Modeling and observer design for recombinant Escherichia coli strain

Madiha Nadri, Isabelle Trezzani, Hassan Hammouri, Prasad S. Dhurjati, R. Longin, Joseph Lieto

1
Université de Lyon, Lyon, F-69003, France; Université Lyon 1;
CNRS UMR 5007 LAGEP (Laboratoire d'Automatique et de GEnie des Procédés),
43 bd du 11 novembre, 69100 Villeurbanne, France
Tel +33 (0) 4 72 43 18 45 - Fax +33 (0) 4 72 43 16 99
http://www-lagep.univ-lyon1.fr/ http://www.univ-lyon1.fr http://www.cnrs.fr

2
Production de Protéines Recombinantes et d'Anticorps (Plate-Forme)
Institut Pasteur de Paris

3
Department of Chemical Engineering,
University of Delaware

Modeling and observer design for recombinant *Escherichia Coli* strain

M. Nadri[†], I. Trezzani[*], H. Hammouri[†‡], P. Dhurjati[‡], R. Longin[*], J.Lieto[†]

Abstract

A mathematical model for recombinant bacteria which includes foreign protein production is developed. The experimental system consists of an E. Coli strain and plasmid PIT34 containing genes for bioluminescence and production of a protein, β_galactosidace. This recombinant strain was constructed to facilitate on-line estimation and control in a complex bioprocess. Several batch experiments were designed and performed to validate the developed model. The design of a model structure, the identification of the model parameters and the estimation problem are three parts of a joint design problem. A nonlinear observer is designed and an experimental evaluation is performed on a batch fermentation process to estimate the substrate consumption.

key-words: Modeling, constant high-gain observer, recombinant bacteria, induction.

[*†]Laboratoire d'Automatique et de Génie des Procédés L.A.G.E.P., UMR CNRS 5007, Université Claude Bernard Lyon I, CPE - Lyon, Bât. 308 G, 43 Bd du 11 Novembre 1918, 69622 Villeurbanne cedex, France.
[‡]Dept of Chemical Engineering, University of Delaware, Newark, Delaware 19716, USA
[*]I. Pasteur, Plateau technique 5, Génomique Structurale, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France.
[♮]Corresponding authors: e-mail:hammouri@lagep.cpe.fr, phone: (33) (0) 4 72 43 18 95, Fax: (33) (0) 4 72 43 16 99

1 Introduction and motivation

One of the primary goals of genetic engineering is the maximization of desired protein production. A model simple enough to use in optimization and control systems, yet sufficiently complex to capture the dynamics of the induced system, is highly desirable. Thus mathematical modeling can be a very efficient tool in the study and use of recombinant systems. Considerable time and money may be saved by examining scenarios mathematically instead of carrying out expensive, time-consuming experiments. The aim of this work is to use genetic engineering to design and construct a new recombinant *Escherichia Coli strain* to facilitate on-line monitoring and control. There has been past research to describe the dynamics of foreign protein production in recombinant bacteria [12, 4, 5, 17, 14] by using a model of the cellular response to induction.

The success of using recombinant cultures for on-line supervision of fermentation, and the pharmaceutic fermentation industry in general, is strongly strain dependent. The luciferase enzyme, which has generated much interest over the past couple of decades, can be used as a biosensor for intracellular product. It provides many attractive features, such as non invasively reporting molecular-level activity, high sensitivity, and a fast response in time of the order of seconds to minutes. These features are conferred by luciferase as a result of its bioluminescent properties. Genetic coupling of the production of luciferase with an induced intracellular product permits one to deduce product levels from bioluminescence measurements. Bioluminescence generates a distinctive "fingerprint" response indicative of the product in the cell.

In this work we propose to use bioluminescence as a macroscopic indicator for the estimation of a microscopic parameter: the intracellular product formation. A recombinant plasmid

containing the pBAD promoter inducible by arabinose, which controls the lacZ gene coding for *β-galactosidase* as a model protein, and the lux CDABE genes coding for bioluminescence was constructed. The bioluminescence and the turbidity were measured on-line by specially designed sensors. The correlations between the bioluminescence and enzyme production were obtained in a minimal medium with glycerol and in a complex medium with higher cell density cultures. The objective was to infer at any time the amount of product produced in the reactor by the cells, using a model that relates the product to the on-line measurements. This initial model opens up the possibility for optimization and control of the fermentation process.

The objective of this work is to develop a dynamic model that adequately describes the this recombinant strain and also to develop methods for the on-line estimation of substrate consumption and enzyme production in complex media.

This paper will be organized as follows: In the next section, we describe the fermentation process and the recombinant strain construction. In section 3, we describe the dynamic model and it's experimental validation in batch fermentation. In section 4, we design a simple estimator that allows us to estimate the substrate consumption.

2 Materials and methods

2.1 Microorganism and recombinant plasmid

The project goal is to monitor a recombinant bacterial product (enzyme, protein) by bioluminescence and turbidity measurements. At the beginning, we use enzymes which are easy to analyze, to build and allow easy testing of the model. The principle is to build a recombinant plasmid with the five lux genes necessary for the endogenous light production, and the gene

coding for the enzyme or the protein, under the control of a unique promoter. Then after the induction the light and the protein are produced.

In this work, we use a recombinant plasmid with the pBAD promoter inducible with arabinose, controlling the production of a protein β-galactosidase and of bacterial bioluminescence coded by 5 lux genes, luxCDABE. This plasmid, named *pIT34*, was introduced into *E. coli MC4100 Dara*, which can not metabolize the arabinose used as inducer. The recombinant strain obtained was called *IT342* [15].

