Modelling and optimization of acetic acid fermentation: Apolynomial-basedapproach

Ines M. Santos-Dueñas[a], Jorge E. Jimenez-Hornero[b*], Ana M. Cañete[a], Isidoro Garcia-Garcia[a]

[a]*Departamentode Ingeniería Química, Universidad de Córdoba, Facultad de Ciencias, Edificio Marie Curie, Campus Universitario de Rabanales, 14071 Cordoba, Spain*

[b]*Departamento de Informática y Análisis Numérico, Universidad de Córdoba, Escuela Politécnica Superior, Edificio Leonardo da Vinci (CTI), Campus Universitario de Rabanales, 14071 Cordoba, Spain*

[*]*Phone: (+34) 957212079, email:jjimenez@uco.es*

E-mail addresses: ines.santos@uco.es (I.M. Santos-Dueñas), b42carom@uco.es (A.M. Cañete), isidoro.garcia@uco.es (I. Garcia-Garcia)

Abstract

Vinegar production is a typical bioprocess in the scope of the agrifood industry. Its optimization requires careful modelling which has so far been addressed by using mainly unstructured first principles models. Because of the difficulties in obtaining these models, black box models, such as those used here, are becoming more frequently used. The polynomial modelsdeveloped in this work accurately reflect the effect of the major and typical operational variables usedin industryfor this process. Also, response surfaces were used to identify the optimum operating conditions with a view to maximizing the mean fermentation rate and productivity. The followed strategy has a huge industrial interest sinceyields a tool that does not only allow finding the best

operational conditions depending on different criteria but also is useful for process control. As far as we know this is the first time that these variables have been correlated in this way.

Keywords: Bioreactors;Modelling; Optimisation; Acetic Acid; Acetobacter; Vinegar

Abbreviations

$(r_A)_{est}$ Estimated mean acetic acid formation rate (g acetic acid·(100 mL·h)$^{-1}$)

C Wine loading rate(L·min^{-1})

E Ethanol concentration remaining at the time the reactor is unloaded (% (v/v))

V Percent unloaded volume (%)

$(P_A)_{est}$ Estimated acetic acid production (g acetic acid·h^{-1})

$(EtOH_{mean})_{est}$ Estimated mean ethanol concentration (% (v/v))

$(HAc_{mean})_{est}$ Estimated mean acetic acid concentration (% (w/v))

$([Total\ Cells]_{mean})_{est}$ Estimated mean total cell concentration (cells·mL^{-1})

$([Viable\ Cells]_{mean})_{est}$Estimated mean viable cell concentration (cells·mL^{-1})

$(V_{mean})_{est}$ Estimated mean volume (L)

HAc_{final} Acetic acidconcentration at the time the reactor is unloaded (% (w/v))

t_{total} Total cycle duration (h)

V_{mean} Mean volume (L)

1. Introduction

The optimization of acetic acid fermentation as a biotechnological process has been the subject of much study in recent times —particularly as regards vinegar production[1-6]. The complex interdependence of the variables influencing growth and activity in acetic acid bacteria[7-10] has led to the development of mathematical models for quantifying the relationships between the major variables. Most such models have a phenomenological or unstructured first principles basis[11-14] and use differential equations to balance substrate and product concentrations, and kinetic equations to define the influence of the different variables[15, 16]. This approach has the advantage of being valid over broad ranges of operating conditions by virtue of its relying on physico–chemical properties of the processes concerned. However, it has the disadvantages that the obtained models are complex and that their kinetic equations have to be constructed from unknown parameters which must be estimated by applying optimization algorithms to experimental values[17]. Also, obtaining accurate, unambiguous estimates requires satisfying the structural and practical identifiability conditions[18-22]. The structural identifiabilityanalysis condition only depends on the mathematical structure of the model equations, whereas the practical identifiabilityanalysis condition additionally considers the amount of data used to estimate parameters and their quality. Checking that both conditions are fulfilled entails using computationally complex algorithms [23, 24], which is an added disadvantage of first principles models.

On the other hand, black box models need not consider the physico–chemical principles behind the target process[25]. Rather, these models seek the simplestpossible relationships between operational and process variables from experimental values obtained under different conditions. As a rule, black box models are easier to construct

than phenomenological models and require no prior identifiability analysis, so they are more practical for process optimization and control. However, black box models are applicable over narrower operational ranges than phenomenological models because they constitute necessarily local approaches.

Polynomial models, which are among the most widely used black box models[26, 27],allow operational and process variables to be correlated via linear or non-linear generalized polynomials of variable order, but usually first or second[28, 29] —the latter tend to be more accurate and widely applicable by effect of their considering interactions between factors (operational variables). However, they require greater numbers of experimental data to fit coefficients; also, the number of experiments needed depends on the polynomial order, the number of factors and the number of levels (values) used to discretize each factor range. Experimental design is used to identify the factors most strongly influencing a process under specific experimental conditions, minimize the effects of uncontrolled factors (perturbations), isolate and assess the effect of each individual factor by statistical analysis[30] and rationalize (reduce) the number of experiments required[31].Experimental design allows obtaining the simplest algebraic equations used to construct polynomial models.

The joint use of polynomial models and response surfaces provides a powerful tool for process optimization[1, 6, 32], as it facilitates identification of the optimum operating conditions of a process considering interactions between individual influential factors.

In this work, we exploited the advantages of these models to construct quadratic polynomials for the process variables of acetic acid fermentation. To this end, we used three different factors, namely: the ethanol concentration remaining in the reactor at the time it was unloaded, the percent unloaded volume and the reactor loading rate, which are the three operational variables most widely used by industry. As far as we know this

is the first time that these variables have been correlated in this way. The ensuing models were used to optimize the process via the response surfaces of the variables and the results compared with those of previously reported first principles models.

2. Material and methods

2.1 Raw material or substrate

The acetification substrate was white wine from the Montilla–Morilesregion, a protected designation of origin in southern Spain[33].The wine had an initial ethanol concentration of 11.7±0.3 %(v/v) and an acidity of 0.2 %(w/v).

2.2 Microorganisms

The inoculum used consisted of 3 L of fermentation broth from an industrial tank in full operation (Deoleo S.A., Córdoba, Spain).

2.3 Fermentation conditions

Experiments were conducted on a fully automated 8 L Frings reactor (Heinrich Frings GmbH & Co. KG, Bonn, Germany), details of which can be found in previous works[14, 24, 34-38].The reactor was operated in a semi-continuous mode to facilitate assessment of the influence of the ethanol concentration at the time it was unloaded, the mean unloaded volume and the wine loading rate on the fermentation rate and acetic acid production. A constant temperature of 31 ºC was used to mimic industrial conditions.

The ethanol concentration at the time of unloading ranged from 0.5 to 3.5% (v/v), the mean unloaded volume from 25 to 75% of the final working volume from 2 to 6 L and the loading rate from 0.01 to 0.06 $L \cdot min^{-1}$. The air flow rate at the time the reactor reached its final volume (8 L) was 7.5 $L \cdot (h \cdot L\ medium)^{-1}$.

The bioreactor was fully equipped to operate in an automated manner, so it was loaded, unloaded and monitored via appropriate computer software. This methodology afforded a high operational reproducibility and exhaustive recording of data.

For estimating the mean acetification rate, the method proposed elsewhere [35], using the variation of the ethanol concentration over the fermentation cycle, was used.

2.4 Experimental design

We used a central composite design (viz., a Box–Behnken design) to simultaneously examine the influence of all factors and reduce the number of experiments needed as a far as possible. A total of 15 different sets experimental conditions were needed to characterize the 3 variables considered (see Tables 1 and 2).In any case, a huge experimental labour has been carried out; Table 2 shows the number of useful replications for each set of experimental conditions (a total number of 176). Additionally, each time the operational conditions were modified, a variable number of adaptation cycles can be necessary until get repetitive results.

