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A LACTOSE FIA-BIOSENSOR SYSTEM FOR MONITORING AND PROCESS CONTROL

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Abstract - In this work an enzymatic lactose biosensor composed of the immobilized enzymes β-galactosidase and glucose oxidase was developed. Oxygen consumption during the reaction catalyzed by these enzymes was detected. The biosensor was integrated into an FIA (flow injection analysis) system that allows measurement of lactose on-line in less than three minutes. This biosensor was used to monitor lactose concentration during the production of β-galactosidase by the yeast *Kluyveromyces marxianus* from cheese whey. The sensor showed good stability after four months and after almost 7000 measurements had been performed. The analytical curve was linear in the range of lactose concentration from 1 to 30 g/L.
Keywords: lactose, biosensor, flow injection analysis, *Kluyveromyces marxianus.*

INTRODUCTION

Culture media are complex systems in which the activity and the state of the biological components are extremely sensitive to changes in the bioreactor's physico-chemical environment. It's necessary to monitor on-line the process variables if an appropriate description of the state of the process is desired. Off-line analyses are not able to carry out that task due to the time delay of some laboratory analyses. Therefore, several biosensors, both integrated and not integrated into FIA (flow injection analysis) systems, have been used. These analytical devices incorporate a biological material (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, etc.) that is closely associated with or integrated within a physicochemical transducer or transducing microsystem, which may be optical, electrochemical, thermometric, piezoelectric or magnetic. They have applications in medicine, environmental diagnostics and in the food and processing industries.

Biosensor characteristics are very attractive: sensitivity, reliability, simplicity and speed. Glucose (Rocha and Ferreira, 2002; Liu and Li, 2000; Liu et al., 1998; Švitel et al., 1998; Folly et al., 1996), sucrose (Salgado et al., 1998; Weigel et al., 1996), ethanol (Salgado, 2001), glycerol (Compagnone et al., 1998), penicillin-G (Hitzmann et al., 1998) and the pathogenic bacterium Salmonella (Pathirana et al., 2000) were previously measured with biosensors.

FIA is a technique that is well suited to monitoring bioprocesses, especially when biosensors are used, because they are easily integrated into sample systems. The advantages of FIA are (1) system components can be substituted during the process without the risk contaminating the medium ; (2) the system can be recalibrated at any time without interference in the process; and (3) several measurements can be done with the same sample,

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when different sensors are working together.

In this work a biosensor is used to monitor lactose concentration during the production of the enzyme lactase. This enzyme is the product of the cultivation of *Kluyveromyces marxianus* yeast using lactose from cheese whey.

Whey is a byproduct of cheese manufacturing and due to the high content of lactose and proteins it can be useful in a variety of ways, such as, as a substrate for enzyme production, concentration of proteins, dairy drinks, biotransformation of lactose into glucose and galactose, ethanol and others. The treatment of cheese whey effluents is difficult due to high BOD (biochemical oxygen demand) that varies from 30,000 to 60,000 mg O_2/L. In Brazil, most of the cheese whey is set aside for animal food, so studies involving other applications for this byproduct must be encouraged.

