Study of Process Variables in Industrial Acetic Fermentation by a Continuous Pilot Fermentor and Response Surfaces

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The modeling and optimization of industrial processes requires an intensive study of the factors involved. In this work, a continuous pilot system for studying the industrial process of acetic fermentation is developed. A Doehlert design is applied to the five variables involved in the pilot process. This experimental design allows reduction of the experimental burden and the maximum amount of information to be obtained, studying the factors at different levels depending on their significance. The experimental system provides a robust measure of the specific growth rate and the rates of substrates consumption and acetic acid production, related to the flow of effluent stream evaluated in the steady state. The results demonstrate the growth-associated kinetics of substrates and product, and the yield factors are calculated with low values of variances for the coefficients, i.e., within the range 1−11%. The specific growth rate suits the quadratic model proposed. The response surfaces generated by the model are applied to explain the behavior of the bacterial growth and, therefore, the effects of the process variables studied over the acetic acid production. Very low levels of ethanol or oxygen make the acetification rate decrease, and a saturation effect with high levels of ethanol or oxygen is also deduced. The effects of the aeration rate, agitation, and overpressure suggest a kind of inhibition of the acetic acid production caused by the oxygen that has not been practically studied before. The temperature strengthens the inhibitory effect of the ethanol and the oxygen. The conclusions of this work consolidate the structure of a hybrid model for the acetic fermentation.

Introduction

Industrial bioprocesses such as industrial acetification are complex systems that require a special effort to study the effects of the variables and the modeling of the process with the minimum experimental charge due to the high cost of the experiments both in terms of economy and time. Furthermore, it is necessary to develop pilot systems to reproduce industrial conditions if a predictive model is to be developed. Working with a proper experimental system is fundamental to evaluating the process variables and inferring the relevance of each variable and its cross-effects.

The relationships among the rates of ethanol and oxygen consumption, bacterial growth, and acetic acid production must be established to infer the kinetics of substrates and product and to calculate the yield factors. Once the relationship between the acetic acid production and the bacterial growth is known, the study of the specific growth rate, which provides the multiplication time of the cells, allows evaluation of the effect of the process variables over the acetic acid production, focusing particularly on the study of optimum conditions and inhibition effects.

Inhibition is one of the main phenomena to study when modeling an industrial bioprocess. Inhibition effects of acetic acid and ethanol on acetic fermentation have been extensively studied by several authors such as Bar et al. (*1*), Park et al. (*2*), Nanba et al. (*3*), Romero et al. (*4*), and Kruppa et al. (*5*). However, most of the time, the models were developed under idealized laboratory conditions and predict inhibition under acetic concentrations lower than 100 g/L, whereas industrial fermentation processes reach concentrations about 150 g/L (*6*). In industrial plants, ethanol is known to have negative effects at concentrations of about 50 g/L, especially when the reactor is not cooled sufficiently, and in industrial batch processes, no more than 50 g/L of initial ethanol is introduced into the fermentor.

It is also well-known that an interruption in the oxygenation longer than 30 s is sufficient to inactivate a large part of the bacterial population (*7*, *8*). On the other hand, the inhibitory effects of the oxygen supply on the acetic fermentation have not been properly considered. Only the effects of the oxygen concentration have been studied (*2*, *4*), proposing optimal concentrations of dissolved oxygen for the acetification rate. However, this approach is not suitable considering that dissolved oxygen is a response of the system due to the balance between oxygen supply and oxygen consumption.

Therefore, the variables involved in the oxygenation system are studied in this work. These variables for the pilot system developed are overpressure, air flow supply, and agitation. Other variables are temperature, which has a great impact on the growth cell kinetic (*9*), and

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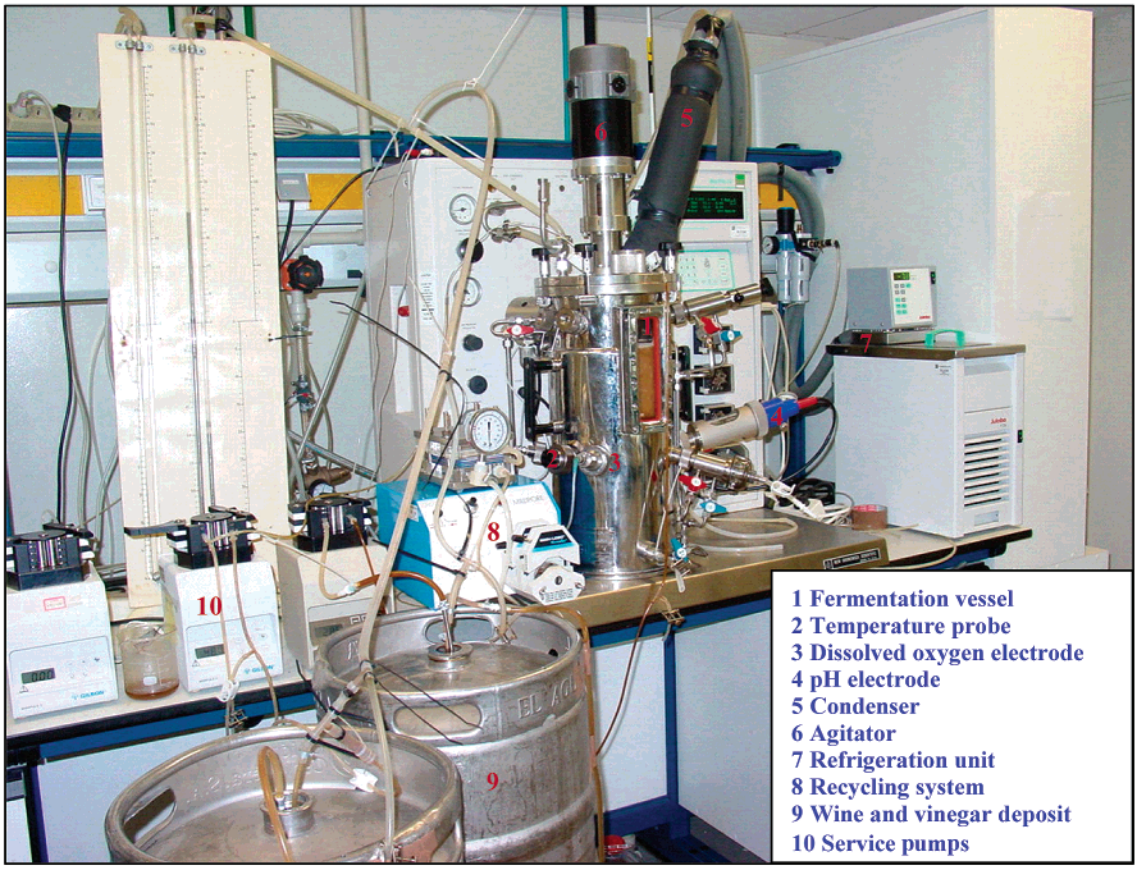

Figure 1. Pilot fermentation system.

ethanol concentration. The observations in industrial plants show that the acetification rate decreases only due to ethanol consumption, while an increase in the acetic acid concentration has no effect on the range of the industrial fermentors, about 120 g/L for wine acetification and about 150 g/L for alcohol acetification. The statistical model calculated for the specific growth rate is used to generate response surfaces that allow explanation of the influence of the process variables over the cell growth rate, inferring the inhibition effect caused by the strong oxygenation conditions. The response surface methodology with response surfaces built by quadratic models (*10*) has been successfully applied in different optimization problems (*11*, *12*), including those related to fermentation processes (*13*, *14*).

The robustness in the assessment of the specific growth values support the conclusions inferred for the acetic acid production and the application of the data to build future models for the acetic fermentation.

Experimental System

Pilot Fermentor. The experimental work was made using a New Brunswick (BioFlo IV) fermentor, shown in Figure 1, with a maximum working volume of 10 L, equipped with PID controllers of agitation and temperature in ranges of 50−1000 rpm and 5−85 °C, respectively. The temperature controller is a jacket of water. The sensitivities of the control units are ±1 rpm for the agitation rate and ±0.1 °C for temperature. A sparger on the back of the vessel homogeneously diffuses the air into the vessel. The air valve and the vessel pressure regulator control the air flow and overpressure, respectively. The fermentor is also equipped with a dissolved oxygen electrode and a pH meter.

