On-line monitoring of Mezcal fermentation based on redox potential measurements

P. Escalante-Minakata [a] V. Ibarra-Junquera [b],
R. González-García [c], A. De León-Rodríguez [a] & H.C. Rosu [d]

[a] *Division of Molecular Biology, Potosinian Institute of Science and Technology (IPICyT), San Luis Potosí, S.L.P., Mexico*

[b] *Faculty of Chemical Sciences, University of Colima, Coquimatlán, Col., Mexico*

[c] *Chemical Engineering Departament, UASLP, San Luis Potosí, SLP. Mexico*

[d] *Division of Advanced Materials, IPICyT, San Luis Potosí, S.L.P., Mexico*

Abstract

The paper describes an algorithm for the continuous monitoring of the biomass and ethanol concentrations and moreover the kinetic rate in the Mezcal fermentation process. This algorithm performs its task having only available the on-line measurements of the *redox potential*. The procedure includes an Artificial Neural Network (ANN) that relates the *redox potential* to the ethanol and biomass concentrations. Then a nonlinear-observer-based algorithm uses the biomass estimations to infer the kinetic rate of this fermentation process. The method shows that the *redox potential* is a valuable indicator of microorganism metabolic activity during the Mezcal fermentation. In addition, the estimated kinetic rate can be considered as a direct evidence of the presence of mixed culture growth in the process. Usually, mixtures of microorganisms could be intuitively clear in this kind of processes, however the total biomass data do not provide definite evidence by themselves. In this paper, the detailed design of the software-sensor is presented, as well as its experimental application at the laboratory level.

Key words: Mezcal, mixed-cultures, software-sensor, *redox potential*, kinetic rates

Email addresses: minakata@ipicyt.edu.mx (P. Escalante-Minakata), vrani@ipicyt.edu.mx (V. Ibarra-Junquera), raulgg@uaslp.mx (R. González-García), aleonr@ipicyt.edu.mx (A. De León-Rodríguez), hcr@ipicyt.edu.mx (H.C. Rosu).

1 Introduction

Mezcal, is a Mexican distilled spirit made from the core of the *Agave* plant (the "piñas"). Several species are used for the Mezcal production, i.e. *Agave salmiana*, *potatorum*, *angustifolia*, *tequilana*, etc, and each produces a slightly different Mezcal. Therefore, the tequila can be considered a regional type of Mezcal, restricted to the usage of *Agave tequilana* as raw material (Cedeño (1995)). It is important to mention that during this Mezcal fermentative process the syrup obtained from the juice of cooked "piñas" is left to naturally (spontaneously) ferment. By natural alcoholic fermentations we refer to the ones that start by themselves when a wild mixture of different microorganism starts fermenting. Therefore, the fermentation of *Agave* syrup into Mezcal is a complex biochemical process involving a whole realm of interactions between microorganisms.

During fermentation, the microorganisms employ sugars and other constituents of *Agave* syrup as substrate for their growth, converting them into ethanol, carbon dioxide, higher alcohols and their esters, and other metabolic compounds that contribute to the chemical composition and sensory qualities of the Mezcal (De León-Rodríguez *et al.* (2006)). We also notice that the qualitative and quantitative composition of the microbiota in fermenting musts could depend on the following factors: region of the *Agave* origin, production procedure, initial cell concentration, temperature, and ethanol concentration. As consequence the organoleptic properties are also the result of the diversity and composition of microorganisms and their dynamics and frequency of occurrence. Consequently, this fermentative process is a vital stage in Mezcal making. Thus, it is very important to know more about the dynamics of the entire microflora during the alcoholic fermentation process. In other words, an algorithm that enables a monitoring process could be fundamental for a quality control that ensures, at least, homogeneity in the final product.

Nevertheless, a bottleneck in all biochemical monitoring process is often the lack of sensors for biological variables. Moreover, it is a well-known issue that in order to monitor many biotechnological processes, the problem of kinetic rates estimation represents a strategic feature. That is why several techniques have been developed to estimate on-line the biological variables from the available measurements, which are usually dealing with physicochemical variables. Depending on the obtainable information about the process, there exist many possible types of estimators that can be used (Bastin & Dochain (1986), Locher *et al.* (1992), Farza *et al.* (1998)).