Usually, to improve process operations in all possible applications of lactose, a fast and efficient way to measure lactose concentration is required. For this purpose, some lactose biosensors can be found in the literature. In Amárita et al. (1997) a hybrid biosensor, in which the biological materials were *Saccharomyces cerevisiae* and β-galactosidase, had a high response time, 1 hour. Jäger and Bilitewski (1994) presented an enzymatic electrode based on the technology of compact film commonly referred to as the screen-printed electrode. With this method the lactose can be determined in the range of concentrations from 0 to 1.6 g/L, which can be considered very low for most of the economic applications. Adányi et al. (1999) developed sensors to measure lactose content in milk and other dairy products. Three sensors with different combinations of biological material were tested: (A) β-galactosidase and galactose oxidase, (B) β-galactosidase and glucose oxidase and (C) β-galactosidase, galactose oxidase and glucose oxidase. They were integrated into an FIA system. The enzymes were immobilized in membranes of natural protein and an amperometric electrode measured the hydrogen peroxide produced in the enzymatic reactions. The ranges of the measurements for systems A, B and C were 0.3 – 1.7, 0.7 – 3.4 and 0.3 – 1.7 g/L of lactose and more than 600, 1000 and 800 samples could be measured, respectively. A microdialysis-coupled flow injection amperometric Sensor (muFIAS) was used by Rajendran and Lrudayaraj (2002) to determine glucose, galactose and lactose in milk. The sensor is based on an enzyme-catalyzed reaction in combination with the three well-established analytical techniques, namely microdialysis sampling, FIA and amperometric detection. With the multianalysis sensor it was possible to detect glucose and galactose by sequential injection of their corresponding oxidase enzymes, glucose oxidase and galactose oxidase, while lactose was determined by injection of a mixture of β-galactosidase and glucose oxidase enzymes. The sensor showed a linear response between 0.05 and 10 mM for glucose, between 0.1 and 20 mM for galactose and between 0.2 and 20 mM for lactose. A biosensor attached to an FIA system was also developed by Vega et al. (1998) for the automatic determination of galactoside conjugates and glycerol. Sensitivity of the biosensor was the highest for galactose, followed by raffinose, lactose and glycerol. The sensor showed a linear response between 0.2 and 2 mM for galactose, 0.5 and 6 mM for raffinose, 25 and 250 mM for lactose and 2 and 200 mM for glycerol. In Weigel et al. (1996) glucose, maltose, sucrose, lactose, xylose, sorbose, galactose, fructose and gluconolactone were analyzed by means of immobilized pyranose oxidase as well as by the combination of immobilized glucose oxidase with immobilized glycoamylase, invertase, mutarotase, maltase (α-glucosidase) and glucose isomerase by FIA. For the simultaneous analysis of glucose and other sugars three different flow-injection configurations were applied and compared. The range of measurements for lactose was from 1 to 10 g/L. The average prediction errors in the analyses were less than 3% for the model media and below 6% for the yeast-extract-containing media.

In order to use these biosensors, they must be calibrated. It is shown in Ferreira et al. (2003) that temperature has a strong influence on them, and thus nonlinear models (exponential and neural networks) were used to interpret the biosensor signals.

THE LACTOSE BIOSENSOR AND ITS INTEGRATION INTO THE FIA SYSTEM

The lactose biosensor is composed of two immobilized enzymes that catalyze the following reactions:

$$\text{Lactose} + H_2O \xrightarrow{\beta-\text{galactosidase}} \beta\text{-D-glucose} + \beta\text{-D-Galactose} \quad (1)$$

$$\beta\text{-D-glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2 \quad (2)$$

In agreement with the above equations, both oxygen consumption and peroxide production could be monitored and related to the lactose concentration. It was decided to quantify the consumption of oxygen.

Temperature dependence was studied by Ferreira (2002a) and Ferreira et al. (2002b). In the range from 20 to 30°C, it was observed that an increase in temperature accelerates the reaction of O_2 consumption. It was observed that this happens up to 28°C, where the output signal reaches the maximum; at temperatures higher than that, the signal begins to decrease. Therefore the effects of temperature must be compensated to avoid distortions in measurements. For this reason, a model was used to deal with the effects of temperature on the output signal of the FIA/biosensor system.

Biosensor Construction

Glucose oxidase and β-galactosidase were weighted and dissolved separately in phosphate buffer solution at pH 7 (K_2HPO_4 – 117.74 g/L and KH_2PO_4 – 44.99 g/L). Next, they were carefully transferred to an eppendorf containing the polymeric carrier for covalent bonding (vinyl-epoxy alcohol, 50-200 μm, Riedel-de Haën) and left at room temperature during three days. Finally, the entire mixture was transferred to a plastic cartridge, using phosphate buffer solution at pH 5.8.

