previously studied by the authors (*14*). Data from previous fermentations, where the state variables were analyzed off-line, have been made available to us. The fermentation model and the kinetic parameters have been obtained from this data.

We have used an unstructured model that considers as variables only the substrate and the biomass. The dissolved oxygen balance is not considered, because it was kept constant by the control system. The model is a Monod-like model of the growth rate, with an inhibition term proportional to the biomass concentration. The equations are the following:

$$dX/dt = [\mu_{max} S/(K_s + S)] X - K_i X \quad (8)$$

$$dS/dt = -(1/Y)(dX/dt) \quad (9)$$

where

μ_{max}, K_s, and K_i are kinetic parameters

S represents substrate concentration (g/L of glucose)

X represents biomass (g/L)

Y represents yield (g biomass/ g consumed glucose)

The state vector is (**S, X, Y,** μ_{max}, K_s, K_i) and the measure vector is (**S**). The initial values of state vector parameters were obtained from previous experiments. When it was not possible to obtain them directly, they were estimated using optimization techniques. Thus, the value of μ_{max} was obtained from the slope of the growing curve at the maximum growth rate ($\mu_{max} = 0.27\ h^{-1}$). K_s was obtained from the literature (*15*) ($K_s = 0.01$ g/L). The yield value was obtained from the relation between the produced biomass and the consumed glucose ($Y = 0.24$ g biomass/g glucose). The inhibition constant was calculated using an unidirectional optimization algorithm (*16*) ($K_i = 0.17\ h^{11}$).

The covariance matrices of the state and output noise were chosen as:

$$\mathbf{Q} = \begin{matrix} 2.5\text{ E-3} & & & & & \\ & 7.5\text{ E-4} & & & & \\ & & 1.0\text{ E-6} & & & \\ & & & 1.0\text{ E-5} & & \\ & & & & 1.0\text{ E-5} & \\ & & & & & 1.0\text{ E-5} \end{matrix}$$

$$\mathbf{S} = (0.01)$$

The variances of the state noise in matrix **Q** are based on subjective estimates of the average changes of the generalized state variables between two sampling instants. Matrix **Q** has been chosen considering that the main objective of the filter was to identify the biomass evolution. The selection also took into account the fact that an increase in the values of the **Q** elements accelerates the convergence, but also increases the amplitude of the fluctuations, giving instability in the estimation of parameters. The variance in the covariance matrix of the input noise, **S**, was determined on the basis of the standard deviation corresponding to the errors of the instruments employed. The initial covariance matrix of the state **P** was chosen as zero.

Since the proposed model is not valid in the lag period, the recursive method does not work in this period. Aeration is the criterion used to identify the end of the lag period. Initially, the dissolved oxygen values of the fermentation are very close to the saturation point. When the glucose consumption is considerable, the value of the dissolved oxygen decreases rapidly, reaching values below the set-point (20% of the saturation value). At this point the dissolved oxygen controller starts introducing air into the fermenter. The EKF identification process needs the initial values of the substrate and biomass. The substrate initial value is obtained from experimental measures. This biomass initial value is obtained from the biomass concentration in the inoculum. In order to diminish the possible error between the initial biomass and the biomass at the end of the lag stage, the initial biomass value is incremented by the product of the consumed glucose during the lag stage and the yield.

This paper presents the results obtained in three fermentations. In the first, the initial glucose concentration was 23 g/L. The glucose evolutions obtained by on-line analyses (FIA) and obtained off-line (HPLC) present good agreement with a 3% average deviation. This means that there have been no interferences and modifications in the analysis system during the course of the fermentation. Figure 3A shows how the system can properly estimate the biomass behavior without even modifying the initial parameters' values. This indicates that there has been no variations along the evolution, and that there are not big differences between the use of the recursive method and a simulation using the initial parameters' values.