Previously, various attempts of relating the *redox potential* to fermentation processes have been made taking into account that *redox potential* assesses the life ability of microorganisms, their growth, as well as the physiological activity

in a given environments (Kwong *et al.* (1992), Berovič (1999), van Dijk *et al.* (2000), Cheraiti *et al.* (2005)). Particularly, the practical significance of redox potential and oxygen content at various stages of winemaking was examined by Kukec *et al.* (2002). Many chemical, enzymatic and biological processes in wine are correlated with the oxidative state of the wine.

The Monod kinetics, which were originally derived from laboratory experiments with pure cultures and single substrates, are frequently applied to describe the behavior of undefined mixed cultures growing with single substrates or complex substrate mixtures (Novák *et al.* (1994), Wanner *et al.* (1994), Gujer *et al.* (1995)). In this case, the growth parameters that have been used represent overall values reflecting the growth constants of the many different strains with respect to the multicomponent substrate and the frequencies and concentrations of both the different substrates and microbial strains. Nevertheless, it is well known from control processes that an accurate model leads to better control design and therefore to better closed-loop performance. There is recent progress shedding light on the dynamical processes underlying the growth of mixed culture in a mixture of substrate (Reeves *et al.* (2004), Ibarra-Junquera *et al.* (2006)). However, the exact determination of the kinetic rates, under such complex situations, is still an open subject.

The rest of the paper is organized as follows. Section 2 is devoted to a concise presentation of the fermentation experiment performed to illustrate our approach. The software sensor, which is a combination of an ANN and an adaptive observer scheme, is described in Section 3. The results obtained by applying this software sensor to the Mezcal fermentation process together with brief comments are included in Section 4. Finally, the paper ends up with some concluding remarks.

2 Materials and Methods

2.1 Microorganism and culture conditions

In order to evaluate experimentally the performance of the estimation algorithm, we performed six individual batch experiments using inocula of native microorganisms (without the addition of any commercial strains). The must (*Agave* syrup) was obtained from *Agave salmiana*, a species from the Mexican plateau (or altiplano) of the geographical region of San Luis Potosí. This must was centrifuged at 8000 $rpm \times 10\ min$ and stored in a frozen state at $-20°C$ prior to experiments.

The batch fermentations were carried out in a Bioreactor (Applikon, Schiedam,

the Netherlands) of 1 liter. The bioreactor is equipped with pH (AppliSens, pH Sensor Innovation, Applikon) and redox sterilizable electrodes (Pt-Ring, Applisens, Sensor Innovation, Applikon). The electrodes are connected to a console for data acquisition (Bioexpert, Data Acquisition Control Program, Version 1.1x, Applicon), a device which is connected to a computer where the data are stored and computed. The schematic representation of the process appears in Fig. 1.

The bioreactor was filled with 900 mL of must as a culture medium, 100 mL of the inoculum in its exponential growing phase (biomass 0.1 g/L) and 0.1 % of ammonium sulfate at final concentration. The initial conditions of the fermentation were settled at a temperature of 32.5°C and initial sugar concentration of 70 g/L. The pH does not show a dynamic evolution, maintaining itself at a value of 4 during the whole process. For a schematic representation of the process see Fig. 1.

2.2 Analytical procedures and measurements

The batch processes have been monitored for 14 hours, through sampling under sterile conditions. In order to quantify biomass and ethanol concentrations, 5 mL samples of culture was removed every 30 min. The samples were cleared by centrifugation at 6000 rpm for 5 minutes at room temperature. The next step was to collect the supernatant phase and store it frozen at $-20°C$ prior to be analyzed. The obtained pellet was resuspended in distilled water in order to proceed with biomass analysis.

2.2.1 Biomass and ethanol quantification

The biomass measurements have been performed using spectroscopy UV-Vis (Cary 50-1030, Varian) at 600 nm. The obtained values were interpolated with a standard curve of cell dry weight concentration.

For the determination of the ethanol 1 mL of each sample with a final dilution $1:10$ and 1 mL of 1-butanol in vortex motion for 5 min and followed by centrifugation at 6000 rpm for 5 min. The organic phase was analyzed in gas chromatographic 6890N (Agilent technologies, Wilmington, DE) provided with a capilar column HP-Innowax (30 $m \times 0.25$ mm i.d., 0.25 m film thickness; Agilent technologies, Wilmington, DE) and an auto-sampler 7863 (Agilent technologies, Wilmington, DE) with a split relation of $25:1$.