Six microreactors (MR) with various amounts of enzymes, were constructed. Their compositions are shown in Table 1. Figure 1 shows the calibration curve for each one of the microreactors. The responses are in volts as the amperometric electrode was coupled to a voltage converter (Wheatstone Bridge). The lowest concentration measured was 1 g/L.

Microreactor 6 had the best linear range and the highest response and was used in all experiments presented in this work.

Glucose Biosensor

A small amount of glucose was detected in the cheese whey, enough to disturb the measurement of the lactose. Figure 2 shows the glucose biosensor response to concentrations from 0.25 to 1.5 g/L. For a glucose concentration of 0.25 g/L, the output signal in volts is almost the same as that for lactose concentrations near 3 g/L, so a glucose biosensor was used together with the lactose biosensor and the glucose concentration was subtracted from the lactose concentration. Figure 3 shows a typical glucose concentration obtained during the fed-batch cultivation.

Table 1: Glucose oxidase (GOxid) and β-galactosidase (β-gal) contents in lactose biosensor.

MR number	Glucose oxidase		β-galactosidase	
	m_{ef}[a]	U_{ef}[b]	m_{ef}	U_{ef}
1	2.35	470	5.9	53.1
2	2	400	11.3	101.7
3	2.3	460	17	153.1
4	2.2	440	23.1	207.9
5	3	600	33.6	302.4
6	3	600	22.3	200.7

[a] mass weight, in mg;
[b] Units of enzyme.

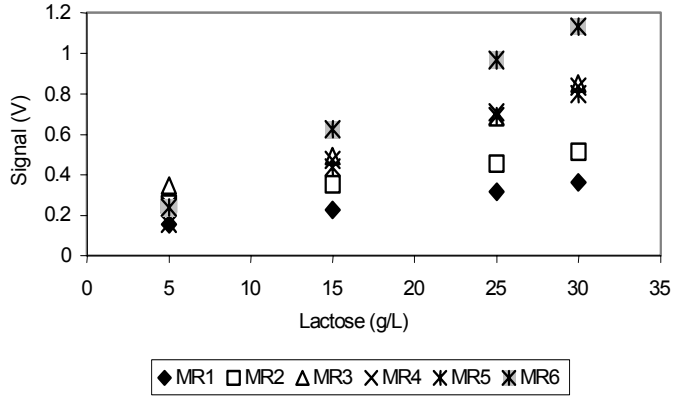

Figure 1: Tests with lactose microreactors.

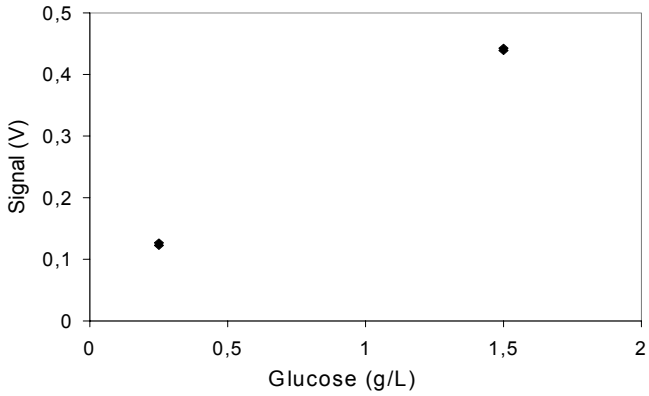

Figure 2: Output signal of glucose biosensor.