The air flow was supplied at standard oxygen and nitrogen concentrations, and a condenser was installed on the vessel to reduce evaporation losses.

Online data of air flow, rpm, temperature, pH, and dissolved oxygen were obtained and saved in the database of the control software provided by New Brunswick.

Analytical Methods. Ethanol, acetic acid, ethyl acetate, and suspended matter concentrations were measured online by near-infrared spectroscopy, NIR (*15*), using a FOSS NIRSystems 5000 Liquid Analyzer.

Air flow measurements were provided by a mass flow sensor, and overpressure was measured with a barometer, both incorporated into the fermentor. Dissolved oxygen concentration was monitored by a sterilizable dissolved oxygen electrode, whereas pH was controlled by a pressurized pH electrode. Both electrodes were introduced into the vessel to obtain online readings.

A gas chromatograph Hewlett-Packard 5890 series II was connected to the gas outlet to measure online the concentration of oxygen, ethanol, acetic acid, and ethyl acetate in the exhaust line. The compounds were analyzed simultaneously by a system with two chromatographic columns and an electro valve. A molecular sieve (13 × 45/60 mesh) was used to separate oxygen and a packed column, with support carbopack B 80/20, to separate ethanol, acetic acid, and ethyl acetate.

Methodology

Statistical Model and Experimental Design. The behavior of *Acetobacter* is complex, and several mechanistic models have been developed to explain acetic fermentation (*1*−*6*). However, a second-order polynomial can be used to represent the impact of the five process variables studied, considering that good response sur-

faces are expected to be obtained. Quadratic models are extensively used in experimental design and higher-order models are used only when quadratic models are clearly inadequate (*9*). Several references have been reported in the Introduction (*11*−*14*) to show that surface methodologies with response surfaces using quadratic models are successfully applied in different optimization problems. Equation 1 shows the quadratic model for five variables:

$$Y = b_0 + \sum_{i=1}^{i=5} b_i X_i + \sum_{i=1}^{i=5} \sum_{j=1}^{j=5} b_{ij} X_i X_j \qquad (1)$$

where X_1 is the overpressure, X_2 is the ethanol concentration, X_3 is the vvm (air flow rate divided by reactor working volume), X_4 is the agitation rate and X_5 is the temperature.

The experimental design applied to study the variables and solve the quadratic model is the Doehlert uniform shell, also known as the Doehlert design (*10*, *16*). The Doehlert design describes a spherical domain with fewer points than a central composite design. This design is also more efficient in screening the experimental space, as it provides a uniform distribution of the experimental points in the space studied and has a high ability to explore the whole domain. Another relevant advantage has to do with the number of levels of each variable. With three variables, X_1, X_2, X_3, the numbers of levels are 5, 7, and 3, respectively. The levels are uniformly spaced and, regardless of the design variables, the minimum number of levels is 3, and then 5, and the rest of the variables take 7 levels. In this work, the levels are 5, 7, 7, 7, and 3. Therefore, the design, except for the two-variable design, is not rotatable, i.e., the variance of the prediction depends not on the direction studied from the central point but only on the distance from the central point. However, the deviation from this property is not excessive, and the different number of levels for each variable allows the levels of the variables to be set according to the criterion and know-how of the researcher.

Another important advantage of the Doehlert design is the possibility of extending or displacing the design in various directions reusing several previous experiments. It also allows addition of more variables reusing all the experiments of the previous stage. These properties have already been applied in sequential optimization (*17*).

The features of the Doehlert design reported in this section justify the choice of the design in this work, due to the characteristics of the experiments in the pilot fermentation, as it is described in following section. Table 1 shows a Doehlert design for five variables, with the variables codified.

Study of the Process Variables by Continuous Fermentation Processes. The following are the variables studied in this work.

Overpressure, vvm, and Agitation. Overpressure, vvm, and agitation are involved in the oxygen transfer, due to the design of the pilot fermentor.

Temperature. The temperature has a large impact on the behavior of the microorganisms (*9*). On the other hand, it also has a great influence on the oxygen dissolution. The optimum range of temperatures for the *Acetobacter* growth is within 25−30 °C (*18*), although there are some differences depending on the species Under 10 °C, growth is hardly possible (*19*), and the maximum temperature is between 35 and 45 °C. The *Acetobacter* is death when the temperature is higher than 55−60 °C. With this behavior, the *Acetobacter* can be classified as a mesophile (*9*).

Table 1. Doehlert Design for Five Variables

experiment	X_1	X_2	X_3	X_4	X_5
1	1.0000	0.0000	0.0000	0.0000	0.0000
2	−1.0000	0.0000	0.0000	0.0000	0.0000
3	0.5000	0.8660	0.0000	0.0000	0.0000
4	−0.5000	−0.8660	0.0000	0.0000	0.0000
5	0.5000	−0.8660	0.0000	0.0000	0.0000
6	−0.5000	0.8660	0.0000	0.0000	0.0000
7	0.5000	0.2887	0.8165	0.0000	0.0000
8	−0.5000	−0.2887	−0.8165	0.0000	0.0000
9	0.5000	−0.2887	−0.8165	0.0000	0.0000
10	0.0000	0.5774	−0.8165	0.0000	0.0000
11	−0.5000	0.2887	0.8165	0.0000	0.0000
12	0.0000	−0.5774	0.8165	0.0000	0.0000
13	0.5000	0.2887	0.2041	0.7906	0.0000
14	−0.5000	−0.2887	−0.2041	−0.7906	0.0000
15	0.5000	−0.2887	−0.2041	−0.7906	0.0000
16	0.0000	0.5774	−0.2041	−0.7906	0.0000
17	0.0000	0.0000	0.6124	−0.7906	0.0000
18	−0.5000	0.2887	0.2041	0.7906	0.0000
19	0.0000	−0.5774	0.2041	0.7906	0.0000
20	0.0000	0.0000	−0.6124	0.7906	0.0000
21	0.5000	0.2887	0.2041	0.1581	0.7746
22	−0.5000	−0.2887	−0.2041	−0.1581	−0.7746
23	0.5000	−0.2887	−0.2041	−0.1581	−0.7746
24	0.0000	0.5774	−0.2041	−0.1581	−0.7746
25	0.0000	0.0000	0.6124	−0.1581	−0.7746
26	0.0000	0.0000	0.0000	0.6325	−0.7746
27	−0.5000	0.2887	0.2041	0.1581	0.7746
28	0.0000	−0.5774	0.2041	0.1581	0.7746
29	0.0000	0.0000	−0.6124	0.1581	0.7746
30	0.0000	0.0000	0.0000	−0.6325	0.7746
31	0.0000	0.0000	0.0000	0.0000	0.0000
32	0.0000	0.0000	0.0000	0.0000	0.0000
33	0.0000	0.0000	0.0000	0.0000	0.0000
34	0.0000	0.0000	0.0000	0.0000	0.0000
35	0.0000	0.0000	0.0000	0.0000	0.0000

Table 2. Variables Studied

variable	range	levels
X_1= overpressure	0−1 atm	5
X_2= ethanol	1−50 g/L	7
X_3= vvm	3−37 h^{-1}	7
X_4= agitation	200−1000 rpm	7
X_5= temperature	26−33 °C	3

Ethanol. It has been observed that, in industrial fermentors, the acetification rate depends only on the ethanol consumption when the rest of the variables are constant. An increase in the acetic acid concentration has no effect on the results reached in industrial fermentors, about 120 g/L for wine acetification and about 150 g/L for alcohol acetification.

Table 2 shows the levels and the range studied for each variable. Ethanol, vvm, and agitation have a larger number of levels because, considering our prior knowledge of fermentation processes, these are the main variables that affect the two substrates of the fermentation process, i.e., ethanol and oxygen. Furthermore, these variables are studied covering a large range of values, and thus more levels are required. Temperature is studied at three levels given that the range studied is narrow and corresponds to the well-known optimum range of temperatures for the *Acetobacter*. Finally, overpressure can only take five levels.

