CHEMICAL ENGINEERING TRANSACTIONS
VOL. 32, 2013
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Chief Editors: Sauro Pierucci, Jiří J. Klemeš

ISBN 978-88-95608-23-5; **ISSN** 1974-9791
DOI: 10.3303/CET1332229

A Procedure for Estimation of Fermentation Kinetic Parameters in Fed-Batch Bioethanol Production Process with Cell Recycle

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The configuration of industrial fermentation in ethanol plants in Brazil is predominantly of the type fed-batch culture with cell recycle. In this operation mode, yeast is exposed through a long period to inhibitors, high cell concentration and fluctuations in the quality of the raw material, with impact on process kinetic and operating performance. In this context, for the implementation of suitable operational strategies, it is necessary to have kinetic models able to describe the process as realistically as possible. Bearing this in mind, in this work the kinetic of the alcoholic fermentation of sugarcane juice was studied in fed-batch fermentation with cell recycle. The accuracy of prediction of a mechanistic kinetic model is evaluated, not only by their precision in describing experimental observations, but essentially by the challenges involved in the estimation of their parameters. The model used to describe the fermentation provided a good prediction of concentration of cell, substrate and ethanol.

1. Introduction

Among all forms of ethanol production, the fermentation route using sugarcane as feedstock is the most economically profitable in Brazil. This fact is due to the geographic location, type of soil, variety of feedstock and possibility of nationwide cultivation (Basso et al., 2011). In fuel ethanol production industry, the fermentation is a biochemical process in which glucose, fructose and sucrose (from sugarcane juice and sugarcane molasses in varying proportion) is metabolized to ethanol by yeast *Saccharomyces cerevisiae* in large production scale (500-3000 m^3/day), which involve high volume fermentors. Yields around 90–92% of the sugar theoretical conversion to ethanol were achieved in the last decade (Della-Bianca et al., 2012). The industrial fermentation process in Brazil is predominantly fed-batch culture with cell recycle, being that 70-80% of distilleries utilize this mode of operation (Brethauer and Wyman, 2010). In this configuration, 90-95% of the yeast cell is reused from successive fermentation (intensive recycling). This allows high cell densities inside the fermentors, which contributes to reduce the fermentation time to 6-11 h (Basso et al., 2008). Although cell recycling enables the adaptation of yeast to adverse process conditions, changes in the kinetic behavior of Brazilian dominant yeast follow an unknown mechanism. Furthermore, recent studies point out the existence of many characteristics of these strains that remain unknown. As a consequence, these yeasts cannot dominate the fermentors of all distilleries; in fact they may be implemented only in 60% of plants (Della-Bianca et al., 2013). Variations on kinetics could be result of inhibitors present in the reaction medium, which changes the yeast physiology. Also variations on the microorganism performance may be correlated to cell stress imposed by process conditions (high ethanol concentration, low pH, high temperature, medium acidification and osmotic stress, among others) and bacterial contamination. Another common source of kinetics fluctuation is related to the variability of feedstock quality. The sugarcane broth quality may vary during the season depending on sugarcane variety, harvest period, climate conditions and sugar cane juice extraction procedure (Junior et al., 2009).

Please cite this article as: Ccopa Rivera E.A., Yamakawa C.K., Herrera Garcia M., Geraldo V.C., Rossell C.E.V., Maciel Filho R., Bonomi A., 2013, A procedure for estimation of fermentation kinetic parameters in fed-batch bioethanol production process with cell recycle, Chemical Engineering Transactions, 32, 1369-1374 DOI: 10.3303/CET1332229

Nowadays, some improvements associated with this type of operation strategy, are required in industrial plants, when optimized operation is a target. Therefore, there is a demand for the development of models and procedures to describe the process in such way that process optimization, control and improved operation techniques may be used. The application considered in this work illustrates the usefulness of model-based methodology to describe the kinetics of a fermentation process. A methodology based on appropriate forms of kinetic rates and an optimization algorithm has been integrated to identify the kinetic parameters. As a consequence, an accurate mechanistic model was obtained, which provides a good description of fed-batch fermentative cycles.