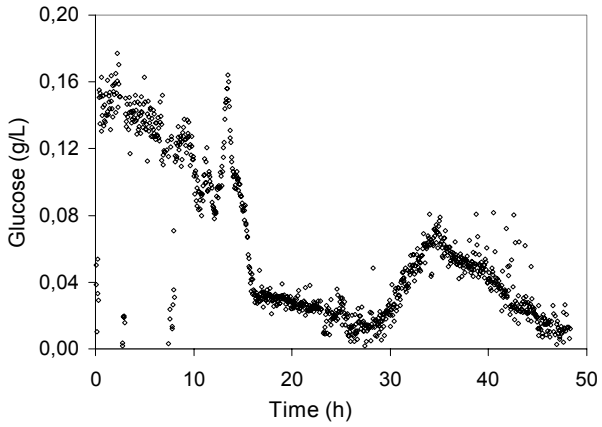

Figure 3: Concentration of glucose during fed-batch cultivation.

FIA System

The FIA/biosensor device is shown in Figure 4.

The system studied is composed of

1) B.Braun Bioreactor (5L);
2) Sterilizable microfiltration membrane (Polypropylene - Eppendorf Sterile In-Line Sampling Probe);
3) Carrier solution (Weigel et al., 1996*)*, phosphate buffer solution at pH 5.8 (K_2HPO_4 – 1.1 g/L, $NaH_2PO_4.2H_2O$ – 5.3 g/L, KCl – 1.8 g/L, EDTA – 1.5 g/L and NaN_3 – 0.065 g/L);
4) Two peristaltic pumps (Flocon 1003) with five channels;
5) Mixer;
6) Selection valve (Knauer) with seven channels;
7) and 8. Injection valves with six channels;
8) Lactose and glucose biosensor;
9) Two oxygen electrodes (Yellow Spring Instruments, 5331) and
10) Two amplifiers (constructed at the Institute for Technical Chemistry, University of Hannover).

The FIA system's capillary tubes, constructed of PTFE polymer, had an internal diameter of 0.8 mm, except for the injection loop (0.5 mm).

CAFCA (Computer-Assisted Flow Control & Analysis – ANASYSCON, Hannover) fully automates the system, controlling the sampling and evaluating FIA output signals. It works in MS-DOS mode using an A/D card (AX5210).

The flow rates were F1 (filter) – 0.55 mL/min, F2 (drain) – 0.77 mL/min, F3 (waste from the biosensor and amplifier) – 1.8 mL/min and F4 (sample line) – 0.67 mL/min. Lactose standard solutions were prepared with the same buffer solution as that of the carrier solution. Chemicals were from Nuclear, Vetec, Merck, QEEL, Grupo Química and Inlab.

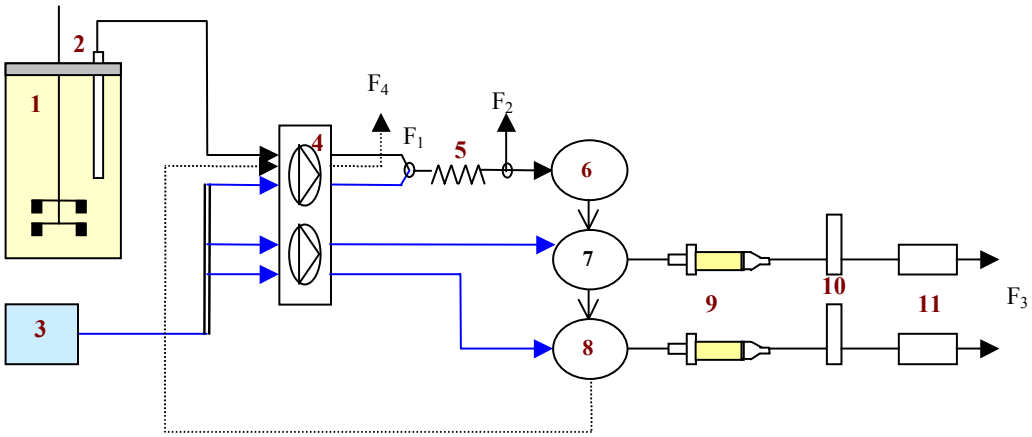

Figure 4: On-line measurement system for lactose.