The experimental plan was covered by continuous processes maintaining constant the values of the variables for each experiment. The experimental system is shown Figure 1. Overpressure, agitation, air flow, and temperature were fixed by the controls provided by the fermentor as it has been described before. The feed

stream was under a proportional, integrative, and derivative (PID) control to hold constant the pH and the concentration of ethanol. The pH set was adjusted for each case to reach a stationary state with an ethanol concentration close to the concentration proposed by the Doehlert design. It is clear that the concentrations of acetic acid and other compounds were also constant when each stationary state was reached and the corresponding ethanol concentration was fixed. The acetic acid concentrations were within the range 57−132 g/L, and the biomass concentrations were within the range 0.21−0.60 g/L; the ethyl acetate concentrations were within the range 0.02−3.6 g/L, and the pH values were within the range 2.5−3. The white wine commonly used by a vinegar company from La Rioja was introduced into the fermentors without any pretreatment. The volume in the fermentor was kept constant during each experiment, within the range 5−9 L, and thus the effluent and feed stream took the same values for the volumetric flow rate, F.

Each experiment was subjected to the same conditions at least for 168 h, until constant values of ethanol, acetic acid, and biomass concentrations, dissolved oxygen, and flow rate, calculated by measuring the wine consumption in a given period, were obtained. At this time, it was considered that a stationary state had been reached. Once the stationary state was reached, the conditions were maintained for 72 more hours, taking measurements of the concentrations of ethanol, acetic acid, and biomass every 1 h and of the rest of the variables (pH, dissolved oxygen, etc.) every 15 min. This implied that the stationary state was assessed and robust measurements of the concentrations in the medium and the average flow rate, calculated with the liters of wine consumed in the 72 h, were obtained.

As it can be inferred, the experimentation was very time-consuming, since a successful experiment took at least 240 h. An intelligent distribution of the experimental burden was needed. Therefore, the application of the Doehlert design is justified.

In the steady state, all the concentrations within the vessel are unrelated to time, and therefore it is possible to apply this balance to any component i of the system:

$$F(C_{if} - C_i) + Vr_{fi} = 0 \tag{2}$$

where F is the volumetric flow rate of feed and effluent liquid streams, C_{if} is the concentration of component i in the feed stream, C_i is the concentration of component i in the bioreactor and in the effluent stream, V is the reactor volume, and r_{fi} is the rate of formation of component i within the reactor.

The expression can be rearranged to the following:

$$r_{fi} = \frac{F}{V}(C_i - C_{if}) = D(C_i - C_{if}) \tag{3}$$

The parameter D is the dilution rate and represents the holding time or processing rate of the continuous reactor.

Use of eq 3 as a starting point allows the acetification production, ethanol consumption, ethyl acetate formation, and growth rates to be evaluated. As it has already been explained, the concentrations and feed stream were robust, and therefore the rates calculated were also robust. The robustness of the results is one of the main advantages of continuous processes applied to kinetic modeling. Each stationary state allows the system to be studied as a snapshot. Ethanol and acetic acid losses by evaporation were reduced by the condenser installed in the vessel of the fermentors. Furthermore, the measures of ethanol and acetic acid concentration in the gas stream allowed to calculate the evaporation rates of the compounds, which were almost negligible thanks to the condenser. The ethyl acetate formation rate, r_{EA}, was used to calculate the ethanol and concentration losses due to the formation of this compound. Therefore, eq 3 was adapted to obtain the rates of ethanol consumption, r_E, and acetic acid production, r_A, due exclusively to the fermentation process:

$$r_{EA} = D(EA - EA_f) - r_{EA-Ev} \tag{4}$$

$$r_E = D(E - E_f) - r_{E-Ev} - r_{E-EA} \tag{5}$$

$$r_A = D(A - A_f) - r_{A-Ev} - r_{A-EA} \tag{6}$$

EA, E, and A are the concentrations of ethyl acetate, ethanol, and acetic acid, respectively, in the bioreactor and in the effluent stream. EA_f, E_f, and A_f are the concentrations of ethyl acetate, ethanol, and acetic acid, respectively, in the feed stream. r_{EA-Ev}, r_{E-Ev}, and r_{A-Ev} are the evaporation rates of ethyl acetate, ethanol, and acetic acid, respectively. r_{E-EA} and r_{A-EA} are the consumption rates of ethanol and acetic acid, respectively, due to ethyl acetate formation. These consumption rates are related to the ethyl acetate formation rate by $Y_{(EA/E)}$ (1.91) and $Y_{(EA/A)}$ (1.47), which are the stoichiometric coefficients of ethyl acetate/ethanol and ethyl acetate/acetic acid, respectively:

$$r_{E-EA} = -\frac{1}{Y_{(EA/E)}} r_{EA} \tag{7}$$

$$r_{A-EA} = -\frac{1}{Y_{(EA/A)}} r_{EA} \tag{8}$$

From an unstructured approach (*9*), the biophase is characterized by the cell mass or the number concentrations. The biomass is then considered as a component in solution, X. The specific growth rate, μ_g, is defined as

$$\mu_g = \frac{1}{X} r_X \tag{9}$$

where X is the biomass concentration in the bioreactor and in the effluent stream, and r_X is the biomass growth rate.

In this work, the suspended matter concentration includes the bacteria cells, in suspension in the medium, and all the suspended solids and colloidal matter that come with the wine. In other works, artificial mediums have been used and the assessment of the biomass concentration was easier, because the suspended matter was only due to the cells. In this case, the NIR system measures all the suspended matter, without any differentiation, but it can be considered that the amount of bacterial cells in wine is negligible, taking into account the storing conditions of the wine. Therefore, the increase in suspended matter in the bioreactor and in the effluent stream is due to the cell growth. From this approach, the biomass concentration is the difference between the suspended matter in the wine and the suspended matter in the bioreactor:

$$r_X = D(SM - SM_f) = DX \tag{10}$$

where SM is the concentration of suspended matter in the bioreactor and in the effluent stream, and SM_f is the concentration of suspended matter in the feed stream.

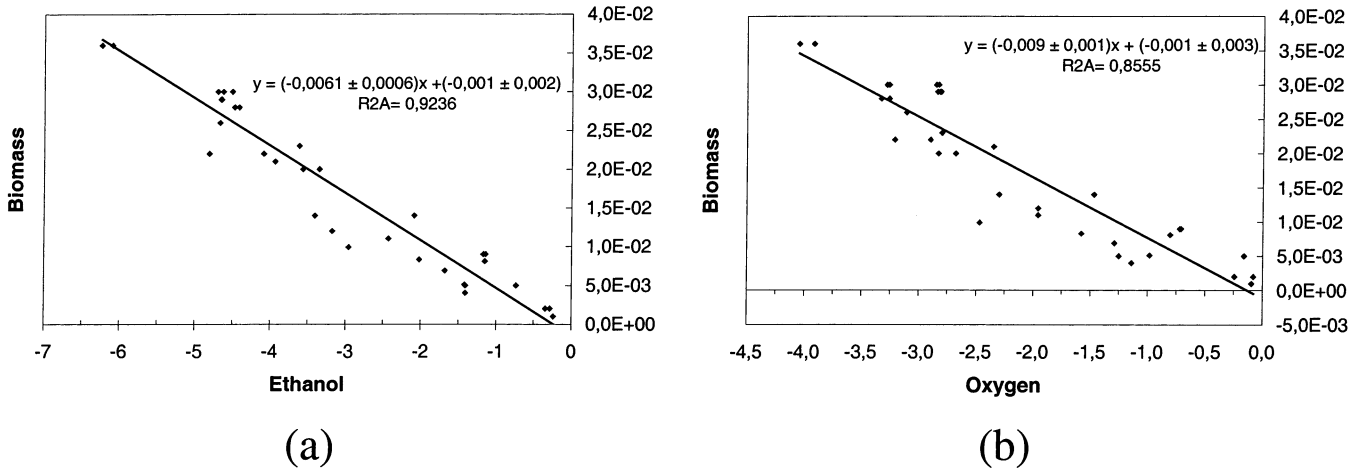

Figure 2. (a) Cell growth rate vs ethanol consumption rate. (b) Cell growth rate vs oxygen consumption rate.

