Proceedings of the 17th World Congress
The International Federation of Automatic Control
Seoul, Korea, July 6-11, 2008

Neural Observer to Trehalose Estimation

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Abstract: These It is generally accepted in yeast production industry that intracellular trehalose is an indicator of yeast fermentation capacity and viability. The disaccharide trehalose is a cytoplasmic compound, so it must be quantified after extraction by means of an off-line analytical method during a biomass production process. Thus, knowing experimental determinations of yeast trehalose content is always delayed; hence no opportune actions can be implemented in order to lead the production process toward a high intracellular trehalose concentration in the produced biomass. An attempt of predicting trehalose concentration in yeast cells through two different mathematical approaches is presented. On the one hand, a biomass and trehalose concentrations estimator was developed with a differential neural network technique. On the other hand, a structured model results are analyzed for explaining the main metabolic events that induce a trehalose accumulation in cells. Our results allow us to think that the coupling of both methods can provide acceptable information aimed at reaching high trehalose content in yeast. Indeed, by integrating the two alternatives, a trehalose-enriched yeast production process could be successfully driven.

1. INTRODUCTION

This Saccharomyces cerevisiae has been largely considered as a paradigmatic model for the study of unicellular eukaryotic systems, and it is also of the most economic importance since it is a paramount microoganism in food industry. This yeast is a central supply for bread production, brewing and wine-making as well (Aranda-Barradas J.S. and Salgado-Manjarrez 2002; Aranda-Barradas J.S. 2002), in a way that the quality of such final products strongly depends on both biochemical composition and kinetic characteristics of the yeast cells involved in the corresponding production techniques. Cell viability and fermentation capacity (CO2 production power) are probably the main properties determining yeast quality for its usage in further food or alcoholic beverages production processes. Disaccharide trehalose (α-D-glucopyranosyl-1,1-α-D-glycopyranoside) is considered as an important factor in preserving yeast resistance to environmental stress conditions (Wiemken 1990; Attfield 1994; Lewis 1997), so increasing cell viability for alcoholic fermentation processes, and also during biomass reactivation in dough, thus providing a good consistency and texture to the mixture through CO_2 release (Jorgensen, Olsson et al. 2002) in bread-making. Several studies report trehalose presenting three metabolic roles in *Saccharomyces cerevisiae*. The first one refers to increased yeast ability to remain viable when confronted to stressful environments (Wiemken 1990; Lewis, Learmonth et al. 1997), such as nitrogen or carbon starvation (Lillie and Pringle 1980; Ertugay, Hamamci et al. 1997; Parrou, Enjalbert et al. 1999; Jorgensen, Olsson et al. 2002), heat-shock conditions (Hottiger, Schmutz et al. 1987; Attfield 1994; Ertugay, Hamamci et al. 1997) or strongly acid culture media (Arneborg, Hoy et al. 1995). The second trehalose metabolic function is related to cell carbohydrate reserves. The disaccharide appears to accumulate in cell plasma under certain culture conditions, and then to enzymatic split into two glucose molecules that incorporate to energy production metabolism through total or partial oxidation in the corresponding biochemical pathways (Parrou, Teste et al. 1997). In the third reported metabolic role of trehalose, it is involved in cell cycle progression, and apparently it also produces a partial regulatory effect on the glycolytic pathway enzyme hexokinase III (Sillje, Paalman et al. 1999). Therefore, trehalose determines in some extent the physiological condition and the biochemical composition of yeast cells regarding their quality and usefulness for some subsequent production process in food industry. This disaccharide has even been considered as an important predicting parameter of yeast viability (Slaughter 1992). Trehalose seems to be a necessary compound of *Saccharomyces cerevisiae* cells in order to assure an acceptable yeast quality for wine and bread making processes, so it should be continuously monitored while the microorganism is being produced. However, in order to follow intracellular trehalose content as a viability and cell physiology indicator during a yeast production process, biomass samples must be taken periodically and trehalose concentration in cells is then obtained from off-line analytical procedures. In order to avoid delayed quantification and knowledge of cytoplasmic trehalose concentration during yeast production, a structured model has been proposed

aiming at predicting trehalose accumulation in Saccharomyces cerevisiae (Aranda 2004). This approach takes account of multiple biochemical events that result in disaccharide accumulation within the cell. Yeast biomass is structured in three cellular compartments, a trehalose compartment defining the disaccharide cytoplasmic content, an enzymatic compartment that includes the enzymes directly involved in trehalose intracellular accumulation, and a cellular compartment with all other cellular components, including genetic material and regulating molecules. This structured model intends a trehalose content prediction during yeast production through computer process simulation.