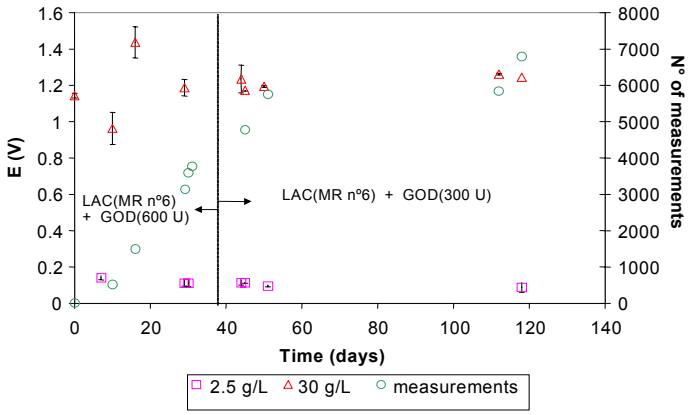

Figure 5: Stability of lactose biosensor.

STABILITY OF THE LACTOSE BIOSENSOR

Figure 5 shows the sensor stability. In addition to measurements for 2.5 and 30 g/L of lactose and their standard deviations, the number of measurements made during this work is also shown.

The figure is divided into two parts; one shows results when a glucose biosensor of 600 units (cartridge A) was used together with the lactose biosensor, and the other shows results when a glucose biosensor of 300 units (cartridge B) was used.

In accordance with Figure 5, the biosensor showed good stability even after 7000 measurements and four months of work. Cartridges filled with the same buffer solution as that used in the FIA system were kept in the refrigerator.

CULTIVATION

Cultivations were conducted in a BIOSTAT B bioreactor (from B. Braun) equipped with a 5L vessel, stirring of 600 rpm and air supply of 8 L/min. Temperature and pH were controlled at 37°C and 5.5.

For precultivation, isolated colonies of yeast were aseptically transferred to an Erlenmeyer flask filled with 60 mL of culture medium. The flask was incubated in a shaker (model NT711/Nova Técnica Equipamentos para Laboratórios, Piracicaba, SP, Brazil) at 200 rpm and 37ºC during 15 to 20 h. The yeast, *Kluyveromyces marxianus* CBS 6556, was from the Biotechnological Development Center, Joinville, SC, Brazil.

The culture medium was composed of cheese whey (7% w/v) from Elegê Laticínios (RS) and yeast extract (0.5% w/v) from Biobrás. The fed medium was cheese whey (28% w/v) and yeast extract (2% w/v).

Cells were measured by absorbance (620 nm) and lactose was also measured off-line by the DNS (dinitrosalicilic acid) method (Chaplin and Kennedy, 1994).

APPLICATION OF THE LACTOSE BIOSENSOR IN CULTIVATIONS

Fed-batch cultivations were done as described below. Initially, a batch was cultivated until lactose consumption was completed. The following fed-batches were started, at a variable time, and the final batch phase was used to consume all the remaining substrate.

The fact that the FIA system continuously removes filtered samples from the bioreactor causes concentration of cells and results in a bioreactor medium with a higher cell concentration . The figures show two sets of cell data, one for the values calculated from the growth curve (X_{at}) and the other (X) deducting the effect of FIA samples, using Equation 3.

$$X = X_{at} \cdot \frac{(V_{in} - F_r \cdot t)}{V_{in}} \qquad (3)$$

where X_{at} is the cell concentration calculated with the growth curve, Vin is the initial volume, Fr is the sampling flow rate and t is the time.

Uncontrolled Fed-Batch Cultivations

In this experiment (Figure 6), the feed rate was constant at 33.5% of the maximum pump flow rate (6.2 mL/min). The total volume of medium fed into the bioreactor was 975 mL. Good agreement between measurement results for the biosensor and the DNS method can be observed.

During the cultivation shown in Figure 6, glucose cartridge A was replaced by glucose cartridge B. This replacement was made because of structural problems with cartridge A – due to the high degree of bed packing, pressure increased causing an opening of the cartridge to occur during cultivation.