The chromatographic conditions were 35°C for 2 min, increased at the rate of 10°C/min up to 80°C, and maintained at the latter temperature for 15 min. The carrier gas was helium at a flow rate of 1.5 mL/min. The temperatures

of the injector and flame ionization detector (FID) were set at $220°C$ and $250°C$, respectively. The ethanol concentration of the samples was determined by means of a calibration curve of known standard solutions of ethanol.

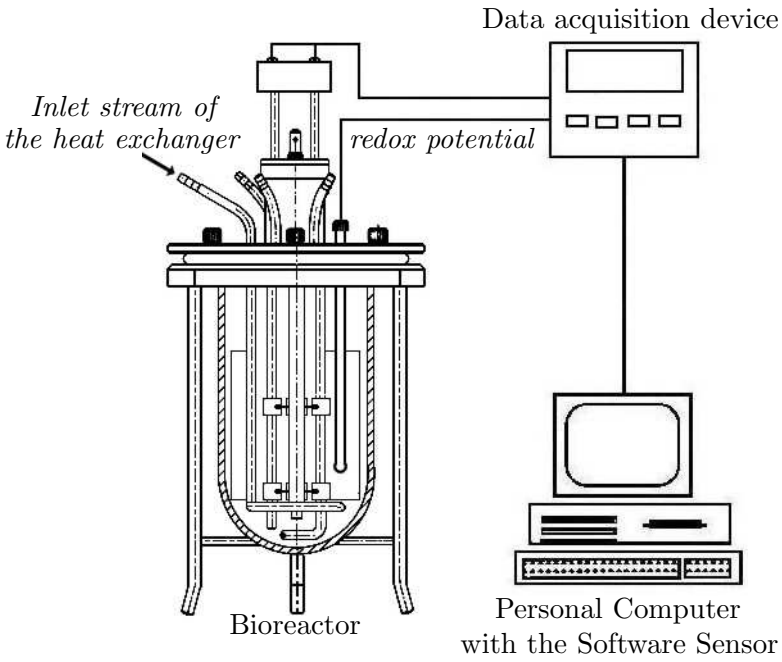

Fig. 1. The figure shows the schematic representation of the experiments carried out in our laboratory.

2.3 Redox potential

The measurement of *redox potential* is relatively fast, accurate and reliable and its values give an insight into the oxidation process as well as the inherent ability of reduction in the process, which is well established in the case of wine (Kukec *et al.* (2002)). We mentioned that improvements in manufacturing processes and equipment have allowed beer, wine, as well as Mezcal producers to increase both the quantity and quality of these products. It is crucial for the commercial competition to maintain good quality control practices. Higher good-quality productions of the manufacturing plants require faster and more sophisticated analytical techniques to achieve them.

The measured values of the *redox potential* can give information on redox reactions in wine, which have an important influence on its quality and stability (Kukec *et al.* (2002)). During storing and aging of wine, oxidation and reduction processes affect the character and taste of wine to a considerable extent (Kukec *et al.* (2002), Cheraiti *et al.* (2005)). We surmise that the same features could occur in the case of the Mezcal making. Checking this assumption is one of the main goals of this research. In the case of tequila, there exist previous efforts to understand the relation between the organoleptic properties and the

stages of the process (Benn & Peppard (1996), López (1999), López *et al.* (2001), Vallejo-Córdoba *et al.* (2004)). However, neither for Tequila nor for Mezcal there exist reports of online-measured variables that allow the monitoring of the fermentation process in real time.

In our experiments, the *redox potential* measurements were acquired periodically each 0.01 hr during 14 hrs, and the data were stored and computed on-line in a PC (see Fig. 1).

3 The Software Sensor

By the software sensor we mean the algorithm generated by the coupling of the ANN and the adaptive observer. In this section we develop step by step this type of algorithm. First, we present the mathematical model that stays as the background of our approach. Then we explain the relationship with the measured output, the *redox potential*, and the ANN scheme. We end the section with a discussion of the adaptive scheme used to infer the kinetic rate function.