2.2 Culture media and bioreactor conditions

Fermentation cultures are inoculated with a 10 hour preculture, stopped in exponential growth phase and kept at 4C during one night. The minimal medium having the following composition: 0.5 mg/l ferrous sulfate, 0.2 g/l magnesium sulfate , 13.6 g/l Di hydrogenous phosphate of potassium, 2 g/l Ammonium Sulfate, 2.8 g/l potassium hydroxyde, 2 g/l glycerol and 0.5 mg./l vitamin B1.

Inoculum was grown in 50 ml of this medium in shake flasks at 37°C for 12 hours ([15]). The same medium with 50 mg/ml ampicillin and 100 mg/l streptomycin was used with about 10% of this inoculum to start the batch culture at an optical density (O.D.) of 0.07-0.08 in the MMF. The Fermentor contained a final volume of 50 ml . The culture was thermostated at 37°C through a dry bath. Oxygen was provided by aeration, with air at 2.57 vvm (154 ml/min). When the O.D. reached 0.7, the culture was induced with 0.2 g/l arabinose, and at the same time, aeration was changed from air to pure oxygen. Figure 2 shows the time course profiles for glycerol, enzymes, biomass and bioluminescence from a batch fermentation experiment. The production of the light and the β-galactosidase began around 5 minutes after the induction.

2.3 Material description

The process was carried out in a specially designed fermentors named "Multi Micro Fermentor" (MMF) with complete monitoring and control instrumentation for batch and continuous experiments. It consists of squared-section glass tubes containing a small culture volume (from 10 to 60 mL), thermostated with a dry bath to keep the optimal temperature. The MMF are equipped with a miniaturized pH probe, turbidity and bioluminescence online sensors which are controlled by a commercially available fermentation software (Labview, National Instruments) running on a microcomputer (see Figure 2).

The turbidity sensor [9] and the light sensor [16] have been designed at the Fermentations Laboratory (Pasteur Institute, Paris). They allow sensitive on-line measurements of biomass and bioluminescence.

The whole fermentation system allows several microbial cultures in parallel with on-line measurements.

3 Mathematical model development

The bioprocess used in this work is a pure recombinant microbial culture (*IT342*) X growing on two substrates glycerol S and dissolved oxygen O_d and yielding a final intracellular product P. The reaction is catalyzed by the inducer I. The bioprocess is assumed to be continuous with a scalar dilution rate D and an input substrate concentration S_{in}.

The classical reaction of bioluminescence is given by:

$$FMNH_2 + O_2 + RCHO \rightarrow FMN_2 + H_2O + RCOOH + h\nu \quad (1)$$

where the catalyst of the reaction is a luciferase enzyme.

The proposed mathematical model consisting of five equations based on component balances was created to simulate the induction of bioluminescence in *E. Coli* strain (PIT34). The bacterial growth, substrate consumption, the oxygen consumption, the inducer degradation, protein production and the bioluminescence were represented in the mathematical model.

Generally, the variables of interest are the cell concentration in the aqueous phase X, the glucose concentration in the aqueous phase S, the intracellular β-galactosidase concentration P, O_d, I as a function of time and bioluminescence.

3.1 General mathematical model

• Cell growth

Bacterial growth was modeled as:

$$\dot{X} = \mu X - DX - \varphi_1(t)X \tag{2}$$

where μ denote the very known specific growth reaction rate and φ_1 represents the death kinetics. This parameter depends on the protein production, which is assumed to be toxic in important concentration.

• Substrate consumption

By a simple mass balance we get:

$$\dot{S} = -y_1\mu X - y_2\nu X - k_m X - D(S_{in} - S) \tag{3}$$

where ν denotes the biosynthesis reaction rate and y_1 and y_2 are yield coefficients.

The third term includes a maintenance rate k_m, which indicates the cells'energy requirements for normal upkeep and repair.

• Inducer degradation

The inducer used to catalyze the bioluminescence reaction can be degraded naturally during the latency period and by recombinant bacteria during the bioluminescence period. We assume it to be:

$$\dot{I} = -\alpha(t)I - DI \tag{4}$$

where the function $\alpha(t)$, which can depend on cell, substrate and product concentrations, represents the degradation effect.

• Protein production

Different batch experiments with ITP34 strain proved that the time response to induction is negligible and cell activity is immediate. However, cell activity is modelled with two terms by:

$$\dot{P}_c = y_3\nu\varphi_2(t) - \mu P_c \tag{5}$$

where y_3 is the yield coefficient, which is assumed to be constant. The first term corresponds to the biosynthesis and the second represents the cell division effect. The function $\varphi_2(t)$ depends on I and represents the inducer effect.

This expression will be multiplied by the cell concentration to obtain the overall production rate of protein in the bioreactor. Then, let us denote by M_c the cell mass in moles and by P the total protein concentration in the MMF. Consequently, we can write:

$$P = \frac{XP_c}{M_c}$$

It follows:

$$\dot{P} = \frac{1}{M_c}(\dot{X}P_c + X\dot{P}_c) \tag{6}$$

Using equations (2) and (5), we obtain:

$$\dot{P} = \frac{1}{M_c}[\nu\varphi_2(t)X - \varphi_1(t)P - DP] \tag{7}$$

where $\varphi_1(t)P$ represents the degradation term.

• **Dissolved Oxygen**

The dissolved oxygen equation was modeled using four terms. The first is analogous to the first term of the substrate utilization equation, inducing a yield term representing the mass of cells formed per mass of oxygen consumed which is assumed to be constant. The second term represents the consumption of oxygen in the light reaction where the oxygen is assumed to be limiting.