Figure 7 shows biosensor recalibrations during the cultivation. Good agreement between them can be observed. Calibration 4 was performed after the problem with the glucose biosensor. It can be seen that the results for this calibration differ slightly from the others. This difference can be attributed to a minimum requirement of time for biosensor adaptation to the new environmental conditions.

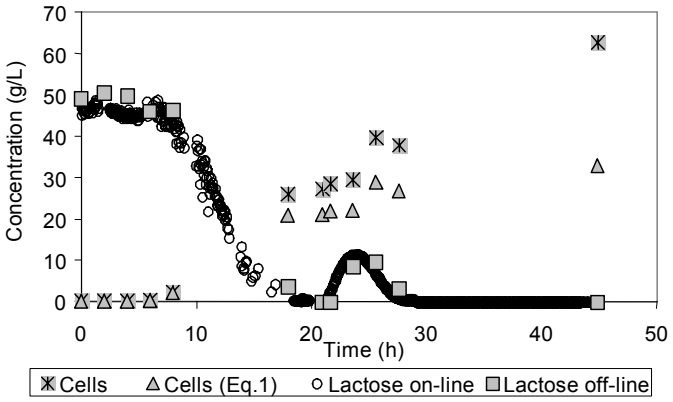

Figure 6: Experimental data for fed-batch cultivation with the following initial conditions: X_0 = 0.2 g/L, S_0 = 49.2 g/L and V_0 = 4L.

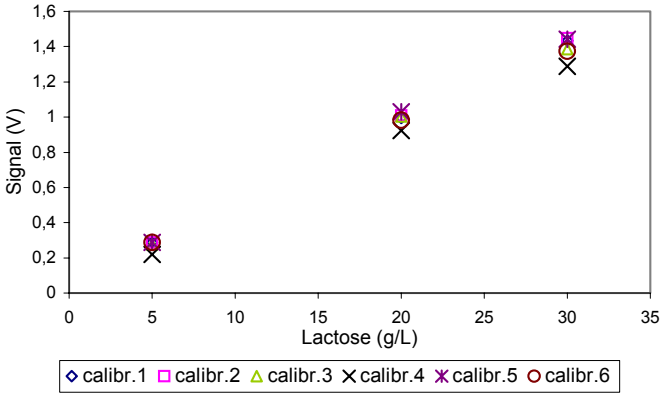

Figure 7: Data on biosensor recalibration during the cultivation.

Controlled Fed-Batch Cultivation

In this experiment lactose concentration was maintained at 2 g/L by manipulating the feed flow rate. That value was chosen because in previous experiments, high lactase activities were achieved with low levels of substrate. A discretized proportional integral (PI) feedback controller (Aström and Hägglund, 1995) was used. Equations (4) and (5) show the PI controller used.

$$P(t_k) = K(y_{sp}(t_k) - y(t_k)) \quad (4)$$

$$I(t_{k+1}) = I(t_k) + \frac{Kh}{\tau_i} e(t_{k+1}) \quad (5)$$

where K is the controller gain, τ_i is the controller-reset time, h is the sampling time and e is the difference between the controlled variable and the set point.

Data for cells and lactose are shown in Figure 8. Lactose measurements, off-line and on-line, during the control phase are detached in Figure 9. The manipulated variable, feed flow rate, is shown in Figure 10.

Control was not very efficient. Two main factors contributed to this behavior: (1) the adjustment of the PI based on a process model (developed for batch cultivations, Secchi et al., 2001) that represented the fed-batch cultivations poorly (results not shown), and (2) sensor noise and faults. These faults mainly occur when air bubbles are removed together with the sample, causing interference with the measurements performed by the oxygen electrode detector. Nevertheless, this is one of the few studies that actually shows results of bioprocess control using a biosensor (see Hitzmann et al., 2000 and Siegwart et al., 1999; for others).