An interesting alternative for obtaining on-line fed-batch fermentation data is process identification and estimation through neural networks, where two different techniques have been applied, the first one is the fermentation process identification by differential neural networks (Cabrera 2002) and the second one is a recurrent neural network to predict biomass concentration (Chen, Nguang et al. 2004). Nevertheless, a classical development of this approach gives only information about the macroscopic process state variables, such as the volume of liquid in the bioreactor or the substrates and products concentrations in the culture medium, so no data regarding the in-cell conditions are available. A modified differential neural network structure, coupled to a state observer, is proposed in the present work. This mathematical technique allowed us to generate very precise real-time estimations of intracellular trehalose, considered as a no-measured state variable. Trehalose estimation is accomplished through a software sensor that consists of a state observer which calculates the no-measured state variable given a real-time dynamical analysis of substrate concentration and liquid volume evolving in a fed-batch yeast production process. Experimental data are used to evaluate the estimator performance and its predicting usefulness as well.

2. THEORETICAL BACKGROUND

2.1 Compartmental and biochemical structured model.

The structured model considers the trehalose-enriched yeast production process depicted by three abiotic variables or states (biomass concentration, substrate concentration and culture medium volume in the bioreactor) and four main biotic components (trehalose concentration in the cell, trehalose phosphate synthesis activity, trehalase activity and cAMP intracellular concentration). Yeast cells are regarded as individually organized in a three compartment constitution, as seen in Fig. 1.

The compartments interact with one another through a set of reactions that could be described as follows. The glucose substrate gets into the cell at rate qs through a membrane phosphorilation reaction producing glucose-6-P, which is normally incorporated to the yeast energetic metabolism for ATP synthesis in the cellular compartment. However, glucose-6-P can also condensate with the glycosil group from an UDPG molecule to form trehalose-P in a biochemical reaction catalyzed by trehalose phosphate synthase (TPS), qST. Trehalose-P then releases inorganic phosphate by a phosphatase activity to produce trehalose, thus increasing the trehalose compartment, wT. The disaccharide can also be dynamically mobilized to provide intracellular glucose to the cell at rate qHT, if the trehalase (TH) enzyme is active in cell plasma. Trehalose content then decreases so leading to a reduction in the trehalose compartment. Under this scheme, enzymes trehalose phosphate synthase and trehalase concomitantly control the trehalose accumulation rate inside the cell. Both enzymes are synthesized from components included in the cellular compartment at rates qES and qEH for trehalose phosphate synthase and trehalase, respectively. These enzymatic activities are supposed to depend on cAMP intracellular concentration, which is linked to the glucose concentration in the culture medium. The enzymatic compartment could eventually be reconverted through proteolysis, qP, into cellular compartment components, i.e. amino acid units.

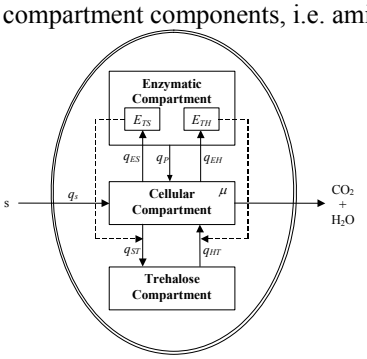

Figure 1. Biomass structure.

2.2 Biomass estimator and trehalose content observer

It should be inferred from the structured model that yeast production and trehalose accumulation in cells are non-linear systems, with a number of kinetic parameters needed for the state estimation of the process. Process estimation through a physical-based model strongly depends on parameters precision, which is usually inaccurate or unknown. Besides, the biotic components of the model are not completely observable states, hence process estimation by means of a dynamical neural network technique provides a good approximation of process numerical indicators, with neither preliminary parameter identification nor exact knowledge of the physical system. To estimate process abiotic states, together with the intracellular trehalose concentration, a dynamic neuro network observer (DNNO) is suggested. This estimation method is based on pervious works, such as Lyapunov-like observers (Slotine 1984), high gain system observation (Nicosia 1989; Giccarella 1993), optimization-based observers (Krener 1983) and reduced-order nonlinear observers (Garcia 1995).