It is obvious by comparing eqs 9 and 10 that, in this experimental system, the specific growth rate is equal to the dilution rate:

$$\mu_g = D \tag{11}$$

The assessment of the specific growth rate is critical for the study of a biosystem, because it evaluates the multiplication time of the average cell in the steady state, making negligible the cell-to-cell heterogeneity, from an unsegregated approach (*9*). The experimental system developed allowed a robust measurement of the volumetric flow rate of wine consumption and vinegar production, F, and therefore of the dilution rate and the specific growth rate. The novelty of the experimental system lies in the fact that the volumetric flow rate is not forced but is the response of the system and adapts to the requirements of the bacterial growth.

Finally, the oxygen consumption rate was calculated using this mass balance:

$$r_{O_2} = \text{vvm}(O_{2(i)} - O_{2(f)}) \tag{12}$$

where $O_{2(i)}$ is the oxygen concentration in the air effluent stream, calculated by a gas chromatograph analyzing on line, and $O_{2(f)}$ is the oxygen concentration in the air feed stream, calculated on the basis of the oxygen concentration in air at 1 atm and 25 °C.

Results and Discussion

The study of a biosystem requires two parts: the relationships among the rate of substrate consumption and the rate by which products are secured, as well as the behavior of the cell growth. These two parts are covered in this section for the acetic fermentation.

Relationships Among Responses: Calculation of Yield Factors. Figure 2a represents the growth rate vs the ethanol consumption rate, and Figure 2b represents the growth rate vs the oxygen consumption rate. Figure 3 shows the acetic acid production rate vs the ethanol consumption rate. Figure 4 shows the relationships between the rates of ethanol consumption and acetification, respectively, and the oxygen consumption rate. The rates were obtained from the pilot fermentor as it was explained in the Methodology Section. The units are g L^{-1} h^{-1}. Other contributions due to the evaporation losses and the production of ethyl acetate were taken into account, and therefore the values of these rates are only due to the biological process. Oxygen consumption was considered to be due only to the fermentation process.

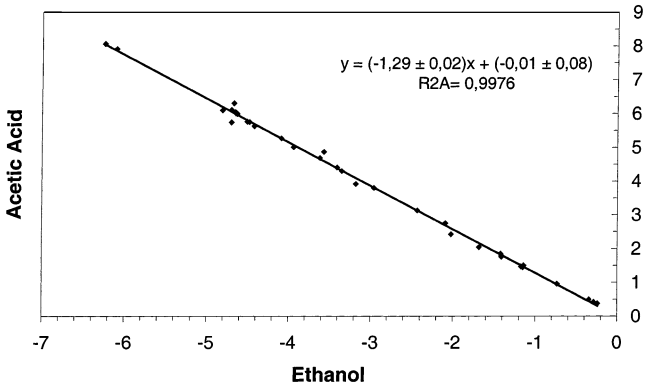

Figure 3. Acetic acid production rate vs ethanol consumption rate.

The distribution of the experimental points, in each graphic, is clearly suited to the linear tendency proposed. The good values obtained for the adjusted correlation rate, r^2A, in each case, show the suitability of the model. Therefore, the consumption rates of ethanol and oxygen, r_E and r_{O_2}, and the production of acetic acid, r_A, due to the fermentation process are linearly proportional to the rate of bacterial growth, r_X:

$$r_E = \left(-\frac{1}{Y'_{X/E}}\right) r_X \tag{13}$$

$$r_{O_2} = \left(-\frac{1}{Y'_{X/O}}\right) r_X \tag{14}$$

$$r_A = Y'_{A/E} r_E = Y'_{A/E} \frac{1}{Y'_{X/E}} r_X \tag{15}$$

where $Y'_{X/E}$ is the yield factor of biomass/ethanol (g of biomass/g of ethanol), $Y'_{X/O}$ is the yield factor of biomass/oxygen (g of biomass/g of oxygen), and $Y'_{A/E}$ is the yield factor of acetic acid/ethanol (g of acetic acid/g of ethanol). The biological support of this approach is the assumption that the bacteria oxidize ethanol with oxygen to meet the energy requirements of the multiplication process.

The values of the yield factors are calculated by fitting the data into the linear model. Table 3 reports the values of the yield factors obtained by the slopes of the linear

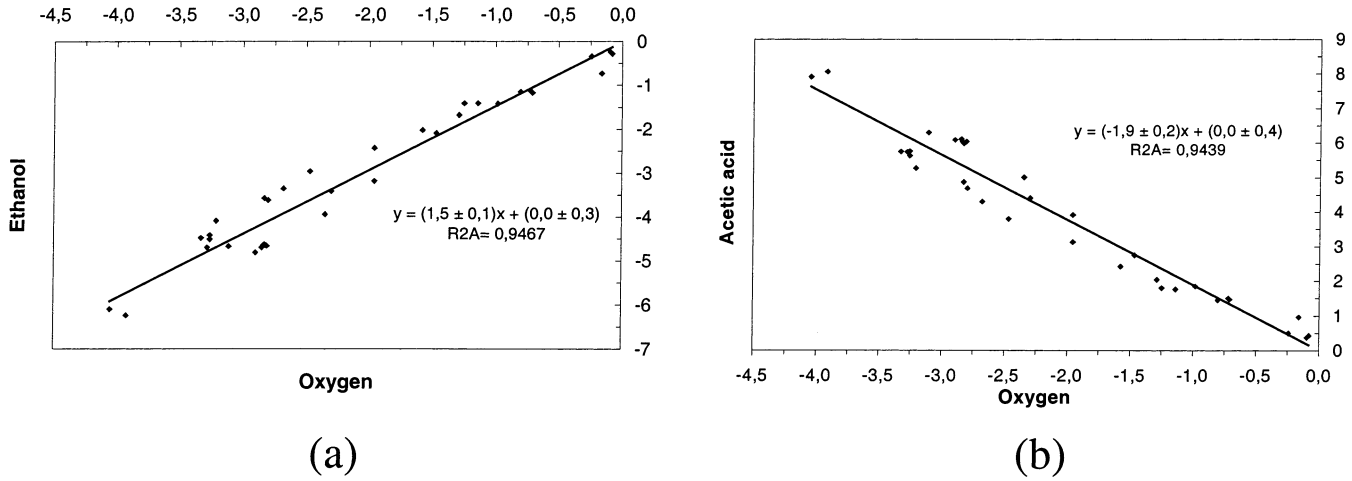

Figure 4. (a) Ethanol consumption rate vs oxygen consumption rate. (b) Acetic acid production rate vs oxygen consumption rate.

Table 3. Summarized Yield Factors

yield factor	value
$Y'_{A/E}$	1.29 ± 0.02
$Y'_{A/O}$	1.9 ± 0.2
$Y'_{E/O}$	1.5 ± 0.1
$Y'_{X/E}$	0.0061 ± 0.0006
$Y'_{X/O}$	0.009 ± 0.001

regressions. The value of the yield factor $Y'_{X/O}$ can also be obtained by the following mathematical relationship:

$$Y'_{X/O} = Y'_{X/E}Y'_{E/O} \quad (16)$$

The 95% interval confidence of the experimental yield factors $Y'_{A/E} = 1.29 \pm 0.2$ g/g, $Y'_{E/O} = 1.5 \pm 0.1$ g/g, and $Y'_{A/O} = 1.9 \pm 0.2$ g/g include the values of the respective stoichiometric coefficients $Y_{A/E} = 1.30$ g/g, $Y_{E/O} = 1.44$ g/g, and $Y_{A/O} = 1.88$ g/g. Provided that the rates calculated were only due to the fermentation process, yield factors that showed the stoichiometric rates of the oxidation of ethanol with oxygen to give acetic acid were expected. No oxygen consumption due to cell respiration or ethanol consumption due to the maintenance requirements of the cells can be inferred from these results, taking into account that, in all the regressions, the 95% confidence interval of the *y*-intercept includes the zero value. In fact, it is reasonable to consider that the energy requirements of the bacteria in the acetic fermentation are basically due to the multiplication process and those other consumptions are insignificant (*6*, *20*). The value calculated for $Y'_{X/E}$, 0.0061 ± 0.0006 g/g, is close to the value obtained by Bar (*1*), i.e., 0.006 g/g.

The values of the yield factors are very useful to model the acetic fermentation, since the variances of the coefficients are very low, i.e., 1.6% for $Y'_{A/E}$, 11% for $Y'_{A/O}$, 6.7% for $Y'_{E/O}$, 9.8% for $Y'_{X/E}$, and 10% for $Y'_{X/O}$, taking into account the complexity of the calculations of this kind of system, especially the coefficients related to biological variables such as *X*.