2. Experiments

2.1 Microorganism, substrate and culture conditions

The *Saccharomyces cerevisiae* strain used in this work is an un-named strain cultivated in the Bioprocess Development Laboratory at CTBE and obtained from the Faculty of Food Engineering, State University of Campinas, originally coming from an industrial ethanol distillery. The strain was maintained on agar plates that were prepared per liter of de-mineralized water: yeast extract, 10 g; peptone, 20 g; glucose, 20 g; and agar, 20 g. Before the inoculum preparation, three slopes from agar plate were transferred to liquid complex medium containing per liter of de-mineralized water: yeast extract, 10 g; peptone, 20 g; and glucose, 20 g. This step named pre inoculums, aimed cell activation that was performed in flask shaker culture for 24 hours at 33°C and 250 rpm.

The complex medium used for inoculum and cultivation contained the following per liter of de-mineralized water: K_2SO_4, 6.6 g; KH_2PO_4, 3 g; $MgSO_4$, 0.5 g; $CaCl_2.2H_2O$, 1.0g; and yeast extract, 5.0 g. After autoclaving at 121°C for 15 min, the medium was cooled to room temperature. Thereafter, filter-sterilized elements were added in the following concentration per liter: urea, 2.3 g; thiamine, 3.0 g; EDTA, 15 mg; $ZnSO_4.7H_2O$, 4.5 mg; $CoCl_2.6H_2O$, 0.3 mg; $MnCl_2.4H_2O$, 0.84 mg; $CuSo_4.5H_2O$, 0.3; $FeSO_4.7H_2O$, 3 mg; $NaMoO_4.2H_2O$, 0.4 mg; H_3BO_3, 1 mg; and KI, 0.1 mg. The carbon source was from sugarcane juice that contained per liter: sucrose, 102.51 g; glucose, 10.99 g; and fructose, 10.01 g or in terms of total reducing sugar (TRS), 129 g. The sugarcane juice was sterilized separately at 121°C for 15 min.

The inoculum culture was performed in Erlenmeyer flask for 24 hours, 33°C and 250 rpm in an orbital shaker incubator (Innova 44 New Brunswick). After that, the inoculum was centrifuged in a Sorvall centrifuge at 8000 rpm for 20 min, then the supernatant was discarded and the cells were suspended in sterilized water up to 200 mL and transferred to the cultivation bioreactor aseptically. The cultivation was performed at 33°C in a bioreactor (Bioflo 115; New Brunswick Scientific) in fed-batch configuration using a cascade control with agitation and air flow to maintain the dissolved O_2 concentration above 60% of saturation with air (Basso et al., 2011). Thereafter, the yeast culture was centrifuged in a Sorvall centrifuge at 8000 rpm for 20 min, and then the supernatant was discarded. The cells were suspended in sterilized water (quantity sufficient for 500 mL) and transferred to the bioreactor for alcoholic fermentation.

2.2 Fed-batch experiments with cell recycle

The substrate used for alcoholic fermentation was formulated with only sugarcane juice that contained per liter: sucrose, 133.01 g; glucose, 16.79 g; and fructose, 14.85 g or in terms of total reducing sugar (TRS), 171.65 g, a typical feedstock for large scale ethanol production plants. The alcoholic fermentation was performed with cell recycling and high cell density in the fed-batch configuration as is usual in industrial Brazilian process (Melle-Boinot). The yeast cells required were obtained previously in the cultivation step. The must batch feeding was performed in four hours (flow of 6.25 mL/min) up to the final volume of 1.5 L and was maintained for two more hours to ensure the uptake of accumulated sugar even though all sugar was consumed. The fermented wine was centrifuged at 8000 rpm for 20 min in a Sorvall centrifuge and then the yeast was suspended with sterilized water and centrifuged again in the same condition. The centrifuged yeast biomass was carried back to the bioreactor for treatment with H_2SO_4 under pH of 3.0 and aeration during one hour. This treatment was performed before each fermentative cycle during yeast cell recycling. The fermentative cycle comprises the fed-batch fermentation, and cell treatment and recycling. In this study were performed two fermentative cycles, i.e., three fed-batch fermentation experiments, and two cell treatments and recycling. All the must used in this work was prepared using sugarcane syrup from da Pedra Mill (Serrana, São Paulo, Brazil).

2.3 Analytical methods

Concentration of sucrose, glucose and fructose were detected by high-performance liquid chromatography (HPLC) Agilent Infinity 1260 with IR detector 50C, Aminex column HPX-87P 300 mm x 7.8 mm at 60°C and 0.5 mL/min of ultrapure Milli-Q water as eluent phase. Ethanol was determined by HPLC Dionex Ultimate 3000 with IR detector Shodex RI-101, Aminex column HPX-87H 300 mm x 7.8 mm at 50°C and

0.5 mL/min of sulfuric acid 5 mM as eluent phase. Measurements of the dry weight mass were carried out in triplicate and determined gravimetrically after centrifuging, washing two times with water and drying at 80°C until constant weight in the analytical balance.