A mass transfer term is included to show that the driving force for oxygen transfer into the system depends on the difference between the oxygen concentration in the batch and the maximum value it can attain, $(O_d)_{sat}$. The last term represents the maintenance for growing cells analogous to the one used in the nutrient equation.

$$\dot{O}_d = -y_4\mu X - y_5\frac{O_d}{k_o + O_d}L + k_l a((O_d)_{sat} - O_d) - m_o X - DO_d \tag{8}$$

where L denotes bioluminescence intensity and $y_4,\ y_5,\ k_l,\ k_o$ are positive constants.

• **Light response**

In most cases, we observe a linear dependence of the light sensitivity an the concentration of luciferase over a wide range. Based on this information, the light production in the first step was taken as directly proportional to the protein concentration variations but depending on the cell activity and related to oxygen as limiting substrate represented here by φ_3:

$$L = y_l \mu \varphi_3(o_d) \varphi_2(I) X P$$

where y_l is a positive constant.

3.2 Simplified mathematical model

When the above model is applied to our batch culture process, numerous operator conditions were required to generate a model simple yet effective for a complex biological process,

- The Aldehyde and $FMNH_2$ were considered as non-limiting substrates of luciferase, since both of these molecules are readily available from the bacteria's normal metabolic processes. Moreover, the lux operon regenerates aldehyde from the carboxylic acid produced in the light reaction (equation 1).
- The arabinose used as inducer is not degraded by recombinant bacteria.
- Carbon source is considered to be the only limiting substrate.
- The batch experiments have show that the growth and biosyntheses reactions. are reduced to a single reaction, i.e. the two specific rates μ and ν are assumed to be identical.

The above assumptions and our operating conditions, give the explicit expressions for the different functions in the general model.

First, we chose for the growth rate portion μ a Monod-type relation that depends on the nutrient concentrations S as follows:

$$\mu = \frac{\mu_m S}{(k_s + S)} \frac{k_i}{(k_i + P)}$$

where μ_m is the maximum specific growth rate for the cell growth in (h^{-1}), k_s is the half saturation constant and k_i is an adjustment enzyme inhibition coefficient.

In equation (2), the φ_1 representing the death kinetics was modeled using an Arrhenius-type expression which depends on the protein concentration by:

$$\varphi_1(P) = k_d \exp(\frac{-k_p}{P})$$

Now, we return to the dynamic protein equation (7). The function $\varphi_2(t)$ representing the limitation by the inducer, is given by a Monod-type expression as :

$$\varphi_2 = \frac{I}{I + k_I}$$

where k_I denotes the saturation constant.

Based on to the assumption three, oxygen is assumed to be a non limiting substrate. Consequently, the function φ_3 related to oxygen as limiting substrate is neglected.

Now, combining the differential equations given above and the general model, the global model can be expressed as follows:

$$\begin{cases} \dot{X} = \mu X - k_d \exp(\frac{-k_p}{P})X - DX \\ \dot{S} = -y_s \mu X - k_m X - D(S - S_{in}) \\ \dot{P} = y_p \mu \dfrac{I}{I + k_I} X - k_d \exp(\dfrac{-k_p}{P})P - DP \\ \dot{I} = -DI \\ L = y_l \dfrac{S}{(k_s + S)} \dfrac{I}{I + k_I} XP \end{cases} \tag{9}$$

Where y_s, $y_p = y_c \dfrac{\mu_c}{M_c}$ and y_l are the yield coefficients that will be identified.

The mathematical equations given above describe the growth, the substrate consumption, the cell activity and the bioluminescence for our experimental strain in different environmental conditions. Consequently, such a model can be used to predict the behavior of the system under different steady-state operating conditions and optimize the reactor conditions.

However, the model parameters identification is an important step in the construction of a predictive model that is able to estimate the behavior of the studied microorganism. To do that, several batch experiments were performed in the same operating conditions (temperature, pH, oxygen rate ...).

4 Validation results in batch mode

The system parameters were estimated through modeling and using the Levenberg-Marquardt algorithm. The proposed procedure was implemented in the software package Matlab to examine the performance of the minimization algorithms in terms of stability and rate of convergence. The rate of convergence refers to the performance of the algorithm at each iteration and the total number of iterations necessary for convergence. It depends on the characteristics of the algorithm itself and/or the objective function.

Parameter estimation has shown fast convergence in this simplest case. The parameters' estimates were almost exact after nine iterations using 40 measurement points for each component. The estimated values of system parameters are listed in Table 1.

Figure 3 and Figure 4, presents the experimental model validation results. As we can see, the mathematical model as outlined by the system (9) represents the behavior of the real process, so it might be used as a guide for developing the design and the operating strategies.

5 Observer synthesis: Substrate estimation

On-line availability of the substrate measurement is very important for the control and particulary for the growth supervision of the biomass and faults detection. Indeed, the bioprocesses are confronted, besides to the usual faults as pump faults and aeration problems, to contamination problems. Furthermore, the plasmid loss is a very common fault in recombinant strain.

As stated before, in the bioprocess studied in this work, the main substrate (Glycose) is not available on-line. One way to overcome this problem is to use "software sensors" to estimate missing state variables on-line. Several works have focused on the development of software sensors for the estimation of component concentrations and reaction rates in bioprocess (see e.g.

[14, 1, 8]). Although these works consist of different approaches, most of them are are based on the Extended Kalman Filter (EKF) approach which generally leads to complex non-linear algorithms difficult to implement and calibrate ([1, 7]). Bastin and Dochain in [1] proposed an asymptotic observer for the estimation of the component concentrations. They introduced a linear change of coordinates which allows to obtain auxiliary variables with open loop stable dynamics. Another approach concerns the classical high gain observer (see for instance [2, 6]). In this case the gain of the observer is obtained through differential equations (Riccati differential equations or Lyapunov differential equations. Generally, the observer gain depends on measured state variables [10, 3, 8]. This causes supplementary constraints when the measurements used are relatively noisy for the calibration of the gain.

