Bioprocess Engineering 18 (1998) 439–444 © Springer-Verlag 1998

Kinetics of substrate consumption and product formation in closed acetic fermentation systems

J.M. Gómez, D. Cantero

Abstract This paper proposes a kinetic model for substrate consumption and product formation, in low alcohol media, of *Acetobacter aceti* in submerged culture. The model considers ethanol consumption for growth of biomass and formation of secondary products by a chemical route. Experimental data were obtained in the laboratory using a variety of discontinuous fermentation apparatus with automatic control, and either open or closed gas recirculation systems. Operating conditions applied were those typical of acetic fermentation process in the food industry. The fit of equations to the experimental data gives high theoretical-experimental determination coefficients.

1
Introduction

The role of *Acetobacter aceti* in the acetic fermentation of wines is widely known; to date, a large number of research studies, together with some excellent reviews, have been published [1, 2]. The bibliography therefore contains much in-depth discussion of the bacterial oxidisation of alcoholic substrates.

The effects of factors such as pH [2] and temperature [3], and concentration of ethanol [2], sulphur dioxide [2], oxygen [2] and other nutrients or inhibitors [2], on the rate of oxidisation of the ethanol by *Acetobacter* have been studied by many authors. Based on the evaluation of the effects of these factors, various kinetic models have been proposed for the growth of *Acetobacter aceti* on alcoholic substrates [4, 5, 6, 7, 8].

In spite of this, very few authors have formulated precise expressions for the consumption of substrate and formation of product in acetic fermentation processes [8], and hence calculated the corresponding yield coefficients. Such parameters are industrially very important, since they enable the concentration of biomass to be related to the concentration of substrate, the concentration of the product, and other essential nutrients of the fermentation process; also importantly, they enable the efficiency of the specific system used to be compared with theoretical values and with the efficiency of other processes.

Moreover, in order to develop accurate kinetic models, it is necessary to know all the factors influencing these parameters, such as evaporation of the components of the fermentation medium, metabolism of the substrate, secondary metabolism, etc.

One of the few models proposed for the consumption of substrate and the formation of product is that of Soo Park et al. [8]. This model was obtained working with a continuous, surface-culture reactor at 30 °C and is mathematically very complex, owing to the special characteristics of this type of culture.

In light of this, this study proposes a kinetic model for the consumption of substrate (ethanol) in closed systems, which is more convenient to use than that proposed by Soo Park et al. Special attention is also paid to calculating the yield coefficients which are so important when it comes to evaluating the efficiency of the fermentation system used.

2
Materials and methods

2.1
Microorganism

One strain of microorganism classified our laboratory as *Acetobacter aceti* UCA1 was used throughout this study; this was isolated from vinegar produced industrially in the Jerez-Xérez-Sherry wine production region. This microorganism, glycerinated to 80%, was stored at −20 °C and used as inoculum for all the fermentations carried out.

2.2
Fermentation substrate

The fermentation substrate used for all the experiments was a complex natural medium consisting of a young wine from the Jerez region (ethanol: 70–90 g/l; total acidity: 15–20 g of tartaric per l; sugars: 1–2 g/l: higher alcohols: 0.5–1.0 g/l; volatile esters: 1–5 mg/l; pH: 2.9–3.1; sulphur dioxide: 60–70 mg/l). This medium was sterilised at 120 °C for 20 minutes, the pH subsequently being set at 4 with KOH 1M,

Received: 7 July 1997

J.M. Gómez, D. Cantero
Biological and Enzymatic Reactors Group,
Department of Chemical Engineering,
Food Technology and Environmental Technologies,
Faculty of Science,
University of Cadiz (UCA),
11510 Puerto Real,
Cádiz, Spain
e-mail: josemanuel.montesdeoca@uca.es

Correspondence to: J.M. Gómez

The authors wish to express their thank to the Pedro Domeq S.A. group of companies for the supply of raw materials and for kindly putting their installations at our disposal.

to provide the most suitable conditions for growth of the microorganism.

2.3 Experimental equipment

The general scheme of the fermentation equipment used in the various experiments is shown in Fig. 1. The fermenter was fitted with a gas recirculation system to enable it to operate as a closed system, preventing loss of volatile compounds in the gas outflow [11]. To compensate for the consumption of oxygen by the biomass, discrete quantities of oxygen were injected into the fermenter's recirculation gas flow. The system was fitted with a dissolved oxygen electrode and an injection controller to maintain the required level of dissolved oxygen. The experiments were conducted fixing this parameter at various different levels within the range possible for the conditions of concentration and ethanol used (between 0 and 9 ppm); accuracy of control was established at ±10% of the set value for oxygen input.