Characterization of the Behavior of the Specific Growth Rate by Response Surfaces. It has been explained in the Methodology Section that the study of the specific growth rate is critical for the understanding of a biosystem, since it evaluates the multiplication time of the average cell in the steady state. In this section, the behavior of the specific growth rate is explained by the surface responses drawn for the specific growth rate over the process variables. The response surfaces have been generated by the quadratic model solved for the specific growth rate and shown in eq 1. The good value, 0.984, obtained for the adjusted correlation rate, r^2A, proves the suitability of the quadratic model proposed.

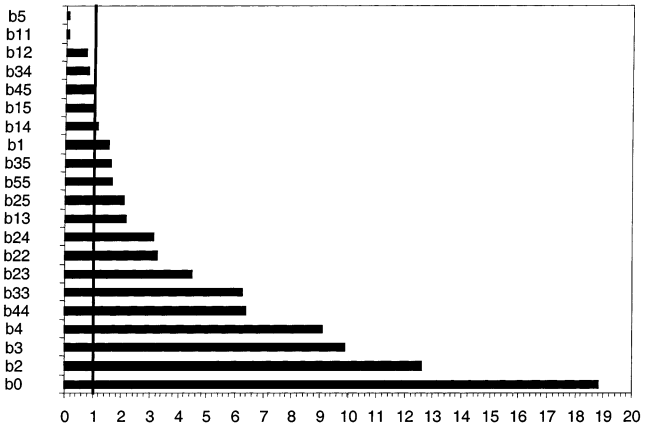

Figure 5. Pareto diagram of the effects of the coefficients of the quadratic model on the specific growth rate.

All the calculations and representations of the response surfaces have been provided by the program NEMROD-W, version 9901.

Figure 5 shows the Pareto diagram (*10*) of the values of the parameter *t*-experimental divided by the value of *t*-student for $\alpha = 0.025$ and 14 degrees of freedom (35 experiments for 21 coefficients). When this estimator is equal to or greater than 1, the coefficient is considered to be statistically significant. The higher the estimator is, the higher the effect of the coefficient on the response. The parameter *t*-experimental is calculated by the software as the value of the coefficient divided by the standard deviation.

The coefficients b_4, b_3, and b_2, which correspond to agitation, vvm, and ethanol concentration, are, as expected, the most relevant ones. In the Methodology Section it has been explained that, due to their significance, these variables were studied at seven levels. The value of the estimator for the coefficient b_5, which corresponds to the variable temperature, is the lowest of the individual coefficients. Figure 5 also shows that the coefficient b_1, which corresponds to overpressure, is the fourth coefficient in terms of significance. In conclusion, the variables studied at more levels are more important, thus justifying the ab initio arrangement of levels.

The linear effect of the temperature is considered to be insignificant according to this criterion. The range of

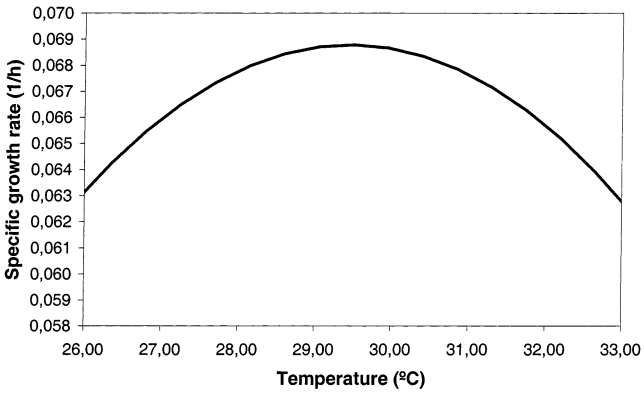

Figure 6. Specific growth rate over temperature. The rest of the parameters are kept constant and equal to the central values.

values studied for temperature is narrow; therefore, its effect on the specific growth rate in this range is limited, and this variable is statistically insignificant for the quadratic model. However, this does not imply that temperature does not have any influence on the specific growth rate. Furthermore, the second-order interactions of ethanol−temperature, vvm−temperature, and overpressure−temperature are very important and statistically significant. Figure 6 shows the specific growth rate over temperature, with all the other parameters constant and equal to the central values. The influence of the temperature on the values of the specific growth rate is limited due to the narrow range, and the response changes less than 0.01 h^{-1} over the whole range. However, it is well-known (*18*, *19*) than temperatures out of this range would cause a decrease in the acetification rate, thus making unnecessary the experimentation with a larger range.

The characterization of a response surface by a quadratic model allows the study of the effect of second-order interactions on the response. The information provided by the second-order interaction can be critical to justifying the behavior of a system. The next second-order interactions are very important for the specific growth rate, taking into account their statistical significance:

Effect of Substrates. The response surfaces of ethanol−vvm and ethanol−agitation are shown in Figure 7. The graphics prove the large influence of the air flow, agitation, and ethanol concentration. Furthermore, the second order interaction ethanol−vvm is the most important, taking into account their statistical significance. The specific growth rate is dramatically reduced when one of these variables is fixed within a low range of values, regardless of the other variables. It must be taken into account that these variables have a direct impact on the supply of the substrates, oxygen and ethanol. The response surface shows a saturation effect with high values of ethanol concentration and vvm or agitation, without a significant increase in the response. In fact, the specific growth rate is almost constant at these levels.

Interactions of Factors Involved in Oxygenation. Inhibition Due to Oxygen. Figure 8 shows the response surfaces of the specific growth rate over pairs of variables that are involved in the oxygenation, i.e., overpressure, vvm, and agitation, for the pilot fermentor. The effects of the oxygen transfer on the bioprocesses have not been studied as extensively as others, and it is more difficult to find references in the literature such as refs 21 or 22.

From the graphics, it can be inferred that an increase in the oxygenation conditions above a maximum value has a negative effect on the specific growth rate. This behavior is particularly clear in the case of overpressure and vvm (Figure 8a). The effect of overpressure on the specific growth rate depends on the value of vvm. High levels of vvm, above 20 h^{-1}, cause overpressure to have a negative effect on the specific growth rate, whereas low levels of vvm cause overpressure to have a positive effect. Figure 9a depicts this behavior in a graphic of the specific growth rate vs overpressure at different levels of vvm. Figure 9b shows the same graphic at different values of