3. Mathematical modeling

This section presents the consideration required to develop a model-based technique for the estimation of the kinetic parameters.

3.1 Kinetic model

The state variables involved in this fermentation process were concentration of total cell mass, X (kg/m^3), substrate, S (kg/m^3) and ethanol, P (kg/m^3).

Experimental observations have shown that cell, substrate and product inhibitions are significant for ethanol fermentation (Rivera et al., 2007). Eq (1) shows the cell growth rate equation, r_x, which includes terms for such types of inhibitions:

$$r_x = \mu_{max} \frac{S}{K_s + S} \exp(-K_i S) \left(1 - \frac{X}{X_{max}}\right)^m \left(1 - \frac{P}{P_{max}}\right)^n X \quad (1)$$

where μ_{max} is the maximum specific growth rate (h^{-1}), K_s the substrate saturation constant (kg/m^3), K_i the substrate inhibition parameter (m^3/kg), X_{max} the cell concentration where the growth ceases (kg/m^3), P_{max} the ethanol concentration where the cell growth ceases (kg/m^3), and m and n are empirical parameters.

In this study, a modified Luedking-Piret expression was used to account for the ethanol formation rate, r_p, as shown in Eq (2). This rate depended on the specific growth rate and cell concentration (X). $Y_{p/x}$ (kg/kg) is the product yield based on cell growth, β_{mp} (kg/kg h) is a parameter associated with maintenance, and $K_{\beta s1}$ (kg/m^3) is a saturation parameter.

$$r_p = Y_{p/x} r_x + \frac{\beta_{mp} S}{K_{\beta s1} + S} X \quad (2)$$

The substrate consumption rate, r_s, was expressed as follows:

$$r_s = (r_x / Y_x) + \frac{\beta_{ms} S}{K_{\beta s2} + S} X \quad (3)$$

where Y_x (kg/kg) denote the limit cellular yield, β_{ms} (kg/kg h) is a maintenance parameter, and $K_{\beta s2}$ (kg/m^3) is a saturation parameter.

3.2 Fed-batch model

Mechanistic models comprise the mass balance differential equations, with microorganism growth, substrate consumption and ethanol formation for a fed-batch reactor described as follows:

Total cell: $$\frac{dX}{dt} = r_x - \frac{F_A X}{V} \quad (4)$$

Substrate: $$\frac{dS}{dt} = \frac{F_A (S_A - S)}{V} - r_s \quad (5)$$

Ethanol: $$\frac{dP}{dt} = r_p - \frac{F_A P}{V} \quad (6)$$

Volume: $$\frac{dV}{dt} = F_A \quad (7)$$

The mass balance differential equations were solved using the LSODE (Livermore Solver for Ordinary Differential Equations) (Radhakrishnan and Hindmarsh, 1993).