The inoculation of the fermentation was in all cases carried out by adding a volume of 10% of the previously-prepared inoculum to the fermentation tank filled to 90% with the sterile medium. The inoculum consisted of a medium with characteristics analogous to that for the fermentation and showed a high rate of growth of *Acetobacter aceti*; it was produced by parallel acetic fermentation processes conducted in an incubation chamber. Before each inoculation the stability of the operational variables and the correct working of the monitoring instruments were checked. The operating conditions were 400 rpm, 26.5 °C and 0.5 vvm.

2.4 Methods of analysis

To monitor the variables previously described during the fermentation process, samples were taken from the fermenter at regular intervals; each sample was submitted to the following determinations: concentration of ethanol and ethyl acetate, by gas chromatography; concentration of the acetic acid, by measuring the total acidity, titrating the sample with NaOH 0.3N to a pH of 7 [9] concentration of viable biomass, by a plate count in a YEPD medium [2]; and concentration of total biomass, by a Neubauer chamber count. To convert the data from the count in CFU/mL into mg DW/L needed for the subsequent numerical calculation, an empirical correlation obtained from previous work [7] was used. Records were made continuously of the pH, the concentration of dissolved oxygen and the temperature of the fermentation medium, using the appropriate equipment.

3 Description of the kinetic model

In principle, the general scheme proposed for this process is represented in Fig. 2. In this scheme, the consumption of substrate for the formation of complex products is not considered, although the consumption for the formation of the secondary product, ethyl acetate, by the chemical route has been induced, since the formation of this does on occasions account for a considerable fall in the levels of ethanol and acetic acid in the fermentation medium.

This model is based on the assumption that the fermentation is controlled by the rate of consumption of the substrate for the growth of new biomass, since it is considered that this rate is much lower than the possible rate of diffusion within the medium. It is considered therefore that microbial growth is the limiting factor in the overall process of conversion of the substrate.

Taking this into account, the model can be divided into three independent expressions which account for the consumption of substrate, the formation of product and the consumption of dissolved oxygen, respectively, which together with the expression for the specific rate of growth proposed by Romero et al. [7], constitute a general model for the oxidisation of alcoholic substrates by *Acetobacter aceti*.

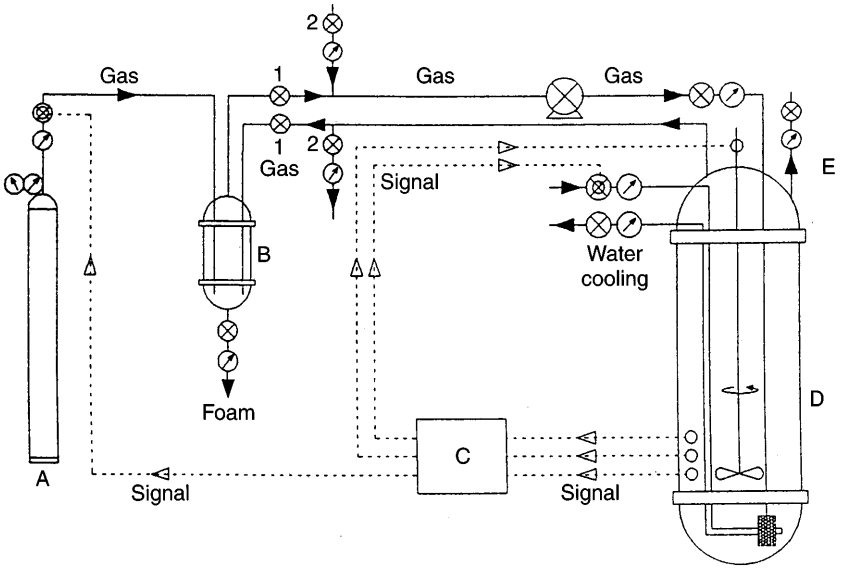

Fig. 1. Schematic diagram of experimental equipment used: A: oxygen supply equipment. B: Gas mixer and foam trap. C: automatic control unit. D: automatic batch fermenter. E: Decompression unit. System open: valves n° 1 closed. System with recirculation of air: valves n° 2 closed