The aim of this section is to design a constant gain observer which does not require the resolution of a dynamical systems or any variable change. The calibration is simple and easy to carry out through the tuning of two parameters. It is synthesized for a large class of nonlinear systems including the mathematical model of the recombinant culture described above. To do so, the observer design is based on the system taking the following form:

$$\begin{cases} \dot{x} = F(x,u) \\ y = h(x) \end{cases} \tag{10}$$

where $F = \begin{bmatrix} F_1(x_1,x_2,u) \\ F_2(x_1,x_2,u) \end{bmatrix}$, $u(t)$ is an input in U a bonded interval of $\mathbb{R}$, the output measurement $y(t) \in \mathbb{R}$ and $x = \begin{pmatrix} x_1 \\ x_2 \end{pmatrix} \in \mathbb{R}^2$ is the state of the system.

The observer construction requires the following hypotheses:

(H1) F is a global Lipschitz, i.e.:

For every $(u, x) \in (U \times \mathbb{R}^2)$, il exist a fixed constant $c > 0$ such that $\|F(x, u) - F(z, u)\| \leq c\|x - z\| \quad \forall x, z \in \mathbb{R},\ \forall u \in \mathrm{U}$

(H2) For every $(u, x) \in (U \times \mathbb{R}^2)$, we have

$$\alpha_1 \leq \frac{\partial F_1}{\partial x_2} \leq \alpha_2 \tag{11}$$

where $\alpha_2 > \alpha_1 > 0$ are two constants.

As in many high gain observer techniques (see for instance [10, 8]), the assumption (H1) can be emitted in the case where the state $x(t)$ is a bounded one for all bounded input (this is the case of most physical processes).

Under the above assumptions, we will show that the following system

$$\dot{\hat{x}} = F(\hat{x}, u) - \rho \Delta_\theta R^{-1} C^T (C\hat{x} - y) \tag{12}$$

forms an exponential observer for system (10).

Where ρ is a positive constant, $\Delta_\theta = \begin{bmatrix} \theta & 0 \\ 0 & \theta^2 \end{bmatrix}$ and R is a symmetric positive definite matrix.

To assure the exponential convergence of the above observer, the matrix R was chosen symmetric positive definite (S.P.D.) and satisfying the following:

$$R = \begin{bmatrix} R_{11} & R_{12} \\ R_{12} & R_{22} \end{bmatrix} \text{ such that: } \begin{cases} R_{11} > 0,\ R_{12} < 0 \\ |R_{12}| > \dfrac{R_{11}^2 \alpha_2^2}{2\rho\alpha_1}, R_{22} > \dfrac{R_{12}^2}{R_{11}}, \end{cases} \tag{13}$$

where α_1, α_2 are the two positive constants given by assumption (H2) and $\rho \geq 1$ is a positive

constant chosen to have the best convergence.

The tuning of the observer (12) is obtained by the calibration of the two parameters ρ and θ. Indeed, choosing R as in (13), $\rho \geq 1$ and θ satisfying $\theta \geq \theta_0 = \dfrac{2\sqrt{2}c\|R\|}{\eta}$, the convergence of the estimation error is exponential. Where $\eta = (1 - \dfrac{R_{11}\alpha_2}{\sqrt{2\rho\alpha_1|R_{12}|}})\min\{\rho, 2\alpha_1|R_{12}|\}$ (see appendix 1) and c is the lipschitz constant given by (H1). (see proof in appendix 2).

In the next section, the performances of the proposed observer are illustrated through the microbial culture described above by the mathematical model (9).

6 Numerical simulations

6.1 Basic equations

In the following, we will show how we can estimate S using the biomass concentration X as the only available time measurement. To do so, we will introduce the following reduced model:

$$\begin{cases} \dot{X} = \dfrac{\mu_m.S}{S + k_s}X - k_d X \\ \dot{S} = -y_s\dfrac{\mu_m.S}{S + k_s}X - k_m X \\ y(t) = X \end{cases} \tag{14}$$

System (14) can be rewritten as:

$$\begin{cases} \dot{x}(t) &= F(x, u) \\ y(t) &= h(x) \end{cases}$$

$$\text{where } x(t) = \begin{bmatrix} X \\ S \end{bmatrix} \quad F = \begin{bmatrix} \dfrac{\mu_m.S}{S+k_s}X - k_dX \\ \\ -y_s\dfrac{\mu_m.S}{S+k_s}X - k_mX \end{bmatrix}; \; h(x) = X$$

Now applying the theoretical result of section 5, an exponential observer for system (14) takes the form:

$$\begin{cases} \dot{\hat{X}} = \dfrac{\mu_m.\hat{S}}{\hat{S}+k_s}\hat{X} - k_d\hat{X} - \rho\theta\dfrac{R_{22}}{R_{11}R_{22}-R_{12}^2}(\hat{X}-X) \\ \dot{\hat{S}} = -y_s\dfrac{\mu_m.\hat{S}}{\hat{S}+k_s}\hat{X} - k_m\hat{X} - \rho\theta^2\dfrac{-R_{12}}{R_{11}R_{22}-R_{12}^2}(\hat{X}-X) \end{cases} \tag{15}$$

where $\rho \geq 1$ is a positive constant, and R is chosen such that

$$R = \begin{bmatrix} 250 & -0.35 \\ -0.35 & 5.10^{-4} \end{bmatrix}$$

6.2 Simulation results

In order to illustrate the performances of estimator (15), we compared the corresponding results with data issued from batch experiments, where the inhibition term is negligible. The numerical values of the system parameters are given in the Table 1.