2.5 Analytical methods

Volume was measured by means of an EJA 110 differential pressure probe (Yokogawa Electric Corporation, Tokyo, Japan).

The ethanol concentration was monitored in a continuous manner by using an Alkosens® probe and an Acetomat® transducer (Heinrich Frings GmbH & Co. KG, Bonn, Germany). The probe was calibrated by determining ethanol with an alcohol meter[39] in media previously obtained by steam distillation.

Acetic acid concentrations were measured by acid–base titration[39].

Total cell concentrations were determined by direct counting in a Neubauer chamber using a light microscope, and viable cell concentrations similarly but using a

LIVE/DEAD BacLight bacterial viability kit and the fluorescence unit of the microscope.

2.6 Mathematical methods

The optimum values of the operational variables were determined by establishing the Karush–Kuhn–Tucker (KKT) conditions[40, 41] to be fulfilled by the optimum points of a non-linear restricted optimization problem. The problems addressed here were defined as follows:

$$\begin{gathered} Max\ f(x_1, x_2, \dots, x_n) \\ s.t.\ g_1(x_1, x_2, \dots, x_n) \le 0 \\ g_2(x_1, x_2, \dots, x_n) \le 0 \\ g_3(x_1, x_2, \dots, x_n) \le 0 \\ \dots. \\ g_m(x_1, x_2, \dots, x_n) \le 0 \end{gathered} \tag{1}$$

where$x_i$denotes decision variables.Potential maxima were obtained from the following Lagrangian function:

$$\mathcal{L} = -f + \sum_{i=1}^{m} \lambda_i\, g_i \tag{2}$$

where$\lambda_i$are the KKT multipliers, and solving the following system of equations:

$$\begin{gathered} \nabla_x \mathcal{L} = -\nabla_x f + \sum_{i=1}^{m} \lambda_i\, \nabla_x g_i = 0 \\ \lambda_i g_i = 0, i = 1, .., m \end{gathered} \tag{3}$$

∇_xbeing the gradient operator with respect tothe decision variables. Only those solutions fulfilling $\lambda_i \ge 0$($\lambda_i > 0$ for those active restrictions at the solution) and$g_i \le 0$ were selected.The latter two conditions and those imposed by Eq. (3) are the necessary first-

order KKT conditions.If $f$is a differentiable, concave function, and all $g_i$restrictions are differentiable and convex, then the points fulfilling the KKT conditions will be maxima of the function. A function is concave if its Hessian matrix with respect to decision variables is negative semidefinite in the operational range of the variables —which can be verifiedsimply by inspecting the eigenvalues—, and convex if the matrix is positivesemidefinite.

3. Results

Table 3 shows the experimentalresults for selected process variables. The results were used to fit the quadratic polynomial model for each variable.

An analysis of variance (ANOVA) of the mean acetic acid formation rate revealed significant differences at the 99.9% probability level. Therefore, this variable was dependent on the operating conditions used and hence amenable to polynomial regression, which was applied in three forms, namely: best subset regression, backward stepwise regression and backward stepwise regression. Together with a Pareto analysis, these procedures revealed which polynomial terms were significant.Equation (4) was obtained for the mean acetic acid formation rate with an error less than 0.01 gacetic acid·(100 mL·h)$^{-1}$:

$$(r_A)_{est} = 0.160 + 0.0443 \cdot E + 3.47 \cdot 10^{-4} \cdot V - 5.84 \cdot 10^{-3} \cdot E^2 - 3.468 \cdot C^2 - 2.33 \cdot 10^{-4} \cdot E \cdot V \quad (4)$$

As can be seen, some operational variables (e.g., the reactor loading rate) were not present as such in the statistically significant terms. Also, some interactions between variables had no influence on the objective function. The ensuing model was valid only locally, within the set ranges of the operational variables.

Figure 1 compares the experimental values with their estimated counterparts. The two sets coincided exactly in 9 cases and differed by 4.5–5.5% in the other 6. An analysis of residuals between experimental and estimated values revealed that they were normally distributed.

Equation (5) was obtained forthe overall production of acetic acid in a cycle with an error less than 0.5 gacetic acid·h^{-1}:

$$(P_A)_{est} = 10.36 + 3.344 \cdot E + 0.118 \cdot V - 0.413 \cdot E^2 - 1.01 \cdot 10^{-3} \cdot V^2 - 0.02 \cdot E \cdot V \quad (5)$$

As can be seen, the acetic acid production was completely independent of the loading rate. Also, a comparison of experimental and estimated values and an analysis of residuals revealed acceptable consistency.

The equations for the other variables in Table 3 were as follows:

$$(EtOH_{mean})_{est} = 0.323 + 0.667 \cdot E + 0.0556 \cdot V \quad (6)$$

$$HAc_{mean} = 9.959 - 0.627 \cdot E - 5.32 \cdot 10^{-4} \cdot V^2 \quad (7)$$

$$([Total\ Cells]_{mean})_{est} = (2.4732 \pm 0.1988) \cdot 10^8\ cells \cdot mL^{-1} \quad (8)$$

$$([Viable\ Cells]_{mean})_{est} = (2.2586 \pm 0.2216) \cdot 10^8\ cells \cdot mL^{-1} \quad (9)$$

$$(V_{mean})_{est} = 7.766 - 1.09 \cdot 10^{-4} \cdot V^2 + 0.163 \cdot V \cdot C \quad (10)$$

Prediction errors for $(EtOH_{mean})_{est}$, $(HAc_{mean})_{est}$ and $(V_{mean})_{est}$were1.2 % (v/v), 1.2 % (w/v) and 0.3 L, respectively.With respect to cellular concentrations, an ANOVA revealed the absence of significant differences among experiments at the 99.9% probability level; this allowed mean values to be represented as constants with a given error.

4. Discussion

After the polynomial models were constructed, response surfaces were used to assess the influence of the operational variables on acetificationperformance and identify their optimum values. In industrial practice, the objective functions to be maximized are those for the mean acetic acid formation rate, $(r_A)_{est}$, and acetic acid production $(P_A)_{est}$.

4.1 Mean fermentation rate

Figure 2 shows the response surfaces of $(r_A)_{est}$ at variable ethanol concentrations at unloading time (0.5–3.5 % v/v) as a function of the percent unloaded volume (25–75%) and loading rate (0.01–0.06 $L \cdot min^{-1}$). As can be seen, the unloaded volume exhibited a marked influence, but always depending on the ethanol concentration. Thus, when an ethanol concentration of 3.5 % (v/v) at unloading time is used, the maximum acetification rate, ca. 0.23 g acetic acid·$(100\ mL \cdot h)^{-1}$, was obtained after unloading 25% of the culture medium; nevertheless if the ethanol concentration is 0.5 % (v/v) a lower maximum acetification rate, ca.a value lightly below0.20 g acetic acid·$(100\ mL \cdot h)^{-1}$, was obtained when a 75% of the culture medium is unloaded. These results clearly indicate that the effects of the operational variables were not independent of one another.

As can also be seen, reducing the ethanol concentrationremaining at the time the reactor is unloaded to 0.5 % (v/v) led to lower mean rates than when the substrate was used to a lesser extent irrespective of the other two operational variables.

In any case, the mean rate was largely dependent on the operating conditions relating to the unloaded volume. Thus, increasing the volume shifted the peak $(r_A)_{est}$ value from an ethanol concentration of 3.5 % (v/v) to one of 2 % (v/v).