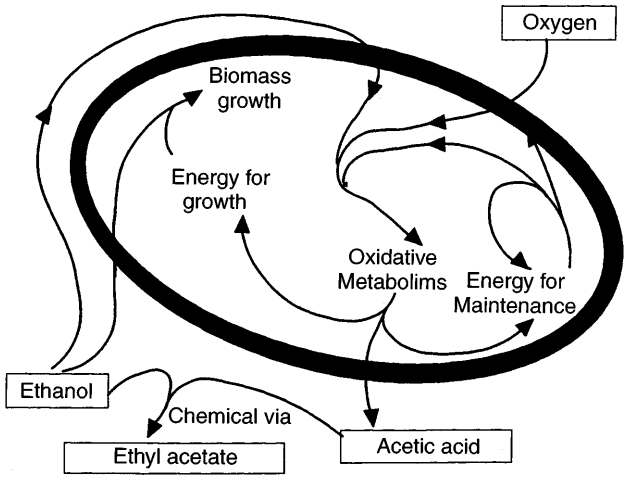

Fig. 2. Simplified model of substrate utilization in the cell, for the acetic fermentation process by batch cultures

3.1
Equation for consumption of the main substrate (ethanol)

Concerning the expression for consumption of substrate (E), in the present model it has been considered that the substrate (ethanol) is exhausted in the medium by three principal mechanisms:

1. *Fermentative consumption* (growth of the biomass). Most of the substrate is metabolised by the biomass (X) for the purposes of its energetic metabolism and, as a consequence, its growth. Thus, the rate of consumption of substrate by fermentation (indicated by the subindex F) is the following:

$$\left(-\frac{dE}{dt}\right)_F = \frac{1}{Y'_{XE}}\left(\frac{dX}{dt}\right) \quad ,$$

   where Y'_{XE} is the coefficient of energy yield of the ethanol, for the growth of the biomass from this substrate. The inverse form of the coefficient is used, as this expresses the grams of biomass generated by means of the energy obtained from each gram of substrate consumed by the fermentation route.
2. *Formation of secondary products.* A large quantity number of secondary products, particularly ethyl acetate (C), are generated from the substrate in acetic fermentation. Taking account of the fact that most of these are only produced at trace levels, the rate of consumption of substrate for the formation of secondary products (indicated by the subindex P) can be calculated from the rate of appearance of ethyl acetate itself, initially ignoring the substrate converted in other possible fermentation products. The relationship between the two rates is reduced to the ratio of molecular weights between the product referred to and the substrate, since the conversion is the result of a simple chemical equilibrium of esterification between the two. Hence, the corresponding mathematical expression is:

$$\left(-\frac{dE}{dt}\right)_P = \frac{1}{Y_{CE}}\left(\frac{dC}{dt}\right) \quad ,$$

   where $1/Y_{CE}$ represents the ratio of molecular weights between ethanol and ethyl acetate, and has a fixed value of 0.523.
3. *Assimilation of the substrate.* In most microbial processes, another possible route for consumption of substrate is assimilation. In the particular case of acetic fermentation, it is considered that this route occurs to only a very limited extend and therefore can be considered negligible.

Consequently, the overall rate of consumption of substrate will be the following:

$$\left(-\frac{dE}{dt}\right) = \left(-\frac{dE}{dt}\right)_F + \left(-\frac{dE}{dt}\right)_P \quad ,$$

$$\left(-\frac{dE}{dt}\right) = \frac{1}{Y'_{XE}}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{CE}}\left(\frac{dC}{dt}\right) \quad .$$

3.2
Equation for the formation of the main product (acetic acid)

The total rate of formation of product (acetic acid), as with that of the consumption of substrate, results from the consideration of two factors:

1. *Fermentative production.* As a result of the consumption of substrate by the fermentation route to obtain energy, the major metabolite of the oxidative route, in this case acetic acid (A), appears. The expression for this is:

$$\left(\frac{dA}{dt}\right)_F = \frac{1}{Y_{EA}}\left(-\frac{dE}{dt}\right)_F \quad ,$$

   where Y_{EA} is the stochiometric coefficient for the conversion of ethanol into acetic acid.
2. *Formation of secondary products.* It has previously been stated that in this type of fermentation, and when certain levels of ethanol and acetic acid are reached, an equilibrium of esterification is established which gives rise to the formation of ethyl acetate. Therefore, part of the acid forming by the energetic route is lost in order to form the ester. As for the substrate, the rate of consumption of product by this phenomenon is calculated from the rate of formation of ethyl acetate (C):

$$\left(-\frac{dA}{dt}\right)_P = \frac{1}{Y_{CA}}\left(\frac{dC}{dt}\right) \quad ,$$

   where $1/Y_{CA}$ is the ratio of molecular weight between acetic acid and ethyl acetate, and has a constant value of 0.682.