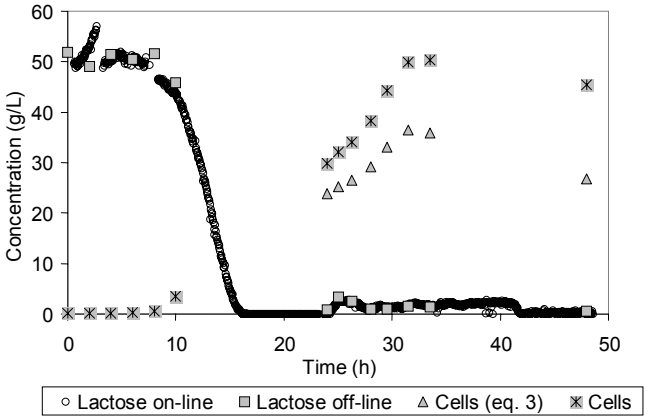

Figure 8: Controlled fed-batch cultivation.

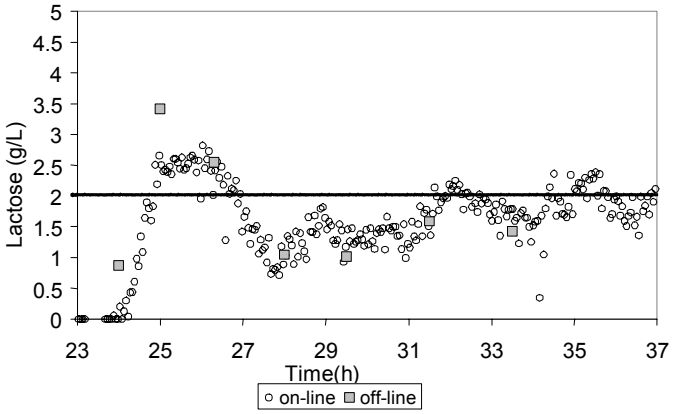

Figure 9: On-line and off-line lactose measurements.

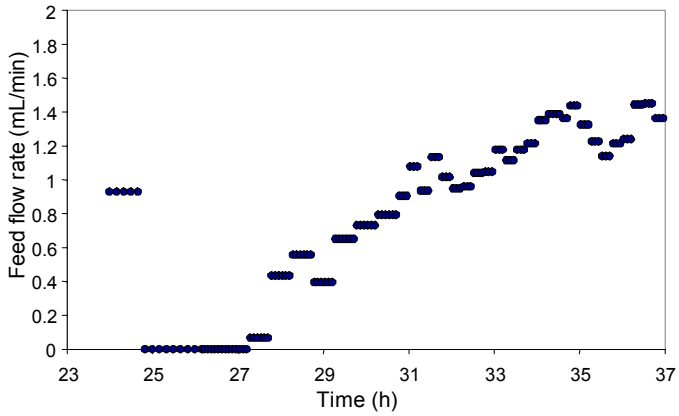

Figure 10: Feed flow rate during the control phase.

CONCLUSIONS

The system studied was shown to be appropriate for measurerement of lactose concentration. Characteristics of linear range and response time are better (for our purposes) than for some other sensors found in the literature (Amárita *et al*., 1997; Jäger and Bilitewski, 1994; Adányi et al., 1999).

Several factors influence the biosensor output signal. Thus, for industrial applications some attention must be paid to (1) verification of the viability of the membrane that recovers the oxygen electrode, (2) automatic recalibrations of the system, (3) preventive methods (e.g., use of filtering membranes) to avoid air bubbles in the system; (4) automatic detection of faults caused by air bubbles, (5) noise filtering and (6) compensation for the effects of temperature or investment in devices to maintain it constant. For fault detection, Kalman filter algorithms or neural networks can be applied to supervise and detect faults (Luo et al., 1999; Liu, 1999; Yu et al., 1999; Xiaoan and Bellgardt, 1995).

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