The simulations were carried out under the following initial conditions:

$$\hat{X}^0 = 0.05g/l \quad \hat{S}^0 = 2.6g/l.$$

In order to show the effects of the initial estimation of R, the value of $\hat{S}$ is initialized with an error of 30% compared to the initial simulated value. The tuning parameter θ given in equations (15) can have different values. In our case, $\theta = 13$, $\rho = 4$ gives a satisfactory estimation.

The corresponding results are presented in Figure 5 As can be seen from these figures, the estimator converges after 1h.

7 Conclusions

A new recombinant *E. Coli* strain was designed and constructed to facilitate control of a bioprocess. A dynamic model for continuous operation of fermonter was developed. The model describes growth of cells, substrate consumption, protein production, with particular focus on the relationship between bioluminescence and enzyme production. The model was experimentally validated in batch mode and used to estimate substrate concentration.

The main characteristic of the proposed estimators lies in their implementation and calibration. Indeed, the gain of these estimators does not depend on state variables. Moreover, its tuning is achieved through the choice of a single constant parameter. Simulation results demonstrate that the given estimator performs well. These results can also be used for diagnostics and faults detection.

References

[1] Bastin G. and Dochain D.(1990) On-line Estimation and Adaptive Control for Bioreactors. Elsevier, Amsterdam.

[2] Bornard G. and Hammouri H. (1991) A high gain observer for a class of uniformly observable systems. Proc. 30th IEEE Conference on Decision and Control, 122:1494-1496, Brighton England.

[3] Busawon K., Farza M. and Hammouri H.(1998) Observer design for a special class of nonlinear systems. *International Journal of Control* 71:405-418.

[4] Cha H.J., Wu C.F., Valdes J.J., Rao G., Bentley W.E. (2000) Observations of green fluorescent protein as a fusion partner in genetically engineered Escherichia coli : monitoring protein expression and solubility. *Biotech. Bioeng.* 67:565-574.

[5] DeLisa M.P., Li J., Rao G., Wiegand W.A., Bentley W.E. (1999) Monitoring GFP-Operon fusion protein expression during high cell density cultivation of Escherichia coli using an on-line optical sensor. *Biotech. Bioeng.* 65:54-64.

[6] Deza F., Busvelle E.,Gauthier J.P. and Rakotopara D. (1992) High gain estimation for nonlinear systems. *Syst. Control Lett.* 18:295-299

[7] Dochain D., Perrier M. and Ydstie B.E.(1992) Asymptotic observers for stirred tank reactors. *Chemical Engineering Science.* 47:4167-4177.

[8] Farza M., Nadri M. and Hammouri H. (2000) Nonlinear Observation of Specific Growth Rate in Aerobic Fermentation Processes. *Bioproc. Eng.* 23:359-366.

[9] Gaillon L., Longin R., Luu T.D. (1997) Appareil et procédé de mesure de propriétés optiques par contrôle rétroactif. French patent application n 9714864 filed on nov. 26th, and foreign counterparts (WO 99/27349).

[10] Gauthier J.P., Hammouri H. and Othman S.(1992) A simple observer for nonlinear systems-Application to bioreactors. *IEEE Trans. on Aut. Control* 37:875-880.

[11] Hammouri H., Targui, B. Armanet, F. (2002) High gain observer based on a triangular structue. *Int. J. Robust and Nonlinear Control* 12:(6)497-518.

[12] Lee J., Ramirez F. (1992) Mathematical modeling of induced foreign protein production by recombinant bacteria. *Biotech. Bioeng.* 29:635-646.

[13] Konstantinov, K.B., Dhurjati, P.(1993) Real-time compensation of the inner filter effect in high-density bioluminescent cultures. *Biotech. Bioeng.* 42:1190-1198.

[14] Shimizu H., Takamatsu T., Shioya S. and Suga K.I. (1989) An Algorithmic approach to constructing the on-line estimation system for the specific growth rate. *Biotechnol. Bioeng.* 33:354-364.

[15] Trezzani I.(2001) Conduite et optimisation de processus de fermentation: quantification en ligne de la synthse de protines recombinantes par mesure de bioluminescence. PH-D Thesis.

[16] Trezzani I., Nadri M., Hammouri H., Lieto J., Dorel C., Lejeune P., Dhurjati P., Bellalou J., Longin R. (2001) Quantification en ligne de la synthèse de molécules recombinantes par mesure de bioluminescence. Rcents Progrés en Gnie des Procds, Nancy 15(87) pp 147-155.

[17] Zhang Z., Scharer J.M., Moo-Young M.(1997) Mathematical model for aerobic culture of recombinant yeast. *Bioproc. Eng.* 17:235-240.

Appendix 1

Obviously R is a S.P.D. matrix. Indeed, let $x \in \mathbb{R}^n$, $x \neq 0$, then

$$\begin{aligned} x^T R x &= R_{11}x_1^2 + 2R_{12}x_1x_2 + R_{22}x_2 \\ &\geq R_{11}x_1^2 - 2|R_{12}||x_1x_2| + R_{22}x_2^2 \\ &\geq (1 - \frac{|R_{12}|}{\sqrt{R_{11}R_{22}}})(R_{11}x_1^2 + R_{22}x_2^2) \\ &\geq 0 \quad \forall x \neq 0 \text{ (from (13))} \end{aligned} \tag{16}$$