Consequently, the overall rate of formation of product will be given by combining these two factors:

$$\left(\frac{dA}{dt}\right) = \left(\frac{dA}{dt}\right)_F + \left(-\frac{dA}{dt}\right)_P,$$

$$\left(\frac{dA}{dt}\right) = \frac{1}{Y_{EA}}\frac{1}{Y'_{XE}}\left(\frac{dX}{dt}\right) + \frac{1}{Y_{CA}}\left(\frac{dC}{dt}\right) \quad .$$

3.3
Equation for the consumption of dissolved oxygen

In this case, the rate of consumption of dissolved oxygen (O) is represented by a single factor: fermentative consumption. The corresponding expression is the following:

$$\left(-\frac{dO}{dt}\right) = \left(-\frac{dO}{dt}\right)_F = \frac{1}{Y'_{XO}}\left(\frac{dX}{dt}\right) ,$$

where Y'_{XO} represents the coefficient of energetic yield of the oxygen, for the growth of the biomass from this component of the medium. This coefficient corresponds to the grams of biomass formed from the energy obtained, per gram of oxygen metabolised by the fermentation route.

4
Experimental results

Figure 3 shows the normalised trends for the concentrations of ethanol, acetic acid, pH, and biomass, in the acetic fermentation experiments carried out. Measurements of the ethanol and acetic acid were performed in triplicate and the resulting average values obtained. The analysis of the biomass was duplicated.

From the experimental data, it is possible to calculate the values of the rates of consumption of substrate $(-r_E)_i$, formation of product $(r_A)_i$, and growth of biomass $(r_X)_i$, at each instant (i). The procedure to follow can be based on methods of numerical or graphical differentiation, depending on the type and number of experimental data gathered. In this case, a procedure of numerical differentiation has been followed corresponding to the following calculation algorithm for the case of the rate of consumption of substrate, and similarly for the rate of product formation and for the rate of biomass growth:

$$(-r_E)_i = \left(-\frac{dE}{dt}\right)_i = \left[\frac{\frac{E_i - E_{i-1}}{t_i - t_{i-1}} + \frac{E_{i+1} - E_i}{t_{i+1} - t_i}}{2}\right] .$$

The results obtained represent the set of values of the rates of substrate consumption, product formation and biomass growth, measured at each instant t_i. Figure 4 shows the values of these rates.

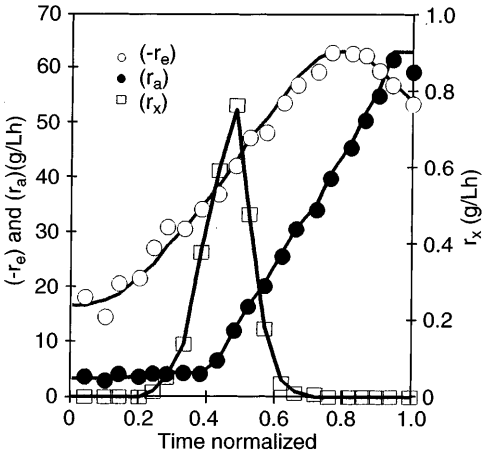

Fig. 4. Representation of the different rates of the acetic fermentation process. $(-r_e)$: rate of ethanol consumption; (r_a): rate of acetic acid formation; (r_x): rate of biomass production

5
Calculation of the coefficients of the model

5.1
Calculation of the coefficient Y'_{XE}

According to the expression for the overall rate of consumption of substrate, it can be stated that the consumption of substrate is proportional to the production of energy in the oxidative process. This energy, in turn, is for the main part used by the biomass to generate new cells; it can therefore be concluded that the consumption of substrate is proportional to the production of biomass. The proportionality factor is $1/Y'_{XE}$, previously defined. The following linear expression can be derived from that equation:

$$\left(-\frac{dE}{dX}\right) = \frac{1}{Y_{CE}}\left(\frac{dC}{dX}\right) + \frac{1}{Y'_{XE}} .$$

Then by plotting $(-dE/dX)$ against (dC/dX), a straight line is obtained, whose slope should be 0.523 and whose intercept gives the value of $1/Y'_{XE}$ (Fig. 5A). From the experimental data obtained, an average value for $1/Y'_{XE}$ of 138.90 is obtained. In other words, $Y'_{XE} = 7.2 \pm 0.8$ mg of biomass generated per gram of substrate consumed.