3.1 Mathematical model of the fermentation process

Batch microbial growth in a completely stirred bioreactor is commonly described by the following differential equations (Bastin & Dochain (1986), Nielsen *et al.* (2003)).

$$\begin{aligned}\frac{\mathrm{d}X_1}{\mathrm{d}t} &= X_1\ \mu\left(t\right)\\ \frac{\mathrm{d}X_2}{\mathrm{d}t} &= -k_1 X_1\ \mu\left(t\right)\\ \frac{\mathrm{d}X_3}{\mathrm{d}t} &= k_2 X_1\ \mu\left(t\right)\end{aligned}$$

where X_1 represents the biomass concentration (g/L), X_2 refers to the substrate concentration (g/L) and X_3 is the product concentration (g/L); in addition, the parameters k_1, k_2 and $\mu\left(t\right)$ represents the biomass yield, the product yield and the growth rate, respectively. The latter one relates the change in biomass concentration to the substrate concentration. As mentioned in the introduction, many different analytical laws have been suggested for modeling $\mu\left(t\right)$ in alcoholic fermentations but here we infer it by means of the software sensor.

3.2 Artificial neural network

In order to relate the redox measurements to the ethanol and biomass concentration an ANN procedure is applied. The methodology that we carried out includes a forward-propagation training algorithm for the ANN using some of our experimental data. In order to perform our task we construct a model of the following form:

$$X_1 = f(X_4) \tag{1}$$

where X_4 represents the *redox potential* measurement data (mV), X_1 is the set of biomass concentration data (mg/L) and the function $f(X_4)$ is approximated by means of the ANN procedure. The ANN architecture is of the standard type (Lapedes *et al.* (1987)) with a single ANN hidden-layer containing 10 units. The same scheme was used for the case of the ethanol but using $X_3 = f(X_4)$ as the ANN model.

Each unit of this network uses a sigmoid function as the activation function. On the other hand, the output contains a linear activation function, in our case the identity. The feed-forward training algorithm considered here is the conjugate gradient method (Rumelhart & McClelland (1986)). Three of the six individual batch experiments were used to provide data for the training process. The ANN after the training gives an error of only 0.0029.

3.3 The adaptive observer

The analysis of the adaptive observer scheme used here is based on the following realistic assumptions:

(A1) The specific growth rate $\mu(t)$ is positive and bounded, that is μ_{max} exists but is unknown, although it is bounded: $0 < \mu(t) < \mu_{max}$
(A2) There is no growth without substrate: $X_2 = 0 \Rightarrow \mu(t) = 0$
(A3) The time derivative of $\mu(t)$ is bounded: $\left|\frac{\mathrm{d}\ \mu(t)}{\mathrm{d}t}\right| \leq M_1$, where $M_1 \in \mathbb{R}_+$.

3.3.1 On-line estimation of $\mu(t)$ from measurements of X_1

Since X_1 is available through the neuronal algorithm mentioned in the previous section, we can now rewrite the output of the system as:

$$y_s = X_1, \tag{2}$$

where y_s is the set of on-line measurements of the system which is available indirectly through ANN means. Then, following Bastin & Dochain (1986), the following algorithm can be used to estimate $\mu(t)$:

$$\begin{aligned} \frac{\mathrm{d}\,\hat{X}_1}{\mathrm{d}t} &= y_s\,\hat{\mu}\,(t) + \mathcal{K}_1\,y_s\left(y_s - \hat{X}_1\right) \\ \frac{\mathrm{d}\,\hat{\mu}}{\mathrm{d}t} &= \mathcal{K}_2\,y_s\left(y_s - \hat{X}_1\right), \end{aligned}$$

where $\hat{X}_1$ and $\hat{\mu}$ represent the estimated value of X_1 and μ, respectively. In others words, $\hat{X}_1$ stands for the estimated biomass concentration. The constants $\mathcal{K}_1$ and $\mathcal{K}_2$ must be chosen such that:

$$0 < \mathcal{K}_2 < \frac{\mathcal{K}_1^2}{4}. \tag{3}$$

The above condition ensures the asymptotic convergence of the observer error to a neighborhood of zero (Bastin & Dochain (1986)).

4 Results and Discussion

In the previous section, the general idea as well as the detailed steps for the construction of the software sensor were been given. To complete the analysis of the approach, this section presents the experimental results obtained at the laboratory level at which the software sensor was tested.

In total, six series of experiments of Mezcal fermentation at temperature of $32°C$ were performed. The temperature has been maintain constant using a heat exchanger device, see Fig. 1. First, we perform three experiments to generate the necessary biomass and ethanol concentration data for the training of the ANN. Once the error given by the ANN goes below the value of 0.003 biomass units, the adaptive scheme was added to complete the software sensor procedure. Then, three more experiments were carried out in order to further testing of the scheme. The results concerned with the performance of the ANN to infer the ethanol and biomass concentrations from the *redox potential* measurements are presented in the Figs. 2-3, where one can appreciate the degree of accuracy of the algorithm. Notice that the Fig. 3 presents several slope changes that do not allows to infer the presence of a mixture of microorganisms during the fermentation process.