Now consider a matrix $M = \begin{bmatrix} 0 & a \\ 0 & 0 \end{bmatrix}$, where $\alpha_1 \leq a \leq \alpha_2$ and set $P = M^T R + RM - \rho C^T C$.
Using a similar argument as above, we obtain:

$$\begin{aligned} x^T P x &= -\rho x_1^2 + 2aR_{11}x_1x_2 + 2aR_{12}x_2^2 \\ &\leq -\rho x_1^2 + 2\alpha_2 R_{11}|x_1x_2| - 2\alpha_1|R_{12}|x_2^2 \\ &\leq -(1 - \frac{\alpha_2 R_{11}}{\sqrt{2\rho\alpha_1|R_{12}|}})(\rho x_1^2 + 2\alpha_1|R_{12}|x_2^2) \end{aligned}$$

From inequalities (13) that we deduce $1 - \dfrac{\alpha_2 R_{11}}{\sqrt{2\rho\alpha_1|R_{12}|}} > 0$

Hence

$$\begin{cases} x^T P x \leq -\eta\|x\|^2 \\ \text{where } \ \eta = (1 - \dfrac{R_{11}\alpha_2}{\sqrt{2\rho\alpha_1|R_{12}|}}) \min\{\rho, 2\alpha_1|R_{12}|\} \end{cases} \tag{17}$$

Appendix 2

We will show that the estimation error $e(t) = \hat{x}(t) - x(t)$ exponentially converges to zero.

Using (12), we obtain:

$$\dot{e}(t) \quad = \quad F(\hat{x}, u) - F(x, u) - \rho \Delta_\theta R^{-1} C^T (C\hat{x} - y) \tag{18}$$

We will use the following decomposition:

$$F(\hat{x}, u) - F(x, u) = \begin{bmatrix} F_1(\hat{x}_1, \hat{x}_2, u) - F_1(\hat{x}_1, x_2, u) \\ 0 \end{bmatrix} + \begin{bmatrix} F_1(\hat{x}_1, x_2, u) - F_1(x_1, x_2, u) \\ F_2(\hat{x}_1, \hat{x}_2, u) - F_1(x_1, x_2, u) \end{bmatrix} \tag{19}$$

From the mean value theorem, we get:

$$F(\hat{x}, u) - F(x, u) = A(\hat{x}, x)e + B(\hat{x}, x)e \tag{20}$$

where $A(\hat{x}, x) = \begin{bmatrix} 0 & \dfrac{\partial F_1}{\partial x_2}(x_1, \hat{x}_2 + \tau e_2, u) \\ 0 & 0 \end{bmatrix}$, and $\tau \in [0, 1]$ is a number which may depend on $(u, \hat{x}, x)$.

Similarly, from the mean value theorem we have:

$$B(\hat{x}, x) = \begin{bmatrix} \dfrac{\partial F_1}{\partial x_1}(\hat{x}_1 + \tau_1 e_1, x_2, u) & 0 \\ \dfrac{\partial F_2}{\partial x_1}(\hat{x}_1 + \tau_2 e_1, \hat{x}_2, u) & \dfrac{\partial F_2}{\partial x_2}(\hat{x}_1, \hat{x}_2 + \tau_3 e_2, u) \end{bmatrix}$$

where $\tau_i \in [0, 1],\ 1, 2.$

Combining (19) and (20), we deduce:

$$\dot{e}(t) = A(\hat{x}, x)e + B(\hat{x}, x)e - \rho \Delta_\theta R^{-1} C^T C e \tag{21}$$

Now setting $\bar{e} = \Delta_\theta^{-1} e$, then, it suffices to show that $\|\bar{e}(t)\|$ exponentially converges to 0 as $t \to +\infty$. To do so, we can easily check that:

$$\dot{\bar{e}} = \theta[A(\hat{x}, x) - \rho R^{-1} C^T C]\bar{e} + \Delta_\theta^{-1} B(\hat{x}, x)\bar{e} \tag{22}$$

Now, consider the following positive definite quadratic function: $V = \bar{e}^T S \bar{e}$. We only need to show that $\dot{V} \leq -\gamma V$ for some constant $\gamma > 0$.

Using (22) we obtain:

$$\dot{V} = \theta \bar{e}^T (A(t)^T S + R A(t))\bar{e} - 2\theta\rho \|C\bar{e}\|^2 + 2R\bar{e}^T \|\Delta_\theta^{-1} B(\hat{x}, x, u)e\|$$

Since $A(t)$ is of the form $\begin{bmatrix} 0 & a(t) \\ 0 & 0 \end{bmatrix}$, where $\alpha_1 \leq a(t) = \dfrac{\partial F_1}{\partial x_2}(x_1, \hat{x}_2 + \tau e_2, u) \leq \alpha_2$, and $\alpha_1,\ \alpha_2$ are fixed constants given by (H2).

From (17), we get:

$$\begin{aligned} \dot{V} &\leq -\theta[\eta\|\bar{e}\|^2 - \rho\|C\bar{e}\|^2] - 2\theta\rho\|C\bar{e}\|^2 + 2\|R\|\|e\|\|\Delta_\theta^{-1} B(\hat{x}, x, u)e\| \\ &\leq -\eta\theta\|\bar{e}\|^2 + 2\|R\|\|e\|\|\Delta_\theta^{-1} B(\hat{x}, x, u)e\| \end{aligned}$$

Now, using the triangular structure of $B(\hat{x}, x, u)$ and the fact that F is a global Lipschitz function (assumption (H1)), we can easily see that for every $\theta \geq 1$ we have: $\|\Delta_\theta^{-1} B(\hat{x}, x)\bar{e}\| \leq \sqrt{2}c\|\bar{e}\|$, where c is the lipschitz constant given by (H1).