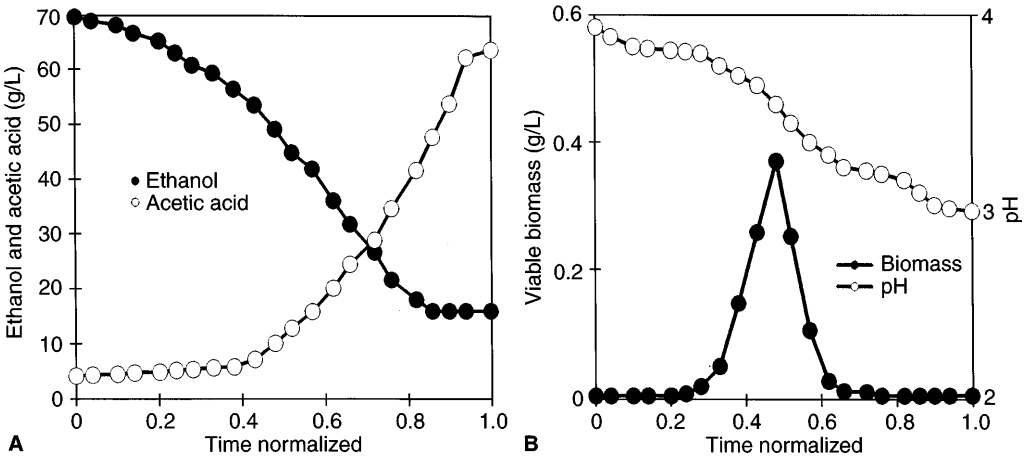

Fig. 3 A,B. Evolution of the main parameters during acetic fermentation by batch culture. **A:** trends of the substrate (ethanol) and the product (acetic acid). **B:** trends of the pH and the total biomass

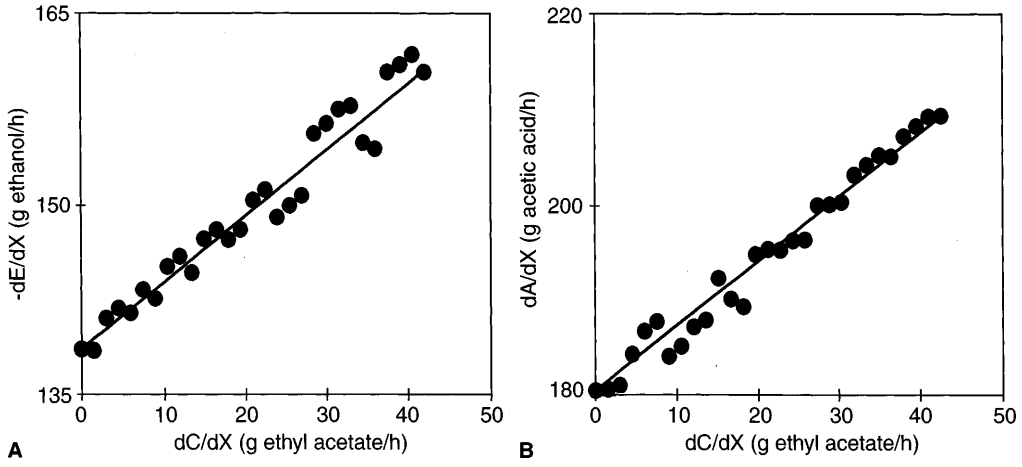

Fig. 5 A,B. Fit of the equations of the model for the calculation of the values of the yield coefficients. **A)** Fit of $-dE/dX$ against dC/dX for the calculation of Y'_{XE}; **B)** Fit of dA/dX against dC/dX for the calculation of the coefficient Y'_{EA}

The value of Y'_{XE} is greater than that obtained in open systems (3.42×10^{-3} g of biomass per g of substrate) [10], mainly because the concentration of ethanol in open systems is lower, as a result of the considerable losses from evaporation suffered by these systems. For similar reasons, the value of Y'_{XE} calculated in this study is also greater than that in systems with recovery of volatile compounds by means of absorption-desorption columns (4.18×10^{-3} g of biomass per g of substrate) [10].