In the second fermentation, the initial glucose concentration was close to the one used in the first fermentation (25 g/L), but as is shown in Fig. 3B, between 23 and 25 h a change in the glucose consumption is observed. In this case a mere reconstruction using the equations of the system would not permit the biomass tracking. The change in the glucose consumption produces a modification in the model parameters. During the fermentation, the value of μ_{max} and K_i were modified by EKF not more than 15% of their initial values, the values of K_s and Y remaining constants. After a short adaptation period, the EKF reconstructs the system. In the final stage of the fermentation, the parameters converge again to values close to the initial ones.

The third fermentation was done with an initial glucose concentration of 19 g/L, which is lower than previous ones. The general behavior of the biomass and the glucose is different from the behavior observed in the previous experiments; *see* Figure 3C. Because of this, the identification method needs to make larger modifications of the parameters' values during a bigger interval. In this case, the algorithm changes the same parameters that were changed in experiment number 2 (K_i, μ_{max}) and again keeps K_s and yield constant. The biomass was described correctly, as in the previous experiments. It is not guaranteed that the modifications of the parameters in each fermentation are caused only by the system's

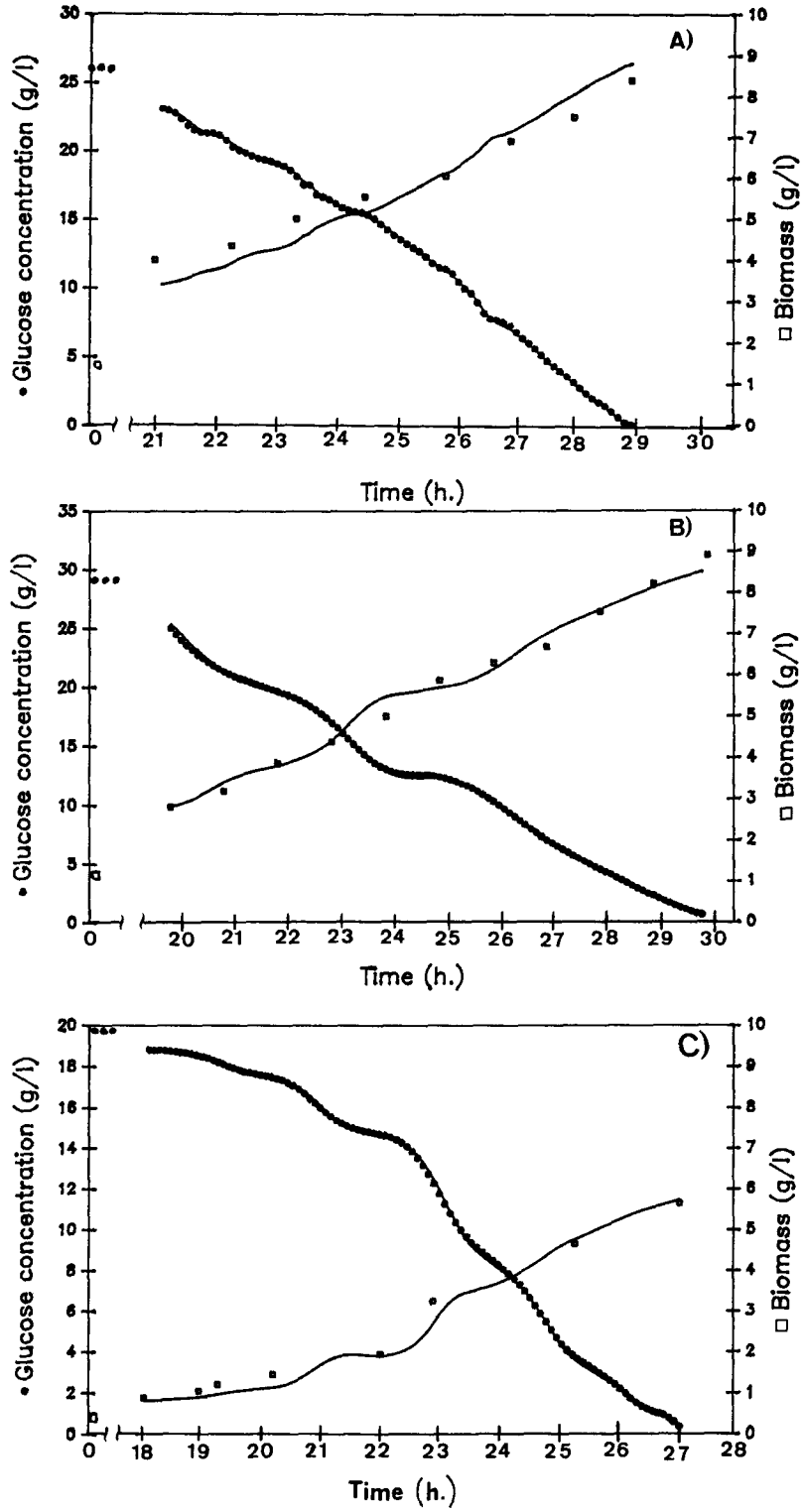

Fig. 3. Experiment results corresponding to (A) an initial glucose concentration of 23 g/L. (B) an initial glucose concentration of 25 g/L. (C) an initial glucose concentration of 19 g/L. In the X-S/t plot, the points correspond to the experimental data, and the continuous line is the filter estimation.

alterations. For example, it should be stressed that the K_s value remains constant along the fermentation, and it is only in the last stage that the recursive method begins to modify it. This fact is in agreement with the observed parameter changes, in the sense that its influence is visible only in a very short interval.