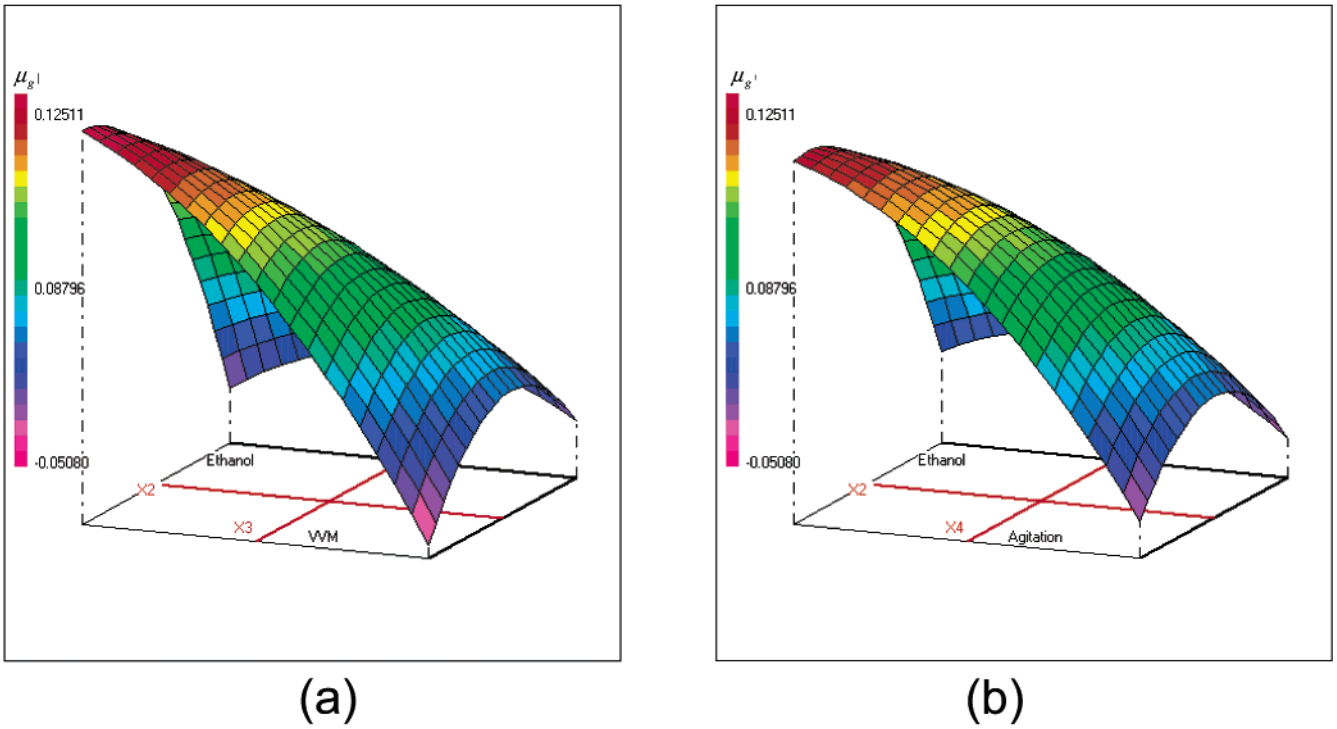

Figure 7. Response surface of the specific growth rate over (a) ethanol and vvm and (b) ethanol and agitation. The rest of the parameters are kept constant and equal to the central values.

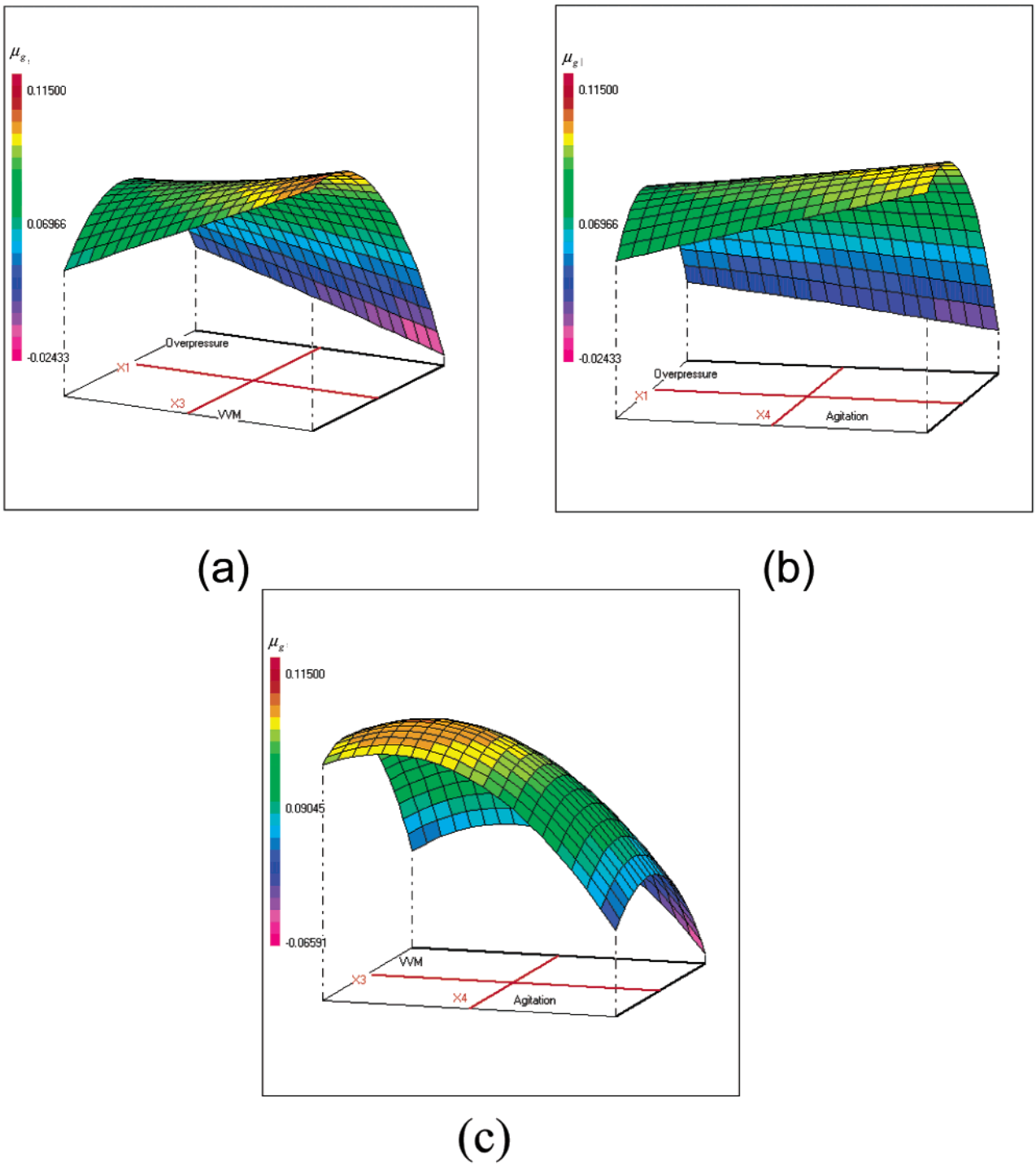

Figure 8. Response surface of the specific growth rate over (a) overpressure and vvm, (b) overpressure and agitation, and (c) vvm and agitation. The rest of the parameters are kept constant and equal to the central values.

agitation. The effect of overpressure on the specific growth rate is similar to the one shown in Figure 9a, and an increase in overpressure has a negative effect on the response when the agitation rate is higher than 400 rpm. The inhibition due to the oxygenation conditions is also reported in Figure 9c, which shows the dependence of the specific growth rate on the vvm at different levels of agitation. There is a maximum between 25 and 30 h^{-1} in all the sequences. The sequences increase their values when the agitation is increased, but those related to agitation rates higher than 644.4 rpm are practically together in Figure 9b and their values of specific growth rates are higher than those of the sequence corresponding to 1000 rpm.

The influence of the variables on dissolved oxygen is also important to understanding the effect of the oxygen transfer over the specific growth. The following mass balance equation describes the variation over time of the dissolved oxygen concentration (DO) in a cell suspension in contact with a gas phase:

$$\frac{dDO}{dt} = OTR - OUR \qquad (17)$$

where DO is the dissolved oxygen concentration in the

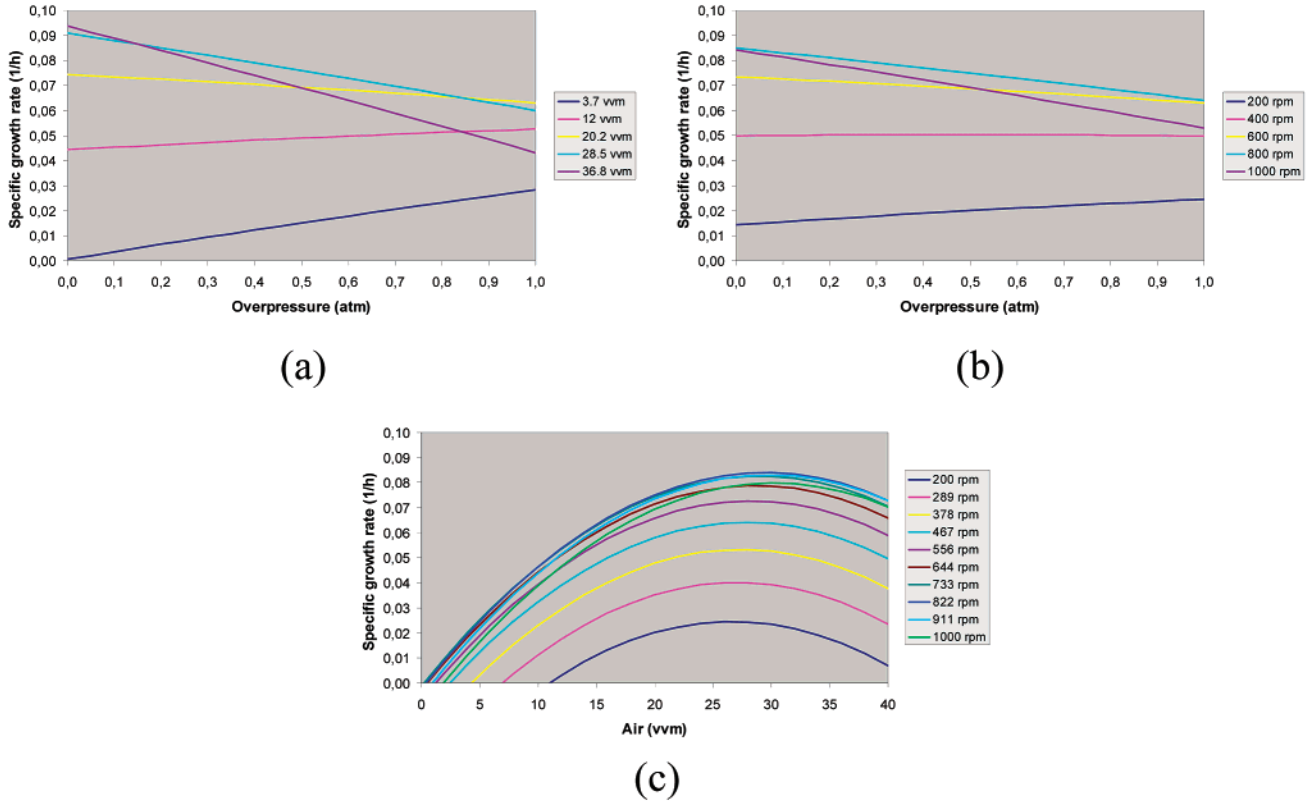

Figure 9. Specific growth rate vs (a) overpressure (for different values of vvm), (b) overpressure (for different values of agitation), and (c) vvm (for different values of agitation). The rest of the parameters are kept constant and equal to the central values.

medium, OTR is the oxygen transfer rate, and OUR is the oxygen uptake rate. Taking into account that the oxygen consumption has been considered before to be due only to the fermentation process, OUR $= -r_{O_2}$, and considering the general relation for the oxygen transfer tate (*23*), then

$$\frac{\mathrm{dDO}}{\mathrm{d}t} = \mathrm{kla}(\mathrm{DO}^* - \mathrm{DO}) - \left(\frac{1}{Y'_{\mathrm{X/O}}}\right) r_{\mathrm{X}} \qquad (18)$$

where DO* is the saturation dissolved oxygen concentration, and kla is the oxygen transfer constant, which depends on the oxygenation conditions, the rheology of the medium, and the features of the turbine.

Obviously, in the steady state, a constant dissolved oxygen concentration is reached, and therefore

$$\frac{\mathrm{dDO}}{\mathrm{d}t} = 0 \qquad (19)$$

$$\mathrm{kla}(\mathrm{DO}^* - \mathrm{DO}) = \left(\frac{1}{Y'_{\mathrm{X/O}}}\right) r_{\mathrm{X}} \qquad (20)$$

Considering these mass balances, when the growth rate and, therefore, the OUR, decrease, due to the values of the process variables, the dissolved oxygen increases because the oxygen supply surpasses the requirements of the bacteria. On the other hand, when the residual oxygen dissolved in the medium is close to zero, the bacteria is consuming all the oxygen provided and the oxygenation can be inadequate to satisfy the cell requirements. Therefore, dissolved oxygen is a response of the system, but not a variable, as it has been traditionally considered. A quadratic model similar to that proposed for the specific growth rate was calculated for the dissolved oxygen, with an adjusted correlation rate of 0.8, and response surfaces similar to those drawn for the specific growth rate were generated by the model. Figure 10 compares these response surfaces. In each case, the response surface for dissolved oxygen is the inverse of the response surface for the specific growth rate. Therefore, the inhibition due to the oxygen increases the residual oxygen (Figures 10d and 10e), as a large amount of nonconsumed oxygen is provided, in the same way that it also increases due to the effect of other variables such as ethanol consumption (Figure 10f).

All in all, the response surfaces generated show an inhibitory effect of the oxygen transferred to the medium over the specific growth rate. This inhibitory effect should be taken into account in future development of new fermentation systems, since the oxygenation is one of the most important energetic consumers in the design of a fermentor. As a matter of fact, most of the industrial fermentors are based on the *Acetator Frings* developed from the original works of Ebner and Hromatka (*24*), the pioneers of submerged acetification. The *Acetator* provides agitation and aeration simultaneously by an autoaspirant system. The air supply depends only on the rate of agitation and, therefore, the energy consumed. In this system, a useless increase in the oxygenation supposes an important waste of energy and, therefore, a significant economic loss.

Influence of Temperature on Inhibitory Effects. The response surfaces are shown in Figure 11. From Figure 11a, it can be inferred that high concentrations of ethanol have a negative effect at temperatures higher than the central point. It has already been explained that it is well-known that ethanol has a negative effect on acetification at concentrations higher than 50 g/L. Indeed, in industrial batch processes, the initial concentration is always under 50 g/L, as higher concentrations hinder the start of the fermentation. The maximum level of ethanol concentration reached during the experi-

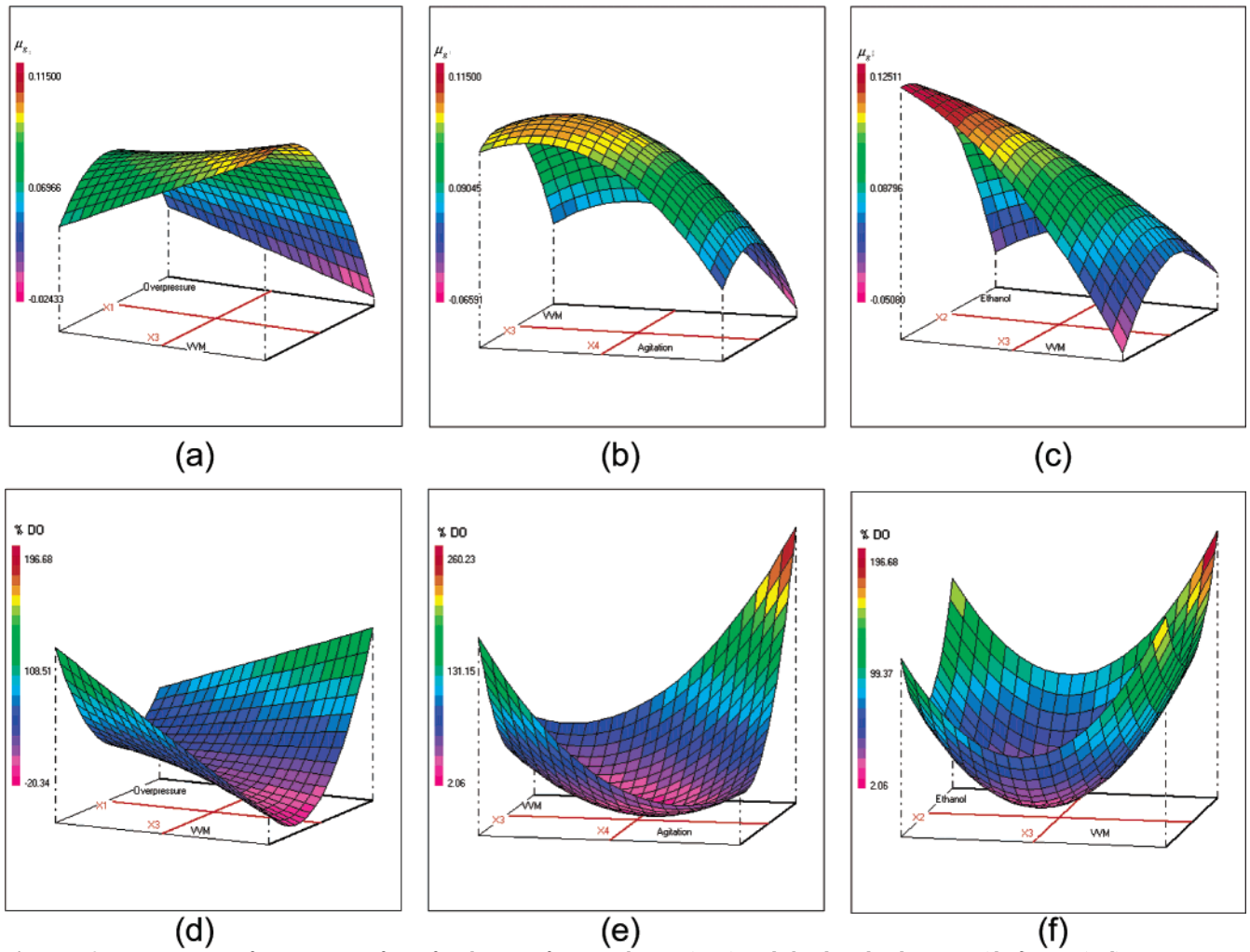

Figure 10. Comparison of response surfaces for the specific growth rate (a−c) and the dissolved oxygen (d−f) over (a,d) overpressure and vvm, (b,e) vvm and agitation, and (c,f) ethanol and vvm. The rest of the parameters are kept constant and equal to the central values.