Artificial neural networks (ANN) represent a good conceptual instrument, when nonlinear systems are trying to be identified (El-Din 2002). There are two known types of ANN: static (SNN) using the back-propagation technique (Narendra 1995) and dynamic neural networks (DNN) (Poznyak 2001). The first one deals with the so-called global optimization problem, trying to adjust the weights of a SNN in order to minimize an identification error. The second approach, exploiting the feedback properties of the applied

DNN (see Fig. 2), permits to avoid many problems related to global search extremum converting the learning-training process to an adequate feedback design. Several effective approaches are known to create the corresponding feedback design. One of them is the variable structure approach (VSA) (Utkin 1992). The corresponding procedures, treated within this theory, usually exploit the so-called signum-type or switching (sliding mode) structures. Despite fruitful research in the variable structure control theory, few authors have considered the application of the sliding mode approach to the problem of observer design for dynamic systems (Utkin 1992; Slotine 1984). In this study we suggest the DNN observer (DNNO), which incorporates a switching type term to correct current state estimates using only available, measurable output data.

The DNN observer corresponding to the scheme given at Fig. 2 is covered by the following ordinary differential equation:

$$\begin{aligned}&\frac{d\hat{x}_t}{dt} = A\hat{x}_t + W_1\sigma(\hat{x}_t) + W_2\varphi(\hat{x}_t)\gamma(u_t) + K_1(y_t - \hat{y}_t)\\ &+K_2 SIGN(y_t - \hat{y}_t)\\ &\hat{y}_t = C\hat{x}_t\end{aligned} \tag{1}$$

Here, $\hat{x}_t$ is the state vector of DNNO representing the current estimates of abiotic states and the observer prediction of intracellular trehalose content as well, $\hat{y}_t$ is the output of DNN corresponding the estimates of measurable abiotic states, *A*, *K1* and *K2* are constant matrices obtained from DNNO training with *K1* being a linear proportional (Luenberger) correction term matrix and *K2* a sliding mode correction term matrix, σ(·) and ϕ(·) are standard sigmoid functions, $\gamma(u_t)$ is the control function applied to the DNNO, C is an output formatting function and the sign function is given by:

$$SIGN(v) := \left(sign(v_1), \ldots, sign(v_n)\right)$$

with,

$$sign(z) := \begin{cases} 1 & if\ z > 0 \\ -1 & if\ z < 0 \\ not\ defined & if\ z = 0 \end{cases}$$

Specifying the x_t and $\hat{y}$ vector components would produce the following straightforward equivalences:
x1 is the biomass concentration variation in the culture medium (abiotic state),
x2 is the substrate concentration variation in the fermentation (abiotic state),
x3 is the working volume in the bioreactor (abiotic state), and $\hat{x}_4$ is the intracellular trehalose content estimate (biotic variable).

The measurable data are two abiotic states, therefore:
$\hat{y}_2$ is the output substrate concentration estimate.
$\hat{y}_3$ is the output liquid volume estimate in the biorreactor.

$$y_t = \begin{bmatrix} 0 & x_2 & x_3 & 0 \end{bmatrix} \tag{2}$$

So, in this case $C = diag\begin{bmatrix} 0 & 1 & 1 & 0 \end{bmatrix}$. The estimated states are the biomass concentration and the intracellular trehalose content.

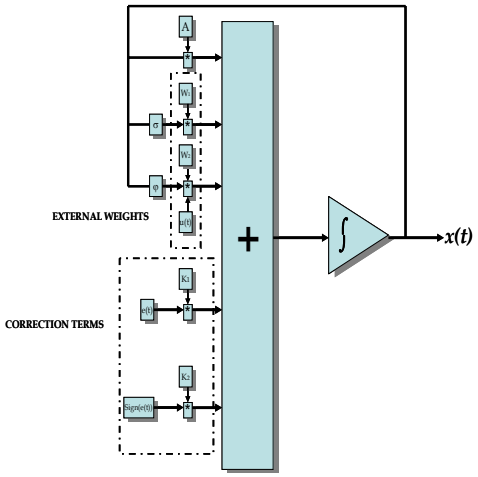

Figure 2. Differential Neuro Observer Structure.