5.2
Calculation of the coefficient Y'_{EA}

Taking into account the accepted mechanism for the general kinetics of the process, the formation of product is proportional to the consumption of substrate by the fermentation route. Hence the coefficient $1/Y_{EA}$ can be calculated from the general equation of rate of formation of product. As in the preceding case, the following is obtained:

$$\left(\frac{dA}{dX}\right) = \frac{1}{Y_{CE}}\left(\frac{dC}{dX}\right) + \frac{1}{Y'_{EA}} \quad .$$

Now plotting dA/dX against dC/dX should give a straight line, whose slope is 0.682 and whose intercept gives the value of $1/Y'_{EA}$ (Fig. 5B). The result should be close to the stochiometric coefficient, provided there are no significant losses of fermentative yield. From the experimental data and by following the procedures of incremental calculus, an average value is obtained for $1/Y'_{EA} = 1.80 \pm 0.06$ g of product per g of substrate consumed by the fermentation route. This coincides with the theoretical value, thus representing a true stochiometric coefficient of conversion of ethanol into acetic acid, for this type of closed system which incurs no losses by evaporation.

5.3
Calculation of the coefficient Y'_{XO}

When oxygen is considered as a substrate for the purposes of kinetics in the case of acetic fermentations, Y'_{XO} becomes a coefficient of yield, of analogous significance to Y'_{XE}.

Oxygen is consumed by the biomass of oxidise the substrate into product and to produce the energy necessary for the synthesis of new biomass. Hence, in several of the experiments conducted, the volume of O_2 injected was monitored with the aim of quantifying the oxygen consumed per gram of biomass formed. From these experimental data and according to the equation for rate of consumption of dissolved oxygen, the following expression can be deduced:

$$\left(-\frac{dO}{dt}\right) = \frac{1}{Y'_{XO}}\left(\frac{dX}{dt}\right) \quad .$$

Applying methods of incremental calculus (Fig. 6), the average value obtained for Y'_{XO} is 2.6 ± 0.4 mg of biomass formed per g of oxygen consumed.

The value for the coefficient of yield, Y'_{XO}, is lower than that proposed by Soo Park et al. [8], because these authors obtain higher concentrations of viable biomass from using a system of reutilisation of cellular population in rechargeable batch fermenters.

6
Conclusions

Given the results obtained, it may be concluded that the values of the coefficients of yield for batch processes of acetic fermentation in closed systems are the following:

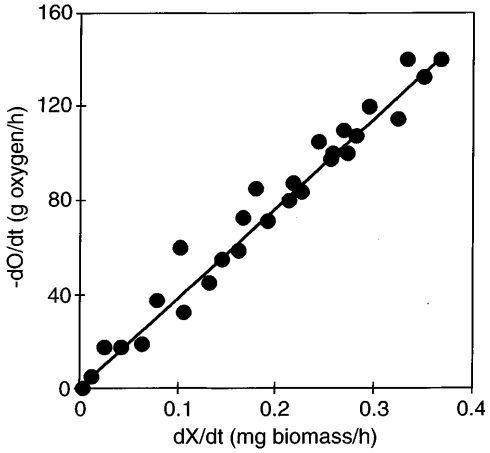

Fig. 6. Fit of the equation proposed for the model, for the consumption of dissolved oxygen and calculation of the coefficient Y'_{XO}

$Y'_{XE} = 7.2 \times 10^{-3}$ g of biomass formed per g of ethanol consumed; $1/Y_{EA} = 1.3$ g of acetic acid formed per g of ethanol consumed; and $Y'_{XO} = 2.6 \times 10^{-3}$ g of biomass formed per g of oxygen consumed. Consequently, it is demonstrated that the coefficients for closed systems are higher than those for open systems, because the absence of losses by evaporation permit a higher yield from the biochemical processes involved.

It may also be concluded that the data obtained constitute an excellent result to be included in an overall model for acetic fermentation, since despite the complex calculations required for this type of coefficient, the variances shown were only 15% for the coefficient Y'_{XO} and 12% for the coefficient Y'_{XE}.

Lastly, it can be stated that the proposed model of consumption of substrate and formation of product, independently of its evident theoretical basis, promises to be extremely useful in industrial design, having a simple mathematical form and is easy to handle and parametrize for different working conditions.

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