Although the main goal is to estimate $\mu(t)$, the biomass estimation is also performed, and the difference between this estimation and the value predicted by

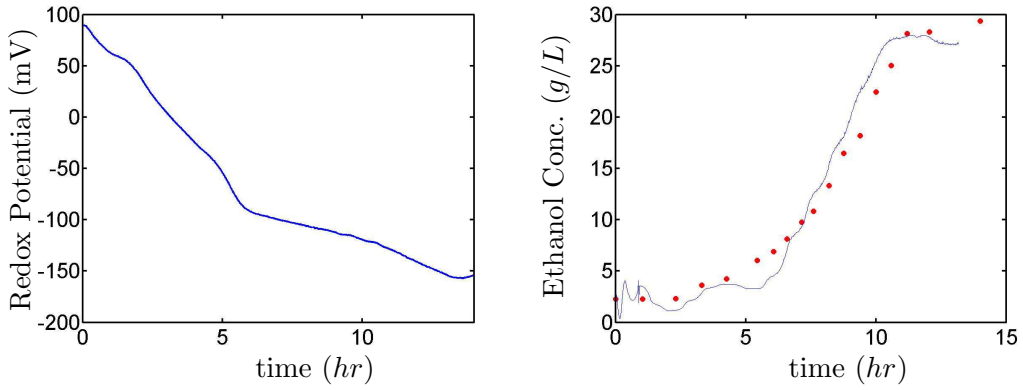

Fig. 2. The right plot illustrates the time evolution of the redox potential in the fermentation process. In the left plot the blue solid lines represent ANN-estimated ethanol concentration and red dots stand for its experimental values.

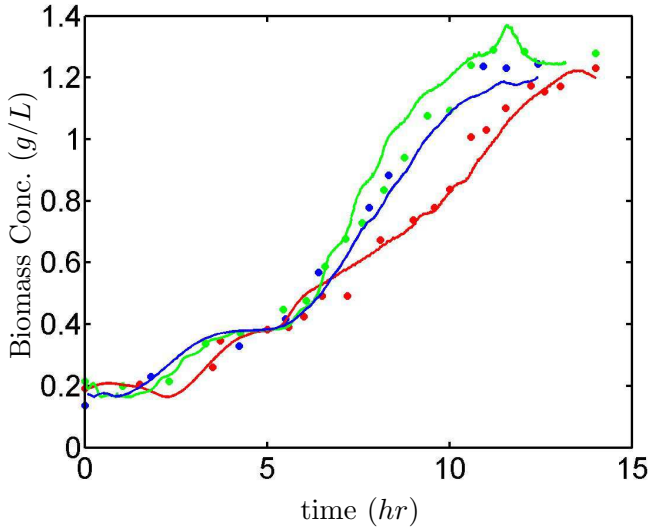

Fig. 3. The continuous lines correspond to the estimated biomass data and the dots to the experimental data. Blue, red and green correspond to each of the experiments carried out in our laboratory.

the ANN procedure is used as a correction term in the algorithm. Fig. 4 shows the ability of the software sensor to rebuild the biomass concentration data. It should be highlighted that the simplicity associated with the implementation of this algorithm and the necessity of a unique measured signal (*redox potential*) are very promising features from the technological and industrial stand points.

From Fig. 5, it is possible to discern three regions, labeled as $\mathcal{A}$, $\mathcal{B}$ and $\mathcal{C}$. In general terms, in region $\mathcal{A}$ one is not able to get any conclusion on the dynamical behavior of the process during this span of time since it corresponds to the transient behavior of the observer. the span of time corresponding to region $\mathcal{A}$ is given by the time that the observer takes up to minimize the