2.2 Training DNNO.

The matrix of weights *Wi (i=1,2)* is the output tuning by a special on-line learning procedure (Poznyak 2001), these matrices are updated with a special learning law is described by the equation:

$$\dot{W}_t = \Phi\left(W_t, \hat{x}_t, u_t, t, y_t \middle| W^{(0)}\right) \tag{3}$$

This learning law is denoted by:

$$\dot{W}_t^{(i,j)} = -k\,\mu_t\, S_t^{(i,j)} sign\left(\tilde{W}_t^{(i,j)}\right);\ i,j = 1,n \tag{4}$$

where St is any matrix with the condition $tr\{S_t\} = 1$, and:

$$\begin{aligned}&\mu_t := \left\| N_\delta P \tilde{W}_t^T \sigma(\hat{x}_t) \right\|_\Pi^2 + 2e_t^T C N_\delta P \tilde{W}_t\, \sigma(\hat{x}_t)\\ &\Pi := C^T \Lambda_{\varsigma_2} C + \delta \Lambda_1, \quad \tilde{W}_t := W_t - W^{(0)*}\\ &e_t := y_t - C\hat{x}_t, \quad N_\delta := \left(C^T C + \delta I\right)^{-1}, \delta > 0\end{aligned} \tag{5}$$

The matrix P is the positive solution for the algebraic Riccati equation given by:

$$P\tilde{A}^{(0)*} + \left(\tilde{A}^{(0)*}\right)^T P + PRP + Q = 0 \tag{6}$$

To guarantee a small enough state estimation error the adequate parameters of DNNO (1) should be selected. The stationary parameters *A, K1, K2* may be tuned during the so-called"training" process. The weights *Wi, (i=1,2)* are quickly adjusted on-line by the special differential learning law. The training procedure may be conducted by using only experimental measurements as a correction criterion of DNNO parameters, as well as for an adequate selection of the initial conditions in the applied learning procedure. The adequate learning of DNNO (1) provides a small enough

upper bound (in an average sense) for the state estimation error $\Delta_t = \hat{x}_t - x_t$.

3. MATERIALS AND METHODS

3.1 Experimental Characteristics.

The used strain in all experiments was a commercial baker's yeast obtained from the market. The micro organism was isolated by triplicate in order to have a one-cell derived colony, and then identified as Saccharomyces cerevisiae. The strain was maintained on slants (glucose 20 g L-1, yeast extract 10 g L-1, agar-agar 20 g L-1) at 4 ^{o}C. Periodic inoculations were made in new slants every three months ca.

Fed-batch yeast production experiments were done on a chemically well-defined fermentation medium [31, 32]. The medium composition is glucose 50 g L-1, KH2PO4 7 g L-1, CaCl2·2H2O 0.25 g L-1, NaCl 0.5 g L-1, MgCl2·6H2O 6 g L-1, mineral solution 10 mL L-1, vitamins solution 10 mL L-1. Five hundred millilitres of mineral solution contain FeSO4·7H2O (278 mg), ZnSO4·7H2O (288 mg), CuSO4·5H2O (7.5 mg), Na2MoO4·2 H2O (25 mg), MnSO4·H2O (169 mg), H2SO4 a few drops, the required for dissolve ferric sulphates. Five hundred millilitres of vitamins solution are prepared with biotin (1.5 mg), calcium pantothenate (20 mg), inositol (125 mg), pyridoxine-HCl (25 mg), thiamine (50 mg).

Inoculums was grown in a 1 L flask containing 500 mL of the synthetic medium at 30 ^{o}C and 150 rpm over 24 h. The bioreactor was inoculated with the obtained biomass and the batch fermentation was carried out on a 6 L work volume. The fed-batch cultivations were initiated after 10 h of the previously established batch cultivation, until a final volume between 12 and 13 L was reached.

A 15 L bioreactor (applikon Z81315 M607) was used for all fed-batch experiments. The experimental conditions were: temperature 30 ^{o}C, pH 5.0, air flow 450 L h-1, dissolved oxygen 10 % of saturation value (0.8 mgO2 L-1 ca). The culture pH was controlled with ammonia-water (20 % v/v) and this solution was the only nitrogen source. The flow of carbon substrate was a function of the respiratory quotient (RQ) of the culture as calculated from effluent gas composition data. The glucose concentration in the working liquid in the bioreactor was always kept near to zero in order to minimize ethanol production. Starving conditions on carbon or nitrogen source were imposed at the end of the culture (last three hours) for inducing intracellular trehalose accumulation. All experiments were triplicate to check out data reproducibility.