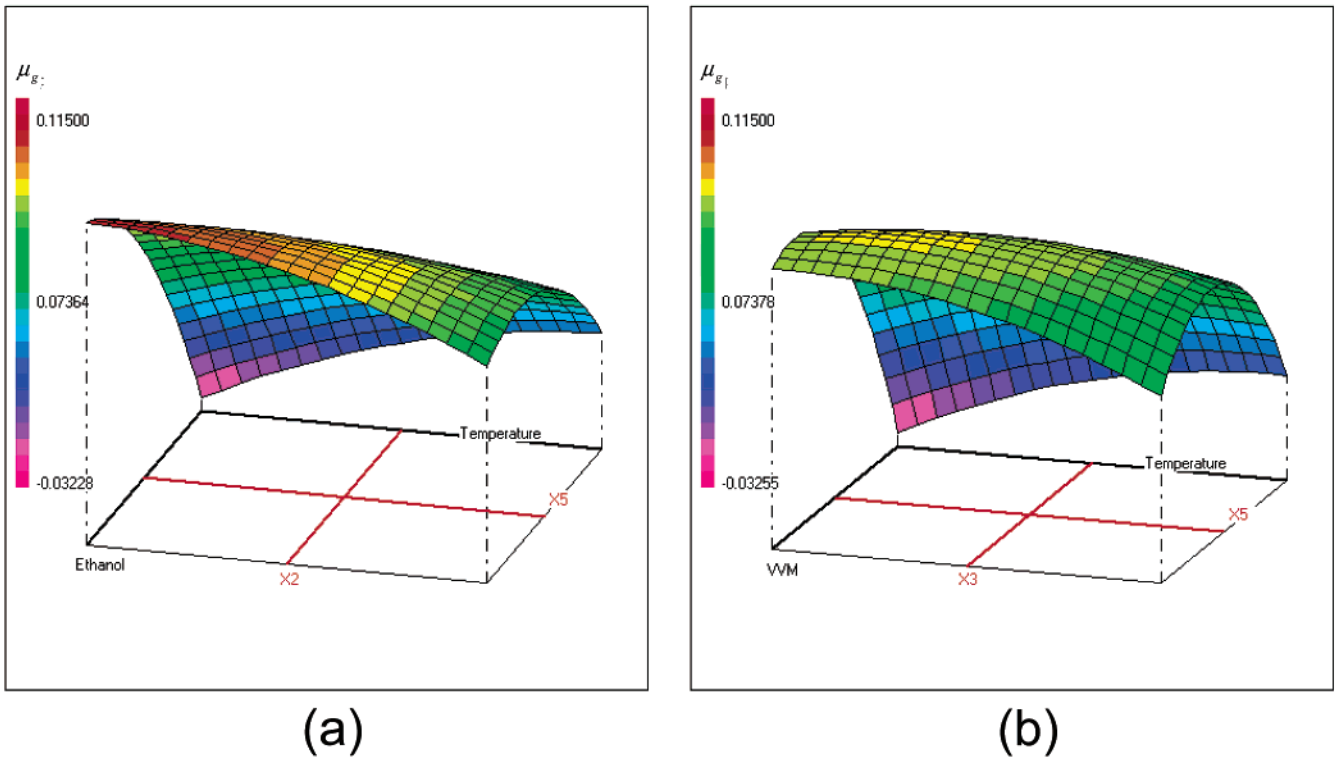

Figure 11. Response surface of the specific growth rate over (a) ethanol and temperature and (b) vvm and temperature.

mentation was 49.2 g/L. This inhibitory behavior of ethanol turns into a saturation effect when temperature is lower than the central point. A similar behavior is inferred for the interaction vvm−temperature showed in Figure 11b.

On the other hand, the effect of temperature on the specific growth rate depends on the values of ethanol and vvm. High levels of ethanol or vvm cause the temperature to have a negative effect on the specific growth rate, whereas low levels of ethanol or vvm cause the temperature to have a positive effect.

In conclusion, the higher the temperature is, the stronger the inhibitory effect of high levels of substrates, ethanol, and oxygen. From a biological point of view, the great capacity for adaptation of the *Acetobacter* is known, although this adaptability depends on the conditions. It is also known that high temperatures strengthen the negative effect of other factors, such as it has been inferred from the response surfaces. In the Introduction, it has been explained that the know-how of acetic fermentation in industrial plants shows that the negative effect of high ethanol concentrations is especially observed when the reactors are not cooled sufficiently. Furthermore, is also known that mediums with high ethanol concentrations can be fermented at lower temperatures.

Finally, it is clearly inferred from the discussion of the results that the effects of the process variables over the specific growth rate cannot be explained individually due to the importance of the interactions among them.

Conclusions

The following are the main conclusions of this work. (1) The pilot system developed, i.e., the continuous fermentor, is an invaluable tool for studying the kinetics of acetic fermentation with guarantees in the assessment of the robustness of the specific growth rates and the other rates evaluated. (2) The experimental cost is high, and the Doehlert design is justified. (3) The values of the yield factors suggest a linear relationship between the growth rate and the rate of substrate consumption and the rate by which product is secured, i.e., growth-associated kinetics of substrates and product. The bacteria oxidize ethanol with oxygen to meet the energy requirements of the multiplication process. Other consumptions of ethanol or oxygen due to the maintenance energy or cell respiration, respectively, are insignificant. The effect of the process variables over the specific growth rate explains the influence of the process variables over the industrial acetic acid production by fermentation. (4) The effect of the substrates (ethanol and oxygen) over the acetic acid production is very important, and very low levels of ethanol or oxygen cause the acetification rate to decrease. On the other hand, a saturation effect with high levels of ethanol or oxygen, without a significant increase in the acetic acid production, is also deduced. (5) The inhibition of the acetic acid production due to the oxygen is proven by the response surfaces of the specific growth rate over the variables involved in the air supply and the behavior of the dissolved oxygen in the medium. (6) The inhibitory effects of high levels of substrates (ethanol and oxygen) increase with high temperatures. (7) From the results of this work is inferred a model for the fermentation process:

$$r_X = \mu_g X \tag{21}$$

$$r_E = \left(-\frac{1}{Y'_{X/E}}\right)\mu_g X \tag{22}$$

$$r_{O_2} = \left(-\frac{1}{Y_{E/O}}\frac{1}{Y'_{X/E}}\right)\mu_g X \tag{23}$$

$$r_A = Y_{A/E}\frac{1}{Y'_{X/E}}\mu_g X \tag{24}$$

The model is a hybrid approach that combines two blocks. The first is the mechanistic relationship between the growth cell and the kinetics of substrate consumption and product formation. The main mechanistic statement inferred is based on the fact that the cells oxidize ethanol to acetic acid to obtain the energy they need to multiply. The model includes the experimental yield factor $Y'_{X/E}$ and the stoichiometric rates of the oxidation, inferred from the values of the yield factors $Y'_{X/O}$, $Y'_{E/O}$, $Y'_{A/O}$, and $Y'_{A/E}$. The stoichiometric constraints allow the model to predict logical and realistic results. Second, the behavior of the specific growth rate is complex, as it is deduced from the response surfaces generated over the process variables. A black-box approach will be necessary to model the specific growth rate. The robustness of the data provided by the pilot system makes the matrix studied in this work very suitable for use in the development of a black-box model.

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