On the other hand, the loading rate was scarcely influential. In fact, this variable was only present in an interaction term in Eq. (4) and the term in question is non-

significant relative to the others in the polynomial. In any case, the peak $(r_A)_{est}$ value was invariably obtained at a loading rate of 0.01 $L \cdot min^{-1}$.

Although the response surfaces exhibited apparently low slopes, the differences between maximum and minimum rates were substantial in some cases. Thus, such differences amounted to 18.2, 8.9 and 13.6% from the minimum values obtained at an ethanol concentration of 3.5, 2.0 and 0.5 % (v/v), respectively —and can result in considerable differences in production costs.

These results are clearly suggestive of an influence of the operating conditions on changes in the culture medium. Specifically, the conditions may have a direct effect on cell concentrations and/or its activity during the acetification cycle. Thus, as can be seen from Figure 3, which shows the variation of the mean concentration of viable cells with the ethanol concentration remaining at the time the reactor is unloaded and unloaded volume, cell concentrations seemingly decreased on reducing the ethanol concentration from 3.5 % (v/v) to 0.5 % (v/v), the differences being statistically non-significant if one provides for experimental errors —for clarity and simplicity, the graph excludes the standard deviation at each point.

Each set of experimental results (viz., that corresponding to an unloaded volume of 25, 50 or 75 %) was represented by an identical mean value shown in the graphs and obtained from Eq. (9). However, the differences between maxima and minima values of viable cells concentration during the acetification cycle decreased with decreasing unloaded volume and was very similar to the mean cell concentration —approximation to a pseudo-steady state. So, these differences at an unloaded volume of 75 % were greater by effect of increased cell renewal.

Thus, when cell concentrations are similar to their mean value at any time (i.e., with a low unloaded volume), the operating conditions have a more marked influence on

acetificationperformance owing to a minor cell renewal. Using operating conditions far from ideal in this situation can have highly adverse consequences on cell behaviour. This may be why the mean acetic acid formation rate obtained with an unloaded volume of 25 % at an ethanol concentration at unloading time of 0.5 % (v/v) was the lowest of all. On the other hand, large cell renewal (i.e., a high unloaded volume) caused fermentation rates to be similar (see Figure 2) and the corresponding slopes to differ between the best (unloading at 3.5 or 2 % (v/v) ethanol) and worst conditions (unloading at 0.5 % (v/v) ethanol).

Ultimately, differences in the above-described results originated from differences in the ethanol and acetic acid concentrations, which were strongly influenced by the operating conditions. As can be seen from Figure 4, which shows the response surface for Eq. (6), reducing the ethanol concentration remaining at the time the reactor is unloaded led to decreased mean ethanol values but increased acetic acid values (Eq. (7)). Also, at each ethanol concentrationremaining at the time the reactor is unloaded, the differences between mean ethanol and acetic concentrations increased with increasing unloaded volume. Thus, when unloading 75% of the medium, the percentages of variation between mean ethanol concentrations respect to the mean minimum values were 68.2, 90.7 and 135.4 % at an ethanol concentration at unloading time of 3.5, 2.0 and0.5 % (v/v), respectively; similarly, the percentages of variation for acetic acid were 56.2, 46.9 and 40.2 %.All these results are just a new confirmation of the well-known influence of ethanol and acetic acid on the growth of acetic acid bacteria [10]. On the other hand, as can be seen from Figure 4, the loading rate had no influence on the mean concentrations of ethanol and acetic acid.

In fact, a comparison of Figures 2–4 suggests that these two compounds influenced cell concentrations and activity, and hence the overall performance of the process. Thus, the

decreased availability of ethanol and increased mean acidity at an ethanol concentration of 0.5 % (v/v) led to markedly decreased mean fermentation rates in relation to other operating conditions. The limiting effects of substrate scarcity and inhibition due to high product concentration were also observed previously with other models[10, 42-45]. However, with less marked ethanol depletion at reactor unloading time, the substrate was available in greater amounts and the acid concentration lower, which seemingly facilitated the process judging by the high mean acetic acid formation rates observed. However, as can be seen from Figure 2, the effect was markedly dependent on the particular unloaded volume. Thus, the rate peaked at an ethanol concentration of 3.5 % with an unloaded volume of 25% but at 2 % (v/v) with one of 75%.

These results can be ascribed to a potentially inhibitory effect of ethanol, which reached increased mean concentrations when allowed to be unloaded at3.5 % (v/v) relative to 2 % (v/v) (see Figure 4); also, theyare consistent with previous results obtained by non-structured modelling of the influence of ethanol and acetic acid on the fermentation rate[2, 10, 14, 24, 46].

Figure 5 suggests that the optimum conditions for microbial development (i.e., those leading to the highest fermentation rates) were those involving a mean ethanol concentration of ca. 4–5 % (v/v) (i.e., an average acetic acid concentration of 7–8 % (w/v)). These results are consistent with those of previous optimization studies[46]. The value of each operating condition to be used in order to maximize $(r_A)_{est}$was calculated more accurately by using a restricted optimization procedure involving application of the above-described KKTconditions (see under Experimental). To this end, the objectivefunction (Eq. (4)), was subjected to the following constraints:

$$0.01 \leq C \leq 0.06$$
$$0.5 \leq E \leq 3.5 \qquad (11)$$

$$25 \leq V \leq 75$$

which can be rewritten as

$$\begin{gathered} 0.01 - C \leq 0 \\ C - 0.06 \leq 0 \\ 0.5 - E \leq 0 \\ E - 3.5 \leq 0 \\ 25 - V \leq 0 \\ V - 75 \leq 0 \end{gathered} \tag{12}$$

As can be seen, the $(r_A)_{est}$ function is differentiable and concave —its Hessian matrix with respect to decision variables is constant and negative semidefinite. Also, the previous restrictions are differentiable and linear with respect to decision variables, so they are convex —and concave as well. Therefore, the solutions to the necessary KKT conditions, if any, should coincide with maxima.

For maximization, Eq. (3) was used to construct the following system of equations in order to identify critical points:

$$\begin{gathered} -(0.0443 - 11.68 \cdot 10^{-3} \cdot E - 2.33 \cdot 10^{-4} \cdot V) - \lambda_3 + \lambda_4 = 0 \\ -(-6.936) \cdot C - \lambda_1 + \lambda_2 = 0 \\ -(3.47 \cdot 10^{-4} - 2.33 \cdot 10^{-4} \cdot E) - \lambda_5 + \lambda_6 = 0 \\ \lambda_1(0.01 - C) = 0 \\ \lambda_2(C - 0.06) = 0 \\ \lambda_3(0.5 - E) = 0 \\ \lambda_4(E - 3.5) = 0 \\ \lambda_5(25 - V) = 0 \\ \lambda_6(V - 75) = 0 \end{gathered} \tag{13}$$

In this way, a total of 21 solutions where obtained of which only one fulfilled the KKT conditions, namely, that involving the following values of the operational variables:

$$E = 3.29\ \%\ (v/v)$$
$$C = 0.01 L \cdot min^{-1} \quad (14)$$
$$V = 25\ \%$$

which led to a peak $(r_A)_{est}$value of 0.23 gacetic·(100 mL·h)$^{-1}$.

4.2 Acetic acid production

Although determining the mean fermentation rate of the process is important to assess the influence of operational variables, in industrial practice it may be more useful to examine the influence on acetic acid production. The conclusions thus reached need not be the same since the mean fermentation rate depends on the mean reactor volume, which is in turn dependent on the operating conditions (see Eq. (10)). In any case, the two are related by the following equation:

$$P_A = \frac{HAc_{final} \cdot V}{t_{total}}$$
$$r_A = \frac{P_A}{V_{mean}} \quad (15)$$

Figure 6 shows the response surface for $(P_A)_{est}$ (see Eq. (5)) over the studied operational ranges. As can be seen, the loading rate had no effect on acetic acid production —this is quite apparent from Eq. (5), which contains no associated term. Also, the maximum production of acetic acid (ca. 17.6 g acetic acid·h^{-1}) was obtained at the highest ethanol concentration at unloading time (3.5 % (v/v)) and lowest unloaded volume (25 %). However, if one considers estimation errors, the unloaded volume maximizing acetic acid production was as high as 40 %.