Trehalose. Samples of 20 mg dry yeast were extracted twice with 3 mL of 0.05 M trichloroacetic acid in continuous orbital shaking during 40 min each time. Trehalose was then determined by anthrone method (Slaughter 1992).

Biomass. The yeast growth was followed by measuring the optical density of the culture at 620 nm with an UV-Vis spectrophotometer (Hitachi U-2000). A correlation between dry weight and optical density was previously established.

Glucose. The glucose concentration was determined by the glucose oxydase method with an automatic analyzer (YSI 2700 Select).

Based on the data base for the feed-batch process dynamic variables of Saccharomyces cerevisiae culture, the DNNO was trained, and then the neuro-observer was implemented to estimate the biomass and trehalose states, these variables were compared with the experimental data obtained from different experimental setups. Finally, the complete evolution for biomass and intracellular trehalose concentrations was plotted for both states (estimate and measure).

4. RESULTS AND DISCUSSION

Substrate depletion, either carbon or nitrogen, at the final three hours of a yeast growth process produces an increase in cytoplasmic trehalose, as shown in Fig. 3. Trehalose content in yeast cells was initially about 0.025 – 0.035 $g_{trehalose}\ g_{biomass}^{-1}$, and intracellular accumulation of the disaccharide has reached a nearly 4-fold increase during the fed-batch processes, attaining final concentrations of about 0.13 $g_{trehalose}\ g_{biomass}^{-1}$ (13 % of biomass dry weight) at the end of the culture.

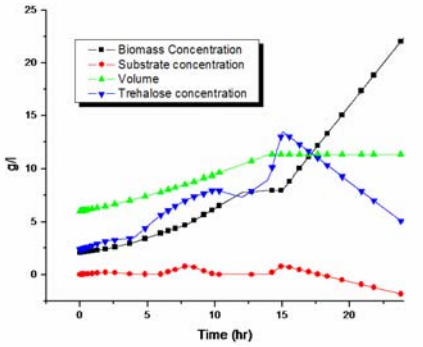

Figure 3. Biomass (x), substrate (s), intracellular trehalose (wT) and culture medium volume (v) as functions of time during a first yeast production process.

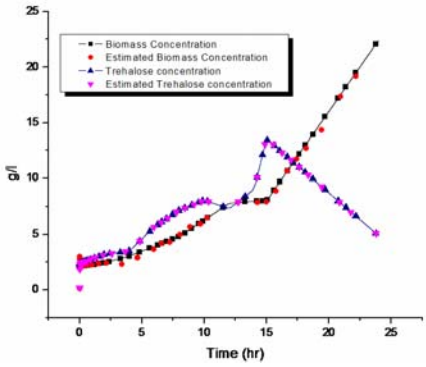

Figure 4. Biomass (x), intracellular trehalose (wT) and their estimate states during a first yeast production process.

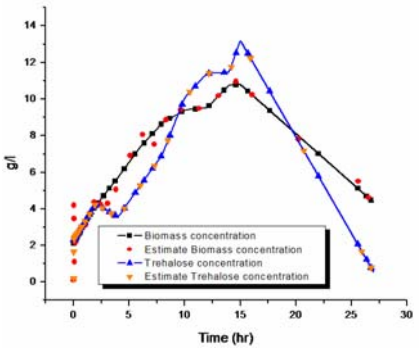

Figure 5. Biomass (x), substrate (s), intracellular trehalose (wT) and culture medium volume (v) as functions of time during a second yeast production process.

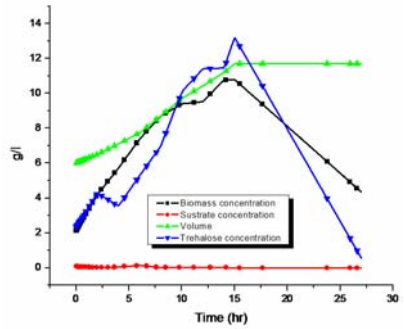

Figure 6. Biomass (x), intracellular trehalose (wT) and their estimate states during a second yeast production process.