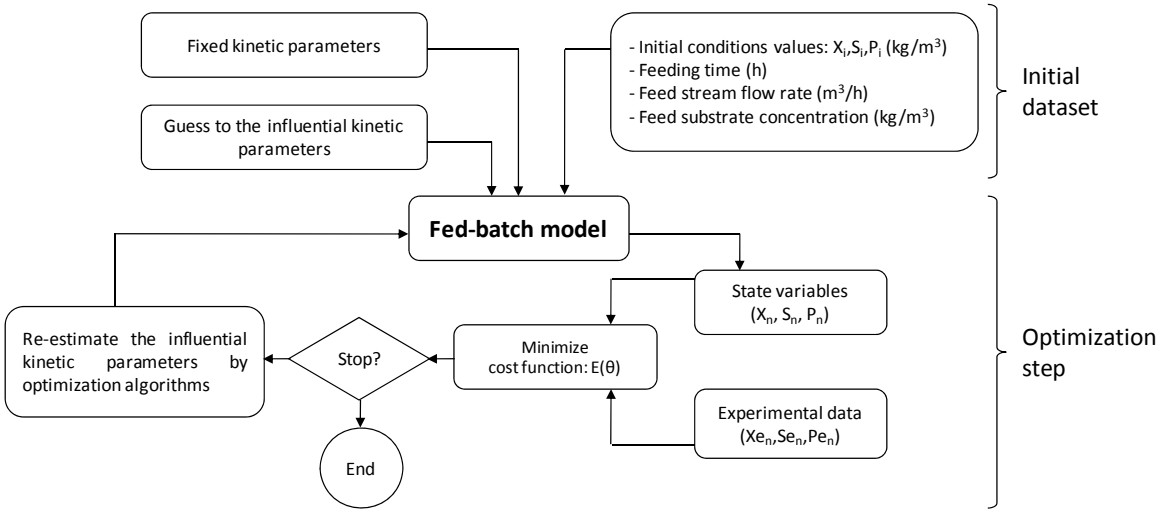

Figure 1: General framework of the model-based approach used to estimate the kinetic parameters

3.3 Parameter estimation method

The proposed method for estimation of kinetic parameters is depicted in Figure 1. First the kinetic parameters are initialized (including fixed parameters and the influential parameters to be estimated) as well as the operational condition values for the fermentation process (feeding time t_F; feed stream flow rate, F_A and feed substrate concentration, S_A). After this step, the proposed method is able to find optimum values for the parameters that produce the best fit between the experimental observations and the simulated response variables by minimizing cost functions, Eq (8), Eq (9).

$$E(\theta) = \sum_{n=1}^{np} \frac{(X_n - Xe_n)^2}{Xe_{max}^2} + \frac{(S_n - Se_n)^2}{Se_{max}^2} + \frac{(P_n - Pe_n)^2}{Pe_{max}^2}; \qquad E(\theta) = \sum_{n=1}^{np} \frac{(X_n - Xe_n)^2}{\left(\frac{X_n - Xe_n}{2}\right)^2} + \frac{(S_n - Se_n)^2}{\left(\frac{S_n - Se_n}{2}\right)^2} + \frac{(P_n - Pe_n)^2}{\left(\frac{P_n - Pe_n}{2}\right)^2} \quad (8\text{-}9)$$

where θ the vector of kinetic parameters is constrained by bounds within a realistic range, i.e., biological means. Xe_n, Se_n and Pe_n are the experimental observations of cell, substrate and ethanol at the sampling time n. X_n, S_n and P_n are the concentration of cell, substrate and ethanol computed by the model at the sampling time n. Xe_{max}, Se_{max} and Pe_{max} are the maximum measured concentration. If a stopping criterion is reached, the estimation is finished. If not, the algorithm re-estimates the parameters using an optimization technique based on Genetic Algorithm (GA) and Quasi-Newton (QN) methods. The determination of the feasible region of the total search space in the multi-parameter optimization of a mechanistic model is not a trivial procedure. For that reason, in this work, the optimization procedure is based on the combination of two optimization techniques. Initially, the potential of global searching of Genetic Algorithm (Cuadros et al., 2012) was explored for simultaneous estimation of the initial guesses for a set of kinetic parameter in the model. Subsequently, the Quasi-Newton algorithm (QN), which converges much more quickly than GA to the optimal values, was used to continue the optimization of the kinetic parameters near to the good local optimum. It was seen that the computational time using the proposed approach is by around 70% smaller than using only the GA. A standard personal computer, Intel® Core™2 Quad Q8400 CPU at 2.66GHz and 4GB RAM, was used to perform these studies.

4. Results and discussion

Fed-batch experiments with cell recycle were performed for estimation of kinetic parameters. The initial values of the state variables (X_i, S_i and P_i) and the operational conditions of these experiments are given in Table 1.

From kinetic rates described by Eq (1) - Eq (3) a set of thirteen kinetic parameters should be adjusted. A sensitivity analysis approach applied to an analogous kinetic system concluded that a sub-set of those parameters differ within a range according to changes in operational conditions and fluctuations in the quality of raw material (Andrade et al., 2009). In this sense, μ_{max}, X_{max}, P_{max}, Y_x and $Y_{p/x}$ are known to be influential parameters in the system, which were estimated using the proposed methodology. In this study β_{mp}, β_{ms}, $K_{\beta s1}$ and $K_{\beta s2}$ also were studied. The estimated numerical values are indicated in Table 2. The remaining ones were fixed in the previous values used in several studies (Andrade et al., 2013), as follows: K_s = 4.1 (kg/m^3), K_i = 0.002 m^3/kg, m = 1.0 and n = 1.5.