If the primary aim is to maximize production, then the above-described operating conditions are quite suitable. However, depending on the particular type of vinegar to be obtained, it may be necessary to impose restrictions on some variables such as the

ethanol concentrationremaining at the time the reactor is unloaded. For instance, unloading the reactor at an ethanol concentration of 0.5 % (v/v) detracts from the maximum possible acetic acid production, obtaining a value ca. 14.8 g acetic acid·h^{-1}. However, this peak value can be obtained over a broad range of unloaded volumes (~ 35–65 %).

As with the mean fermentation rate, we determined the optimum acetic acid production in the operational ranges. The objective function used to this end was that for $(P_A)_{est}$ (Eq. (5)) and restrictions imposed via the equation subset (12) except for the first and second constraint, since the variables were independent of the loading rate. The Hessian matrix for the objective function was constant and negative definite; also, obviously, the conclusions of the constraints were identical with those for the mean fermentation rate.

The equation system used to identify the maxima was constructed as in the previous case and yielded 9 solutions of which only one fulfilled the KKT conditions:

$$\begin{aligned} E &= 3.44\ \%\ (\mathrm{v/v}) \\ V &= 25\ \% \end{aligned} \tag{16}$$

which led to a peak $(P_A)_{est}$value of 17.58gacetic acid·h^{-1}.

4.3 Comparison with other modelling approaches

In previous works, our group developed a first principles model based on balance and kinetic equations[46] that was subsequently used to identify the optimum operating conditions[2, 14, 24]; the conclusions afforded by the results were similar to those reached in this work. The approach used in previous work has the advantages that it provides a deeper knowledge of the underlying mechanisms of the process and holds over broader operating ranges. Unlike the polynomial approach, however, it has the disadvantage that the model parameters are much more difficult to examine, determine and handle. Thus, ensuring that the model parameters could be unequivocally obtained

from the equation structure required verifying that the model possesses structural identifiability. This entailed using complex algorithms[14, 47]. Also, the model had to be checked for practical identifiability, which required additionally assessing the amount of experimental data available and their quality. The proposed method was found not to be globally, but only locally, identifiable[2]. This, however, did not make it useless for representation and optimization purposes within specific operational ranges. These results further validate the previous models, which were constructed via rather different approaches. Therefore, based on the high consistency between the estimations of the two types of models compared, the polynomial models are to be preferred for practical purposes by virtue of their simplicity and easier mathematical processing.

5. Conclusions

In this work, we examined the influence of the major operational variables of industrial interest (viz., ethanol concentration at the time the reactor was unloaded, unloaded volume and unloading rate) on the acetic fermentation process with a view to optimizing the operating conditions in terms of mean fermentation rate and acetic acid production. To this end, an experimental design, that allowed black box models relating process and operational variables via polynomial equations to be developed, was used. The target variables additionally included the mean ethanol and acetic acid concentrations,the mean volume and the mean total and viable cell concentrations. The presence of interaction terms in the resulting equations testifies to the mutual relationships between variables. The optimum values of the variables were identified by examining their response surfaces and the results compared with those of a mathematical approach under optimality conditions.

The maximum mean rate of acetic acid formation, ca. 0.23 g acetic acid·(100 mL·h)$^{-1}$, was obtained at an ethanol concentration at unloading time of 3.5 % (v/v), an unloaded volume of 25 % and a loading rate of 0.01 L·min^{-1} —the last variable, however, was scarcely influential.

The acetic acid production was independent of the loading rate. Thus, production peaked at ca. 17.6 g acetic acid·h^{-1}, which was obtained at a near-maximal ethanol concentration (3.5 % (v/v)) and a low unloaded volume (25%).

The proposed modelling approach and the optimum values it provided were compared with previous studies involving an unstructured approach based onbalance differential equations, kinetic equations and equilibrium equations. The coincidence of their predictions provides further validation for the two models. In practice, however, polynomial models will facilitate mathematical processing (particularly as regards parameter estimation). It should be noted that the target process is a typical example, so the ensuing conclusions may apply to other, similar biotechnological processes.

Acknowledgements

The authors wish to acknowledge funding of this research by Spain's Ministry of Science and Technology (MCyT) and Ministry of Education and Science (MEC) in the framework of Project AGL2002-01712, PET2006_0827. Also, they wish to thank the firm Deoleo S.A. (Cordoba, Spain) for valuable help and advice.

References

[1] D. Garrido-Vidal, C. Pizarro, J.M. Gonzalez-Saiz, Study of process variables in industrial acetic fermentation by a continuous pilot fermentor and response surfaces, Biotechnol. Prog., 19 (2003) 1468-1479.

[2] J.E. Jimenez-Hornero, I.M. Santos-Duenas, I. Garica-Garcia, Optimization of biotechnological processes. The acetic acid fermentation. Part III: Dynamic optimization, Biochem.Eng. J., 45 (2009) 22-29.

[3] L. Yuemei, B. Weidong, L. Zhoumin, Z. Hao, Optimization of acetic acid fermentation parameters for production of persimmon vinegar, Transactions of the Chin. Soc. of Agric.Eng., 24(4) (2008) 257-260.

[4] J. Yihong, Technology Optimization of Acetic Acid Fermentation of Greengage Vinegar [J], Transactions of The Chin. Soc. of Agric. Machinery, 36(7) (2005) 85-88.

[5] Z. Qi, H. Yang, X. Xia, Y. Xin, L. Zhang, W. Wang, X. Yu, A protocol for optimization vinegar fermentation according to the ratio of oxygen consumption versus acid yield, J. of Food Eng., 116 (2013) 304-309.

[6] Y. Zhang, K. Li, K. Yang, X. Fan, M. Zhang, Optimization of vinegar-steaming process for Wuweizi (Fructus Schisandrae Chinensis) with response surface method, J. of Traditional Chin. Med., 33 (2013) 682-685.

[7] F. Nieto, Algunos aspectos de la tecnología de la fermentación acética, in: C. Llaguno, M.C. Polo (Eds)El vinagre de vino, CSIC, Madrid, España, 1991, pp. 69-103.

[8] F. Emde, State-of-the-art technologies in submersible vinegar production, in: I. García (Eds) Segundas Jornadas de I+D+I en la Elaboración de Vinagres - Second Symposium on R+D+I for Vinegar Production,Servicio de Publicaciones, Córdoba, 2006, pp. 101-109.

[9] S. Sellmer-Wilsberg, New strategies in process control for the production of wine vinegar, in: I. García (Eds) Segundas Jornadas de I+D+I en la Elaboración de Vinagres - Second Symposium on R+D+I for Vinegar Production,Servicio de Publicaciones, Córdoba, 2006, pp. 127-132.

[10] M. Gullo, E. Verzelloni, M. Canonico, Aerobic submerged fermentation by acetic acid bacteria for vinegar production: Process and biotechnoogical aspects, Process Biochem., 49 (2014) 1571-1579.

[11] J.M. Gonzalez-Saiz, C. Pizarro, D. Garrido-Vidal, Evaluation of kinetic models for industrial acetic fermentation: Proposal of a new model optimized by genetic algorithms, Biotechnol. Prog., 19 (2003) 599-611.