CONCLUSIONS

The methodology and the tools mentioned above made possible the development of an on-line glucose analysis system. Also, they allow the tracking of the biomass behavior in systems where it is difficult to analyze biomass (e.g., systems that present agglomeration, low growth rates, biomass growing on particles).

ACKNOWLEDGMENTS

This work has been sponsored by the Commission of the European Communities in the framework of the Biotechnology Action Program (BAP), and by the Spanish Biotechnology Program under contract CICYT Bio 135/88. We also acknowledge partial support of a NATO collaborative research grant.

REFERENCES

1. Williams, D. (1987), *Process Biochem.* **xx,** 49.
2. Ruzicka, J. and Hansen, E. H. (1988), *Flow Injection Analysis,* 2nd ed., Wiley, New York.
3. Gibson, T. D. and Woodward, J. R. (1988), *Anal. Chim. Acta* **213,** 61.
4. Stephanopoulos, G. and San, K-Y. (1984), *Biotechnol. Bioeng.* **26,** 1176–1188.
5. Caminal, G., Lafuente, J., López-Santín, J, Poch, M., and Solà, C. (1987), *Biotechnol. Bioeng.* **29,** 366.
6. Nahlik, J. and Burianec, Z. (1988), *Appl. Microbiol. Biotechnol.* **28,** 128.
7. Takamatsu, T., Shoya, S., Yokoyama, K., Kurome, Y., and Morisaki, K. (1981), *Proc. 8th IFAC World Congress, Kyoto,* **22,** 146.
8. Bellgardt, K. H., Meyer, H. D., Kuhlmann, W., Schugerl, K., and Thoma, M. (1984), *3rd. Europ. Cong. Biotech. Verlag Chemie,* Weinheim, **2,** 607.
9. Cheruy, A. (1988), Resultats et perspectives en Automatique, Hermès Ed. Paris.
10. Dochain, D. and Bastin, G. (1984), *Automatica.* **20,** 621.
11. Ota,Y., Miyairi, S., and Yamada, K. (1968), *Agric.Biol. Chem.* **32,** 1476–1478.
12. F. Valero et al. (1989), *Biotechnol. Bioeng.*
13. Kalman, R. E. and Bucy, R. S. (1961), *Trans. ASME J. Basic Eng.* **108,** 95.
14. Valero, F., Ayats, F., López-Santín, J., and Poch, M. (1988), *Biotechnol. Lett.* **10,** 741.

15. Adkisson, B. and Mavituna, F. (1983), Biochemical Engineering and Biotechnology Handbook, Nature Press, New York.
16. Himmelblau, D. M. (1972), *Applied Nonlinear Programming*, McGraw-Hill.