Hence,

$$\begin{aligned} \dot{V} &\leq (-\eta\theta + 2\sqrt{2}c\|R\|)\|\bar{e}\|^2 \\ &\leq -(\eta\theta - 2\sqrt{2}c\|R\|)\lambda_{min}(R)V \end{aligned}$$

where $\lambda_{min}(R)$ denotes the smallest eigenvalue of R.
To end the proof, it suffices to chose θ such that $\theta \geq \theta_0 = \dfrac{2\sqrt{2}c\|R\|}{\eta}$.

List of symbols

X	biomass concentration (g/l).
S	substrate concentration (g/l).
P	protein concentration in the reactor (g/l).
P_c	protein concentration in the cell (g/l).
L	light intensity (V).
I	inducer concentration (g/l).
O_o	oxygen concentration (g/l).
μ	specific growth reaction rate $(1/h)$.
ν	biosynthesis reaction rate $(1/h)$.
y_i	yield coefficients.
M_c	cell mass in moles (g/mole).
φ_1	death kinetics (l/h).
φ_2	inducer effect.
φ_3	oxygen limitation.
α	Inducer degradation effect $(1/h)$.
k_m	maintenance constant associate to the substrate.
m_o	maintenance constant associated to the oxygen.
k_s	saturation constant associated to the oxygen.
k_o	saturation constant associated to the substrate.
$K_l a$	oxygen saturation constant $(1/h)$.
$(O_d)_{sat}$	dissolved oxygen saturation concentration (g/l).
S_{in}	substrate concentration in the input stream (g/l).
D	dilution rate (h^{-1}).

FIGURES CAPTION

Figure 1. Recombinant plasmid construct PIT34.

Figure 2. Schematic presentation of the Multi-Micro-Fermentor with the deferent sensors.

Figure 3. Comparison between model simulation (solid line) and experimental data (markers) in Batch process.

Figure 4. Comparison between bioluminescence intensity given by simulation model (line) and experimental data (markers) in Batch process.

Figure 5. Comparison between measured concentrations (markers) and there estimates with the nonlinear observer (line).

k_d	0.002	$g.l^{-1}$
k_m	0.21	$g.l^{-1}$
k_I	0.03	$g.l^{-1}$
y_s	0.75	
y_p	0.32	
y_l	13	

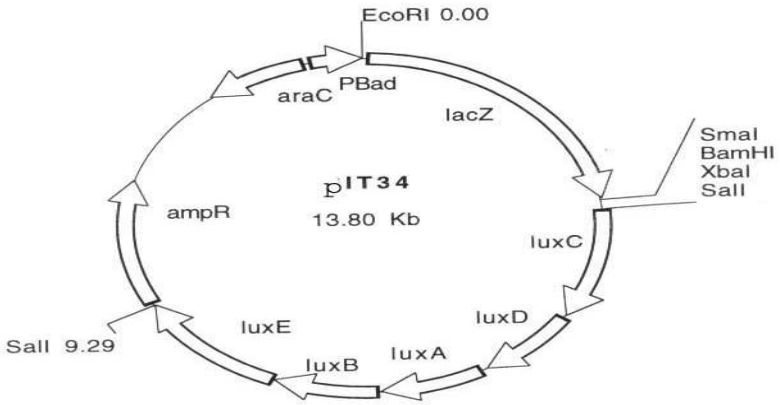

Figure 1: Recombinant plasmid construct *pIT34*.

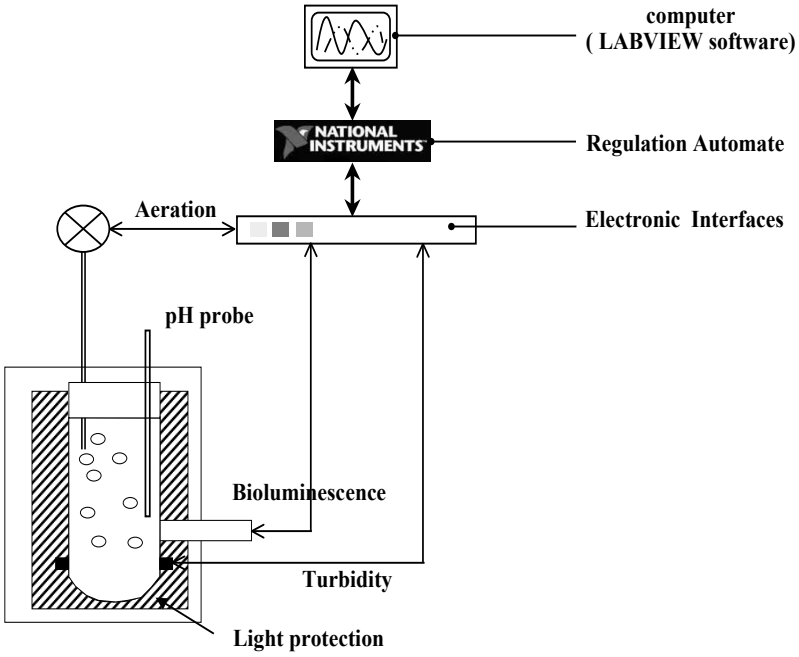

Figure 2: Schematic presentation of the Multi-Micro-Fermentor with the deferent sensors.