[12] J.M. Gonzalez-Saiz, D. Garrido-Vidal, C. Pizarro, Modelling the industrial production of vinegar in aerated-stirred fermentors in terms of process variables, J. of Food Eng., 91 (2009) 183-196.

[13] C. Pizarro, J.M. Gonzalez-Saiz, D. Garrido-Vida, Kinetic modelling of acetic fermentation in an industrial process by genetic algorithms with a desirability function, J. of Chemometrics, 17 (2003) 453-462.

[14] J.E. Jimenez-Hornero, I.M. Santos-Duenas, I. Garcia-Garcia, Optimization of biotechnological processes. The acetic acid fermentation. Part I: The proposed model, Biochem.Eng. J., 45 (2009) 1-6.

[15] M. Tobajas, A.F. Mohedano, J.A. Casas, J.J. Rodriguez, Unstructured kinetic model for reuterin and 1,3-propanediol production by Lactobacillus reuteri from glycerol/glucose cofermentation, J. of Chem.Technol. and Biotechnol., 84 (2009) 675-680.

[16] B.S. Chang, Z.G. Sue, Kinetic model for simultaneous cell disruption and aqueous two-phase extraction, J. of Chem.Technol. and Biotechnol., 81 (2006) 454-460.

[17] I. García-García, I.M. Santos-Dueñas, C. Jiménez-Ot, J.E. Jiménez-Hornero, J.L. Bonilla-Venceslada, Vinegar engineering, in: L. Solieri, P. Giudici (Eds.) Vinegars of the World, Springer, Italy, 2009, pp. 97-120.

[18] R. Bellman, K.J. Åström, On structural identifiability, Mathematical Biosci., 7 (1970) 329-339.

[19] K. Godfrey, J. DiStefano III, Identifiability of model parameters, Identifiability of parametric models, (1987) 1-20.

[20] K. Versyck, J. Claes, J. Van Impe, Optimal experimental design for practical identification of unstructured growth models, Mathematics and computers in simul., 46 (1998) 621-629.

[21] B. Petersen, K. Gernaey, P.A. Vanrolleghem, Practical identifiability of model parameters by combined respirometric-titrimetric measurements, Water Sci. and Technol., 43 (2001) 347-356.

[22] N. Noykova, T.G. MuÈller, M. Gyllenberg, J. Timmer, Quantitative analyses of anaerobic wastewater treatment processes: identifiability and parameter estimation, Biotechnol. and bioeng., 78 (2002) 89-103.

[23] J.E. Jimenez-Hornero, I.M. Santos-Duenas, I. Garcia-Garcia, Structural identifiability of a model for the acetic acid fermentation process, Mathematical Biosci., 216 (2008) 154-162.

[24] J.E. Jimenez-Hornero, I.M. Santos-Duenas, I. Garcia-Garcia, Optimization of biotechnological processes. The acetic acid fermentation. Part II: Practical identifiability analysis and parameter estimation, Biochem. Eng. J., 45 (2009) 7-21.

[25] M.A. Mazutti, M.L. Corazza, F. Maugeri, M.I. Rodrigues, J.V. Oliveira, H. Treichel, F.C. Corazza, Hybrid modeling of inulinase bio-production process, J. of Chem.Technol. and Biotechnol., 85 (2010) 512-519.

[26] N.K. Nguyen, J.J. Borkowski, New 3-level response surface designs constructed from incomplete block designs, J. of Statistical Plan. and Inference, 138 (2008) 294-305.

[27] M.A. Bezerra, R.E. Santelli, E.P. Oliveira, L.S. Villar, L.A. Escaleira, Response surface methodology (RSM) as a tool for optimization in analytical chemistry, Talanta, 76 (2008) 965-977.

[28] A.G. Abilov, V.S. Aliev, M.I. Rustamov, N.M. Aliev, K.A. Lutfaliev, Problems of control and chemical engineering experiment, Boston/Cambridge, Massachusetts, USA, 1975.

[29] G.E. Box, W.G. Hunter, J.S. Hunter, Statistics for Experimenters.Through Description of Experimental Planning with Linear Models, John Wiley and Sons, New York, 1978.

[30] N. Miller, C. Miller, Statistics and chemometrics for analytical chemistry, Harlow, England; New York: Prentice Hall, 2000.

[31] R. Castro Mejias, R. Natera Marin, M. de Valme Garcia Moreno, C. Garcia Barroso, Optimisation of headspace solid-phase microextraction for analysis of aromatic compounds in vinegar, J. of chromatography A, 953 (2002) 7-15.

[32] H. Gao, G.F. Xu, Y.Y. Fan, H.J. Nan, S.F. Fu, Optimization of Fermentation Process of Pear Vinegar by Response Surface Methodology, Advanced Mater.Res., 201 (2011) 2513-2516.

[33] BOJA 249, ORDEN de 30 de noviembre de 2011, por la que se aprueba el Reglamento de funcionamiento de las Denominaciones de Origen <<Montilla-Moriles>> y <<Vinagre de Montilla-Moriles>>, así como sus correspondientes Pliegos de Condiciones., in: B.O.d.l.J.d. Andalucía (Eds.)Sevilla, 2011, pp. 144-166.

[34] S. Baena-Ruano, C. Jimenez-Ot, I.M. Santos-Duenas, D. Cantero-Moreno, F. Barja, I. Garcia-Garcia, Rapid method for total, viable and non-viable acetic acid bacteria determination during acetification process, Process Biochem., 41 (2006) 1160-1164.

[35] I. Garcia-Garcia, D. Cantero-Moreno, C. Jimenez-Ot, S. Baena-Ruano, J. Jimenez-Hornero, I. Santos-Duenas, J. Bonilla-Venceslada, F. Barja, Estimating the mean acetification rate via on-line monitored changes in ethanol during a semi-continuous vinegar production cycle, J. of Food Eng., 80 (2007) 460-464.

[36] O. Maestre, I.M. Santos-Duenas, R. Peinado, C. Jimenez-Ot, I. Garcia-Garcia, J.C. Mauricio, Changes in amino acid composition during wine vinegar production in a fully automatic pilot acetator, Process Biochem., 43 (2008) 803-807.

[37] S. Baena-Ruano, C. Jimenez-Ot, I.M. Santos-Duenas, J.E. Jimenez-Hornero, J.L. Bonilla-Venceslada, C. Alvarez-Caliz, I. Garcia-Garcia, Influence of the final ethanol concentration on the acetification and production rate in the wine vinegar process, J. of Chem.Technol. and Biotechnol., 85 (2010) 908-912.

[38] S. Baena-Ruano, I.M. Santos-Duenas, J.C. Mauricio, I. Garcia-Garcia, Relationship between changes in the total concentration of acetic acid bacteria and major volatile compounds during the acetic acid fermentation of white wine, J. of the Sci. of Food and Agriculture, 90 (2010) 2675-2681.

[39] J.G. Cazorla, M.X. Vayreda, R.A. Romero, Técnicas usuales de análisis en enología, Panreac Química, España, 2005.

[40] W. Karush, Minima of functions of several variables with inequalities as side constraints, Master's thesis, Dept. of Mathematics, Univ. of Chicago, 1939.

[41] H. Kukn, A. Tucker, Nonlinear programming, Proceedings of second Berkeley symposium on mathematical statistics and probability, 1951, pp. 481-492.

[42] H. Ebner, S. Sellmer-Wilsberg, Vinegar, acetic acid production, Encyclopedia of Bioprocess Technology.

[43] A. Alian, I.L. Rabatnova, P.I. Nikolaev, V.A. Ivanov, Submerged culture of acetic acid bacteria under different aeration conditions, Mikrobiologiya, 1963, pp. 703-710.