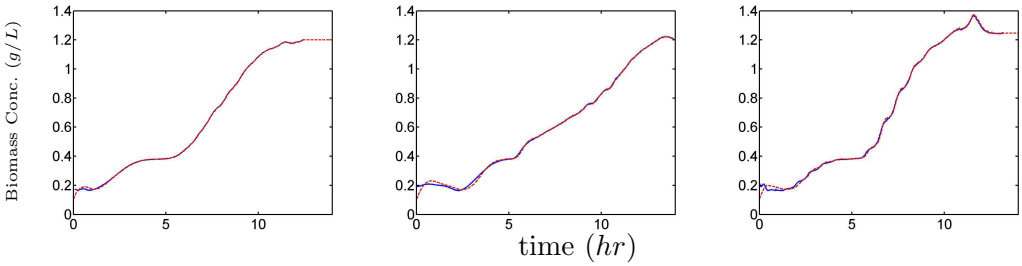

Fig. 4. (Blue) solid lines represent the estimated biomass concentration given by the software sensor and (red) dash-dotted lines stand for the ANN-predicted biomass. From the left to the right appears the plots corresponding to the three experiment performed to teste our approach are displayed.

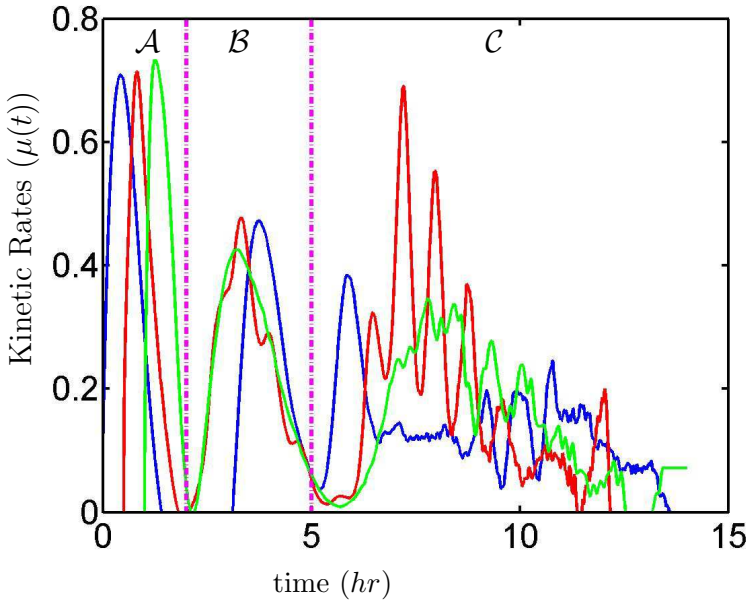

Fig. 5. The figure shows kinetic rates as estimated by the adaptive algorithm. The blue, red an green lines correspond to the estimation results for each of the experiments carried out in the bioreactor.

error between its estimated biomass concentration and that given b the ANN procedure.

On the other hand, we associate regions $\mathcal{B}$ and $\mathcal{C}$ with the presence of two different groups of microorganisms, namely bacteria and yeasts. This assumption is based on the fact that the growth rate of bacteria is faster than that of yeasts. Thus, from Fig. 5, the presence of a mixed culture growth comes out naturally, a fact which is not so obvious when one examines only the biomass data given in Fig. 3. Note in addition that the end of the fermentation process is quite clear in Fig. 5.

5 Concluding Remarks

In this work, we have shown that in the Mezcal fermentation process the *redox potential* could give relevant information on the microorganism metabolism, including both ethanol and biomass concentration. Besides, the problem of estimating the specific kinetic rates in the Mezcal fermentation process is treated. Moreover, the strategy here presented clearly detects the end of this fermentative process. The latter fact is quite relevant from the production point of view since it is the piece of information by which one can save time and avoid the degrading process due to the conversion of the ethanol to acetic acid, improving in this way the quality of the product. In addition, the computational scheme gives an very appropriate tool for quality control, helping to ensure the homogeneity of the final product. The methodology presented in this paper is general and can be also used for automatic control applications.

References

Cedeño, M., (1995), *Tequila Production*, Crit. Rev. Biotechnol., 15, 1-11.

De León-Rodríguez, A., González-Hernández, L., Barba de la Rosa, A.P., Escalante- Minakata, P., López, M.G. (2006), *Characterization of volatile compounds of mezcal, an ethnic alcoholic beverage obtained from Agave Salmiana.* J. Agric. Food Chem., 54, 1337-1341.

Bastin, G., Dochain, D., (1986) *On-line Estimation of microbial specific growth rates.* Automatica Vol. 22, No. 6, 705-709.

Locher, G., Sonnleitner, B., Fiechter, A., (1992) *On-line measurement in biotechnology: Techniques*, Journal of Biotechnology, 25, 23-53.