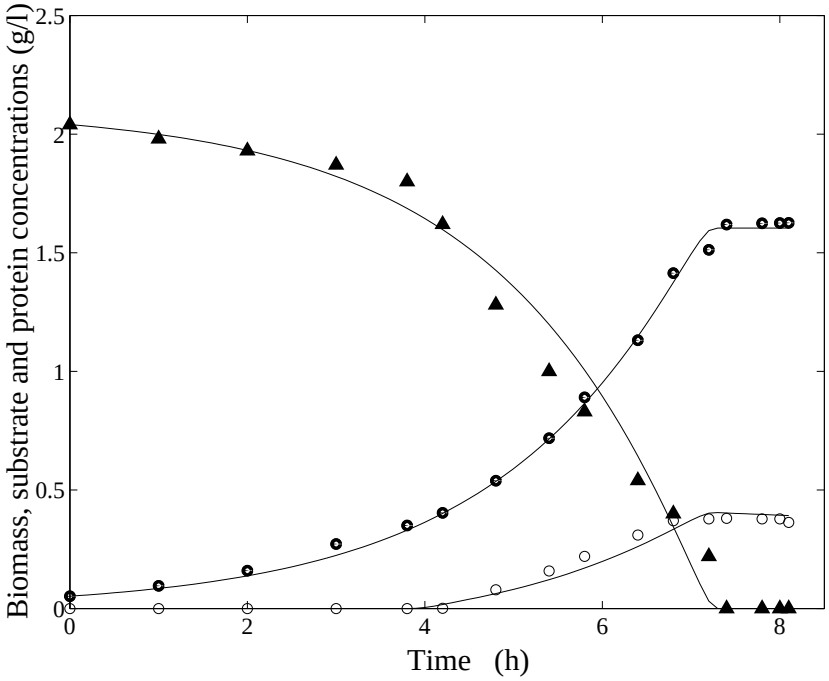

Figure 3: Comparison between model simulation (solid line) and experimental data (markers) in Batch process.

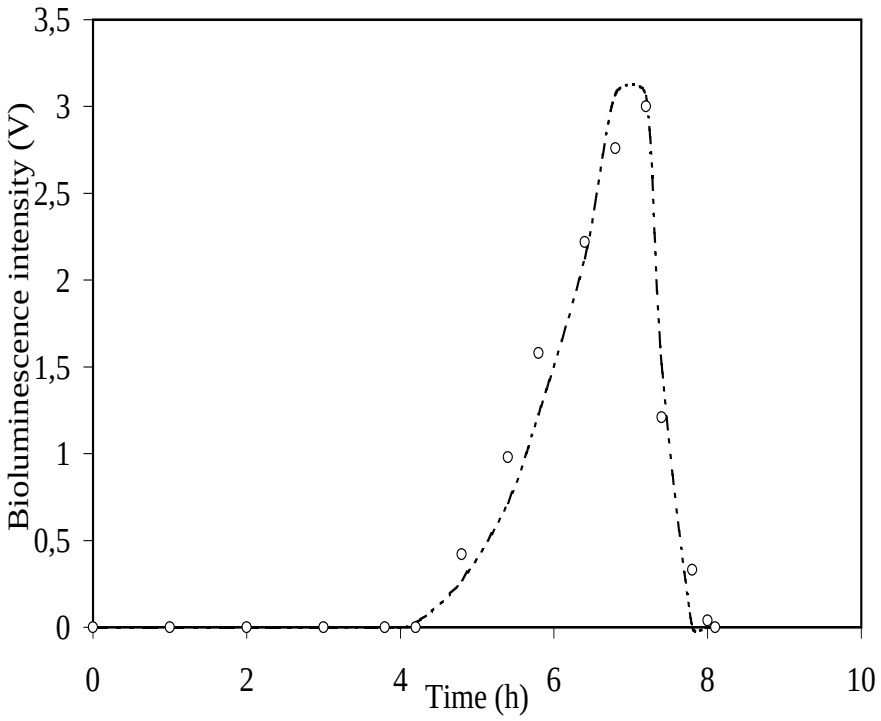

Figure 4: Comparison between bioluminescence intensity given simulation model (line) in Batch process.

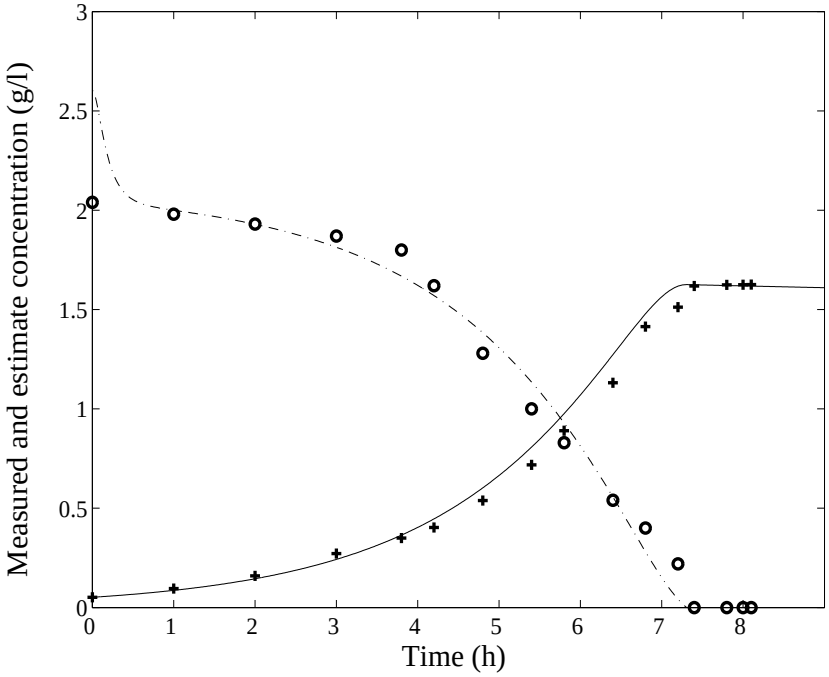

Figure 5: Comparison between measured concentrations (markers) and there estimates with the nonlinear observer (line).