[44] A. Mori, N. Konno, G. Terui, Kinetic studies on submerged acetic acid fermentation .1. Behaviors of Acetobacter-Rancens cells toward dissolved oxygen, J. of Fermentation Technol., 48 (1970) 203-212.

[45] A. Mori, G. Terui, Kinetic studies on submerged acetic acid fermentation 2. Process kinetics, J. of Fermentation Technol., 50 (1972) 70-78.

[46] J.E. Jiménez-Hornero, Contribuciones al modelado y optimización del proceso de fermentación acética, Universidad Nacional de Educación a Distancia, España, 2007.

[47] I.García-García, J.Jiménez-Hornero, Estudio de la identificabilidad estructural de un modelo para el proceso de la fermentación acética, in: I. García (Eds) Segundas Jornadas de I+D+I en la Elaboración de Vinagres - Second Symposium on R+D+I for Vinegar Production, Servicio de Publicaciones, Córdoba, 2006, pp. 186-192.

Figure 1. Estimated versus experimental mean acetification rate, and95 % confidence and prediction intervals

Figure 2. Estimated mean acetification rate as a function of the unloaded volume and loading rate at a final ethanol concentration of 3.5 (black grid), 2.0 (solid) or 0.5 %(v/v) (white grid)

Figure 3. Viable cell concentrations at different unloaded volumes and final ethanol concentrations

Figure 4. Mean ethanol concentration as a function of the unloaded volume and loading rate at a final ethanol concentration of 3.5 (black grid), 2.0 (solid) or 0.5 %(v/v) (white grid)

Figure 5. Estimated mean acetification rate as a function of the mean ethanol and acetic acid concentrations at a loading rate of 0.01, 0.35or 0.06 $L \cdot min^{-1}$

Figure 6. Estimated production rate as a function of the loading rate and unloaded volume at a final ethanol concentration of 3.5 (black grid), 2.0 (solid) or 0.5 %(v/v) (white grid)

Table 1. Control factors used in the Box–Behnken experimental planand their levels.

Factor	Code	Level		
		(–1)	(0)	(1)
Ethanol at unloading time, %(v/v)	E	0.5	2	3.5
Unloadedvolume, %	V	25	50	75
Loadingrate, $L \cdot min^{-1}$	C	0.01	0.035	0.06

Table 2. Box–Behnken experimental plan and responses at different factor levels

Exp	*E*, %(v/v)	*V*, %	*C*, $L \cdot min^{-1}$	Number of replications
1	3.5 (+1)	75 (+1)	0.06 (+1)	8
2	3.5 (+1)	75 (+1)	0.01 (–1)	10
3	3.5 (+1)	25 (–1)	0.06 (+1)	13
4	3.5 (+1)	25 (–1)	0.01 (–1)	21
5	0.5 (–1)	75 (+1)	0.06 (+1)	12
6	0.5 (–1)	75 (+1)	0.01 (–1)	8
7	0.5 (–1)	25 (–1)	0.06 (+1)	7
8	0.5 (–1)	25 (–1)	0.01 (–1)	7
9	3.5 (+1)	50 (0)	0.035 (0)	15
10	0.5 (–1)	50 (0)	0.035 (0)	10
11	2 (0)	75 (+1)	0.035 (0)	7
12	2 (0)	25 (–1)	0.035 (0)	16
13	2 (0)	50 (0)	0.06 (+1)	19
14	2 (0)	50 (0)	0.01 (–1)	11
15	2 (0)	50 (0)	0.035 (0)	12

* The numbers in brackets are normalized values of the variables

Table 3. Experimental values of the dependent variables

Experiment	$(r_A)_{est}$ g acetic acid·(100 mL·h)$^{-1}$	$(P_A)_{est}$ g acetic acid·h^{-1}	$(EtOH_{mean})_{est}$ % (v/v)	$(HAc_{mean})_{est}$ % (w/v)	$([Total\ cells]_{mean})_{est}$ 10^{-8} cells·mL^{-1}	$([Viable\ cells]_{mean})_{est}$ 10^{-8} cells·mL^{-1}	$(V_{mean})_{est}$ L
1	0.20 ± 0.01	15.4 ± 0.4	6.9 ± 1.7	4.7 ± 1.7	2.65 ± 0.98	2.50 ± 0.89	7.8 ± 0.4
2	0.21 ± 0.01	15.1 ± 0.4	6.4 ± 1.5	5.2 ± 1.4	2.74 ± 0.85	2.55 ± 0.78	7.1 ± 0.3

3	0.22 ± 0.01	17.8 ± 0.3	4.5 ± 0.5	7.2 ± 0.5	2.65 ± 0.36	2.44 ± 0.34	7.9 ± 0.2
4	0.23 ± 0.01	17.4 ± 0.6	4.1 ± 0.4	7.5 ± 0.3	2.57 ± 0.28	2.36 ± 0.27	7.7 ± 0.2
5	0.18 ± 0.01	14.4 ± 0.4	5.2 ± 2.1	6.5 ± 2.0	2.41 ± 0.97	2.18 ± 0.83	7.9 ± 0.3
6	0.20 ± 0.01	14.3 ± 0.3	4.9 ± 2.3	6.7 ± 2.2	2.35 ± 0.90	2.11 ± 0.80	7.4 ± 0.4
7	0.17 ± 0.01	13.6 ± 0.3	2.0 ± 0.8	9.5 ± 0.8	2.35 ± 0.24	2.15 ± 0.25	8.0 ± 0.2
8	0.18 ± 0.01	13.8 ± 0.2	1.8 ± 0.7	9.3 ± 0.8	2.29 ± 0.36	2.07 ± 0.33	7.7 ± 0.1
9	0.21 ± 0.01	16.3 ± 0.4	5.5 ± 1.1	6.2 ± 1.1	2.66 ± 0.57	2.47 ± 0.52	7.8 ± 0.3
10	0.20 ± 0.01	15.5 ± 0.2	3.5 ± 1.6	8.2 ± 1.5	1.99 ± 0.56	1.74 ± 0.48	7.9 ± 0.1
11	0.20 ± 0.01	15.1 ± 0.5	6.0 ± 2.1	5.6 ± 2.0	2.60 ± 1.02	2.44 ± 0.91	7.7 ± 0.4
12	0.22 ± 0.01	17.3 ± 0.4	3.1 ± 0.6	8.6 ± 0.6	2.26 ± 0.32	1.99 ± 0.30	7.9 ± 0.2
13	0.21 ± 0.01	16.7 ± 0.5	4.4 ± 1.3	7.2 ± 1.3	2.50 ± 0.55	2.29 ± 0.49	7.9 ± 0.3
14	0.22 ± 0.01	16.5 ± 0.6	4.0 ± 1.1	7.6 ± 1.0	2.56 ± 0.50	2.29 ± 0.44	7.5 ± 0.3
15	0.22 ± 0.01	17.1 ± 0.5	4.2 ± 1.3	7.3 ± 1.3	2.52 ± 0.61	2.30 ± 0.54	7.8 ± 0.3