Farza, M., Busawon, K., Hammouri, H., (1998) *Simple Nonlinear Observers for on-line estimation of kinetic rates in bioreactors*, Automatica Vol. 34, No.3, 301-318.

Kwong, S.C.W., Randers, L., and Rao, G. (1992) *On-line assessment of metabolic activities based on culture redox potential and dissolved oxygen profiles during aerobic fermentation.* Biotechnol. Prog. 8, 576-579.

Berovič, M., (1999) *Scale-up of citric acid fermentation by redox potential control*, Biotech. and Bioeng., vol. 64, No. 5, sep 5.

van Dijk, C., Ebbenhorst-Selles, T., Ruisch, H., Stolle-Smits, T., Schijvens,

E., van Deelen, W., Boeriu, C., (2000) *Product and redox potential analysis of Sauerkraut fermentation.* J. Agric. Food Chem., 48, 132-139.

Cheraiti, N., Guezenec, S., Salmon, J.M., (2005) *Redox interactions between Saccharomyces cerevisiae and Saccharomyces uvarum in mixed culture under enological conditions*, Applied and Environmental Microbiology jan. 2005, p. 255-260.

Kukec, A., Berovič, M., Čelan, S., Wondra, M.,(2002) *The Role of On-line Redox Potential Measurement in Sauvignon blanc Fermentation*, Food Technol. Biotechnol. 40 (**1**), 49-55.

Novák, L., Larrea L., & Wanner, J. (1994), *Estimation of maximum specific growth rate of heterotrophic and autotrophic biomass: a combined technique of mathematical modelling and batch cultivations*, Water Sci. Technol. 30, 171-180.

Wanner, J. (1994). *Activated sludge bulking and foaming control*, Technomic Publishing Co., Inc., Lancaster, Pa.

Gujer, W., Henze,M., Mino, T., Matsuo, T., Wentzel, M. C., & Marais, G.R.. (1995), *The activated sludge model no. 2: biological phosphorus removal*, Water Sci. Technol. 31, 1-11.

Reeves, G.T., Narang, A., & Pilyugin, S.S., (2004), *Growth of mixed cultures on mixtures of substitutable substrates: the operating diagram for a structured model.* Journal of Theoretical Biology 226, 143-157

Ibarra-Junquera, V., Escalante-Minakata, P., Murguía-Ibarra, S., Rosu, H.C., (2006), *Inferring mixed-culture growth from total biomass data in a wavelet approach.* Physica A, 370 (**2**), 777-792.

Nielsen, J., Villadsen, J., Liden, G., *Bioreaction Engineering Principles* Kluwer Academic/Plenum Publishers (2003).

Benn, S. M. & Peppard T. L., (1996) *Characterization of tequila flavor by instrumental and sensory analysis.* J. Agric. Food Chem., 44, 557-556.

López, M.G., *Tequila aroma. In Flavor Chemistry of Ethnic Foods.* Shahidi and Ho Eds. Kluwer Academic/Plenum Publishers, New York, 1999; 211-217.

López, M.G.; Dufour, J.P. *Tequilas: Charm analysis of blanco, reposado, and aejo tequilas. In Chromatography-Olfactometry. The state of the art.* Leland, J.V., Schieberle, P., Buettner, A., Acree, T.E., Eds.;ACS

Sympusium Series 782; American Chemical Society: Washington, DC 2001; pp 60-72.

Vallejo-Córdoba, B.; González-Córdoba, A.F.; Estrada-Montoya, M.C., (2004) *Tequila volatile characterization and ethyl ester determination by solid phase microextraction gas chromatography/mass spectrometry analysis.* J. Agric. Food Chem., 52, 5567-5571.

Lachenmeier, D.W.; Sohnius, E.M.; Attig, R.; López, M.G. (2006), *Quantification of selected volatile constituents and anions in Mexican Agave spirits (Tequila, Mezcal, Sotol, Bacanora).* J. Agric. Food Chem., 54, 3911-3915.

Lapedes, A.S. & Farber, R.M., (1987). *Nonlinear signal processing using neural networks: Prediction and system modeling*, Los Alamos Report LA-UR-87-2662.

Rumelhart, D. & McClelland J., (1986). *Parallel Distributed Processing: Explorations in microstructure of cognition, Vol. 1, Foundations.* MIT Press, Cambridge, MA., 318-362.