Table 1: Initial values and operational conditions of the experiments

Initial values	Fermentation 1	Fermentation 2	Fermentation 3
X_i (kg/m^3)	40.55	44.83	44.65
S_i (kg/m^3)	17.95	15.52	4.22
P_i (kg/m^3)	9.84	14.56	11.13
Operational conditions			
V_i (m^3)	0.5	0.5	0.5
S_A (kg/m^3)	171.7	171.7	171.7
F_A (m^3/h)	0.43	0.22	0.23
t_F (h)	2	3	3

Table 2: Estimated parameters values

Parameter	Unit	Estimated value
μ_{max}	h^{-1}	0.33
P_{max}	kg/m^3	85.7
X_{max}	kg/m^3	56.2
$Y_{p/x}$	kg/kg	3.56
Y_x	kg/kg	0.0909
β_{mp}	kg/kg h	0.229
$K_{\beta s1}$	kg/m^3	0.001
β_{ms}	kg/kg h	0.201
$K_{\beta s2}$	kg/m^3	0.001

The performance of the model in describing the experimental observations for Fed-batch fermentation with cell recycle is shown in Figure 2 and quantified through the RSD(%) (Residual Standard Deviation) and R^2 (correlation coefficient) (Rivera et al., 2010). From these criteria, it was concluded that the model described the experimental data accurately, as evaluated by RSD(%). Also, in all cases R^2 was close to unity, indicating a good fit of the model, as can be seen in Table 3. Results showed that it is possible to accurately infer concentration in fed-batch fermentation with intensive recycling.

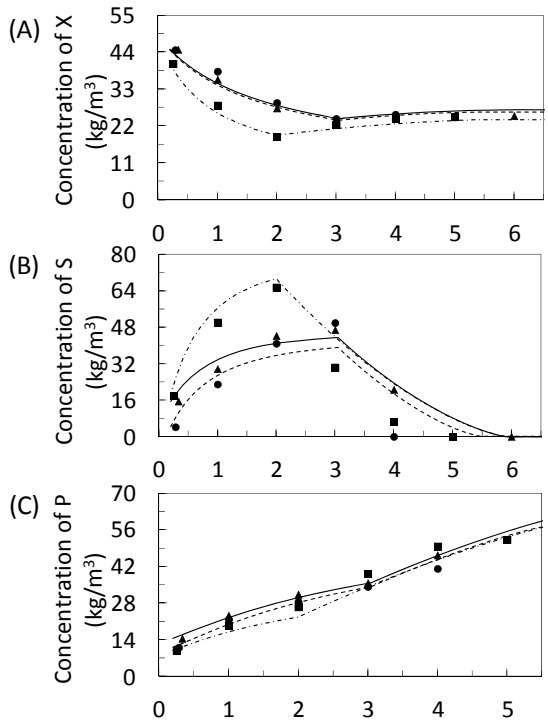

Figure 2: Experimental (Ferm. 1 (■); Ferm. 2 (▲) and Ferm. 3 (●)) and model prediction (Ferm. 1 (-·-·-); Ferm. 2 (——) and Ferm. 3 (----)) for concentration of (A) Cell, (B) Substrate and (C) Ethanol

Table 3: Statistical criteria to characterize the prediction quality of the fed-batch model

Fermentation	X (kg/m^3)		S (kg/m^3)		P (kg/m^3)	
	RSD(%)	R^2	RSD(%)	R^2	RSD(%)	R^2
N^{o}1	6.25	0.99	35.24	0.98	10.54	0.98
N^{o}2	3.73	0.98	11.06	0.98	15. 60	0.95
N^{o}3	6.59	0.99	35.73	0.99	5.67	0.99

5. Conclusions

This work presents results from the development and testing of a modeling approach for the estimation of kinetics parameters in fed-batch fermentation with high cell densities intensively recycled. Even considering that the kinetic rate expressions are known in the mechanistic model, the estimation problem is complex and time consuming. This suggests that using a model in a situation where frequent parameter re-estimation is necessary could be a limitation, such as the studied system. In this work, a model-based approach has been developed using a mechanistic model and optimization algorithms that have been widely used for modeling and optimization purposes in engineering application. Based on this approach, a mechanistic model was obtained and its performance in describing the dynamic behavior of concentration of cell, substrate and ethanol during fermentation was assessed. Model predictions using the experimental observations provided acceptable performance measures (RSD and R^2). Finally, it can be said that the use of this approach enables a rapid determination of a mathematical description of fed-batch fermentation processes that can be used for optimization and control.

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