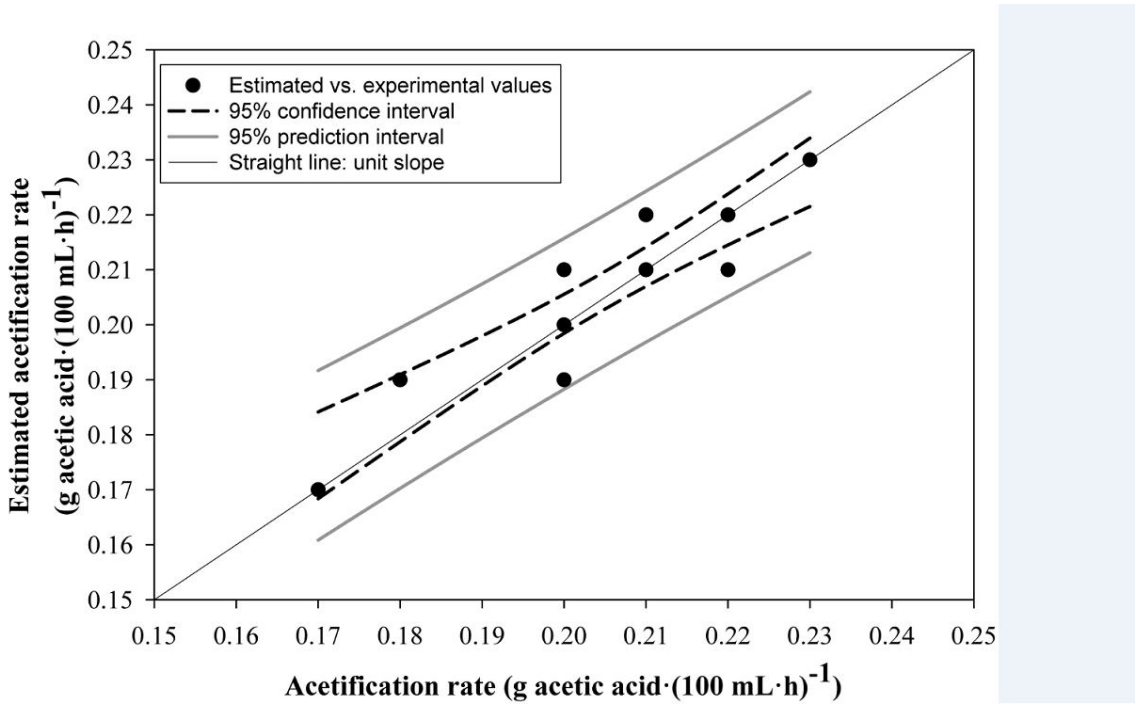

Fig.1

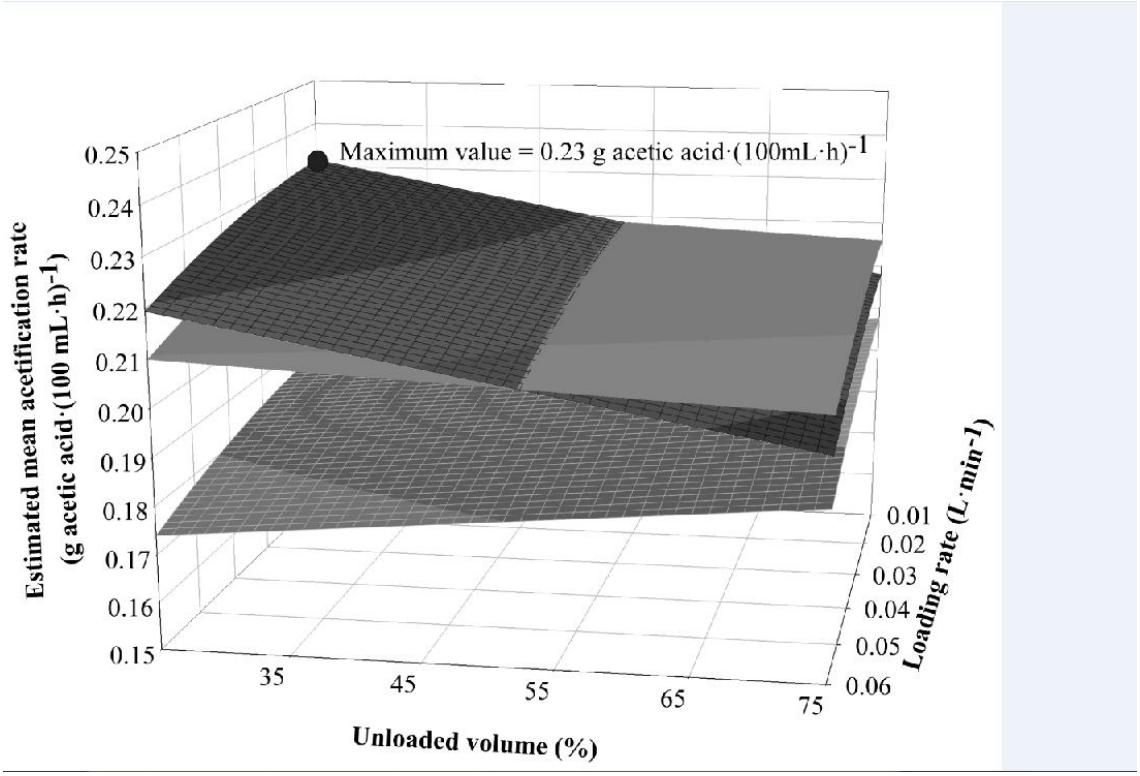
Maximum value = 0.23 g acetic acid·(100mL·h)-1
Estimated mean acetification rate (g acetic acid·(100 mL·h)-1)
0.25
0.24
0.23
0.22
0.21
0.20
0.19
0.18
0.17
0.16
0.15
35
45
55
65
75
Unloaded volume (%)
0.01
0.02
0.03
0.04
0.05
0.06
Loading rate (L·min-1)

Fig.2

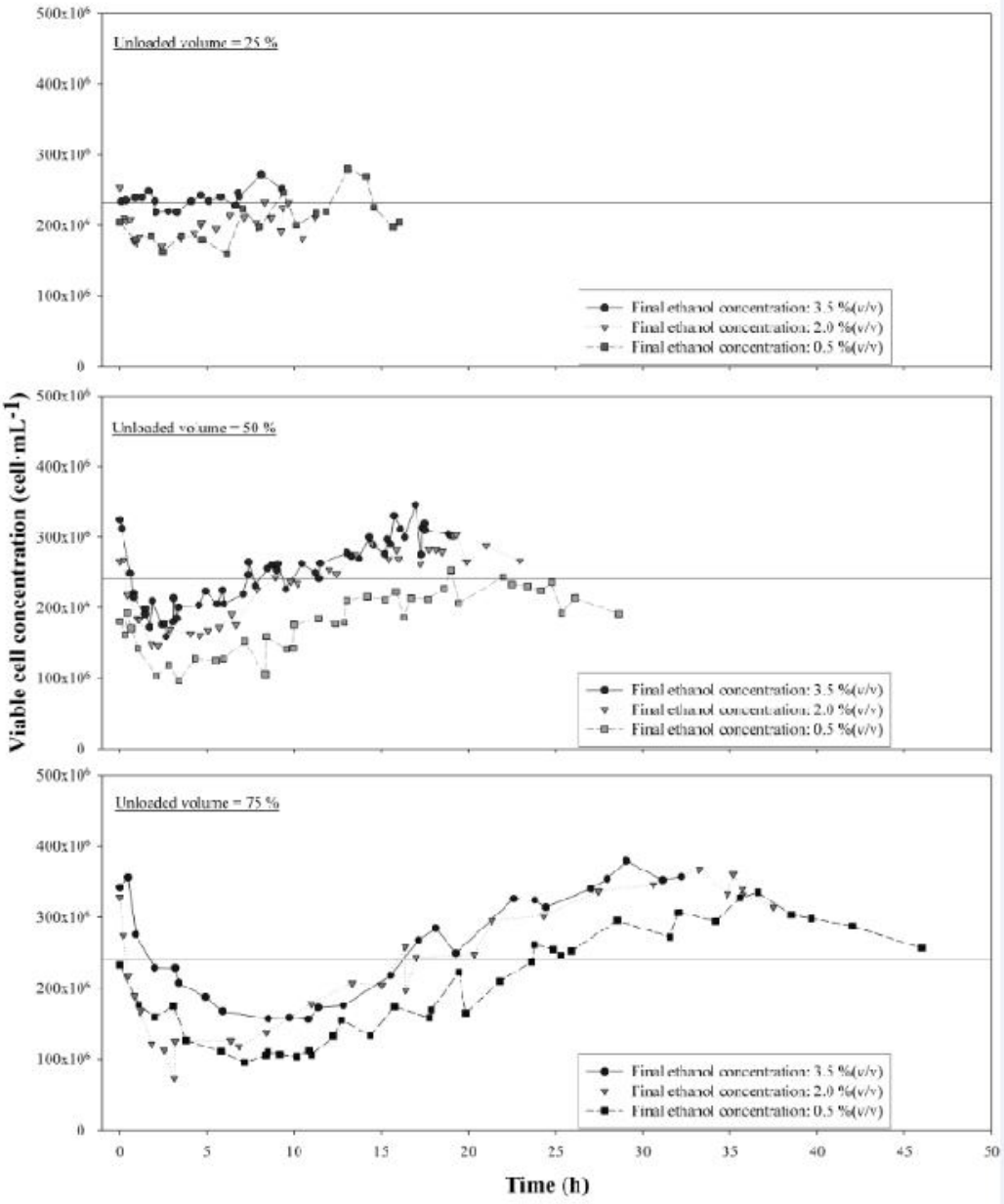
Unloaded volume = 25 %
Unloaded volume = 50 %
Unloaded volume = 75 %
Final ethanol concentration: 3.5 %(v/v)
Final ethanol concentration: 2.0 %(v/v)
Final ethanol concentration: 0.5 %(v/v)
Viable cell concentration (cell·mL-1)
Time (h)