This fact seems to be explained by some molecular and metabolic events happening in the cytoplasm. Since the trehalose accumulation depends on the enzymatic activity of trehalase and trehalose phosphate synthase, and these enzymatic activities rely upon cAMP intracellular concentration as stated before, it can be assumed that nitrogen starvation produces an important modification of cAMP in the cell. The hypothesis is supported by the reported evidence that adenyl cyclase (the enzyme that catalyses the cAMP synthesis in the cell) is enhanced when a membrane associated Ras system is active (Werner-Washburne M 1993). Like some other G-proteins, the activity of the Ras proteins is controlled by the guanine nucleotide being inactive when bounded to GDP and active when it joins to GTP (Barbacid 1987). While yeast is taken to a nitrogen-depeted culture medium, guanine nucleotides synthesis is stopped (Moat 2002), and then the Ras system becomes inactive. As a consequence, cAMP synthesis could be stopped or, at least, decreased so giving low intracellular levels of cAMP. Under these conditions, trehalose phosphate synthase activity is increased with a concomitant lessening of trehalase activity, thus yielding a significant increment of trehalose in cells cytoplasm.

Intracellular cAMP concentration dependence on the carbon source availability seems to be more direct, since the extra cellular glucose determines the energy charge, that is, the total adenosine phosphates concentration in the cell. This means that a lack of glucose in the culture medium will produce a plasmic shortage of adenosine phosphates, including cAMP. This condition leads to an increasing TPS activity and to a diminished TH activity, jointly with the intracellular trehalose accumulation.

The proposed structured model requires kinetic parameters estimation in order to be applied to the cytoplasmic trehalose prediction during the yeast production process. The model parameters estimation has been accomplished using an error function evaluated through a least squares criterion as a maximum likelihood estimator (Aranda 2004). Prediction of trehalose content in cell plasma by means of the structured model is presented in Fig. 4, for both carbon and nitrogen source limitations.

It can be seen that, as a general trend, the model follows the experimental trehalose accumulation in yeast cells, so this structured model could be a helpful tool for predicting trehalose cells content in the course of a yeast production process, as long as similar experimental conditions are established in such a process. However, model predictions accuracy may not fulfil process simulation expectations because of the too large variations in the estimated yeast trehalose content. This accurateness problem won't allow applying the appropriate correcting measures to shift a yeast production process toward the intended purpose of reaching a high intracellular trehalose concentration. The origin of simulations inaccuracy is the lack of precision in parameters numerical values, and also the incomplete knowledge of the complex metabolic phenomena taking place in the yeast cells. The structured model is nevertheless useful because it captures some essential traits of yeast growth and trehalose biosynthesis, and explains the biosynthesis intensification within the cells while the carbon or nitrogen sources are exhausted in the culture medium.

Yeast production with a high intracellular trehalose needs a better forecast method for yeast trehalose content than the estimations given by a bio chemically structured model. DNNO estimates of biomass and intracellular trehalose concentrations are shown in Fig. 5 and Fig. 6. These numerical results were developed using the DNNO parameters of Table 3.

DNNO estimate for abiotic state biomass and experimental data of two different yeast production processes are represented in Fig. 5. In this graphs it can be seen that the estimate states present an oscillating behavior around the experimental values, however the DNNO estimated values approximate sufficiently enough the experimental states so estimations are considered acceptably correct to depict the biomass evolution in the bioreactor. As shown in Fig. 6, DNNO intracellular trehalose estimations give a more regular behavior following the experimental results. These estimated values of trehalose content provide a more reliable basis for decision-making in producing yeast biomass with a high yield in cytoplasmic trehalose.

The presented trehalose and biomass estimator is based on the differential neural network theory applied to a fed-batch production process of Saccharomyces cerevisiae. The obtained results suggest the possibility of developing a new class of sensors called soft sensors. These sensors only need the experimental data for a few process variables in order to accomplish the estimation of some other no measurable process variables. Of course, soft sensors performance is

restricted to a certain experimental conditions, those used in the learning-training procedure.

5. CONCLUSIONS

Two alternatives have been analyzed in this paper; the first is dealing with the biochemical fundamentals of trehalose synthesis, and the second one aiming at trehalose content prediction through a dynamical neural network observer. The combination for both techniques shown a good performance described by the dynamical evolution between the estimate states and the experimental states. The biochemical model contributes to correct the estimate states given by the neuro estimator and this technique could be the basis to develop basic algorithms to soft sensors.

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