Fig.3

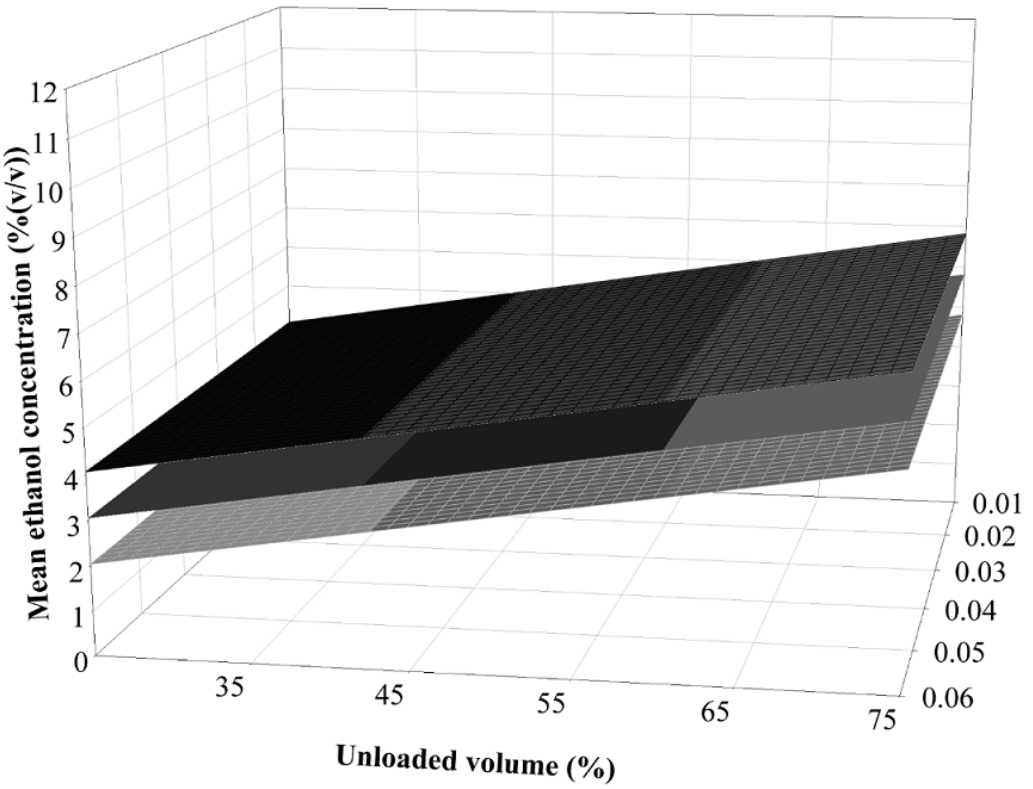
Mean ethanol concentration (%(v/v))
12
11
10
9
8
7
6
5
4
3
2
1
0
35
45
55
65
75
Unloaded volume (%)
0.01
0.02
0.03
0.04
0.05
0.06

Fig.4

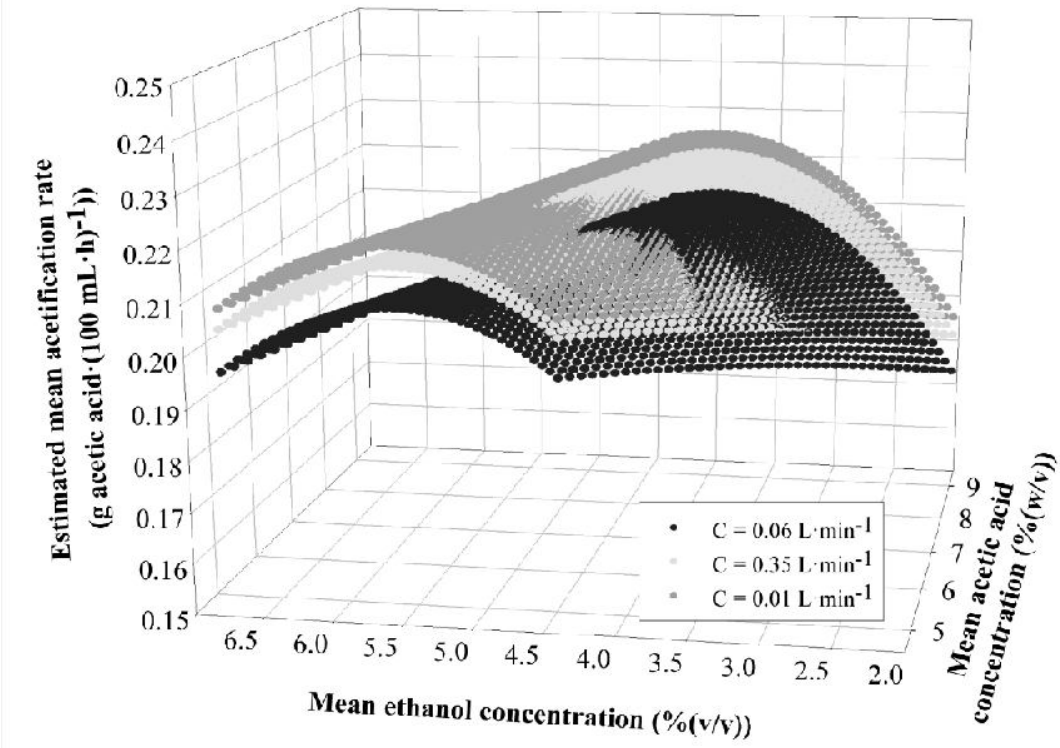
Estimated mean acetification rate
(g acetic acid·(100 mL·h)-1))
0.25
0.24
0.23
0.22
0.21
0.20
0.19
0.18
0.17
0.16
0.15
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Mean ethanol concentration (%(v/v))
C = 0.06 L·min-1
C = 0.35 L·min-1
C = 0.01 L·min-1
9
8
7
6
5
Mean acetic acid concentration (%(w/v))

Fig.5

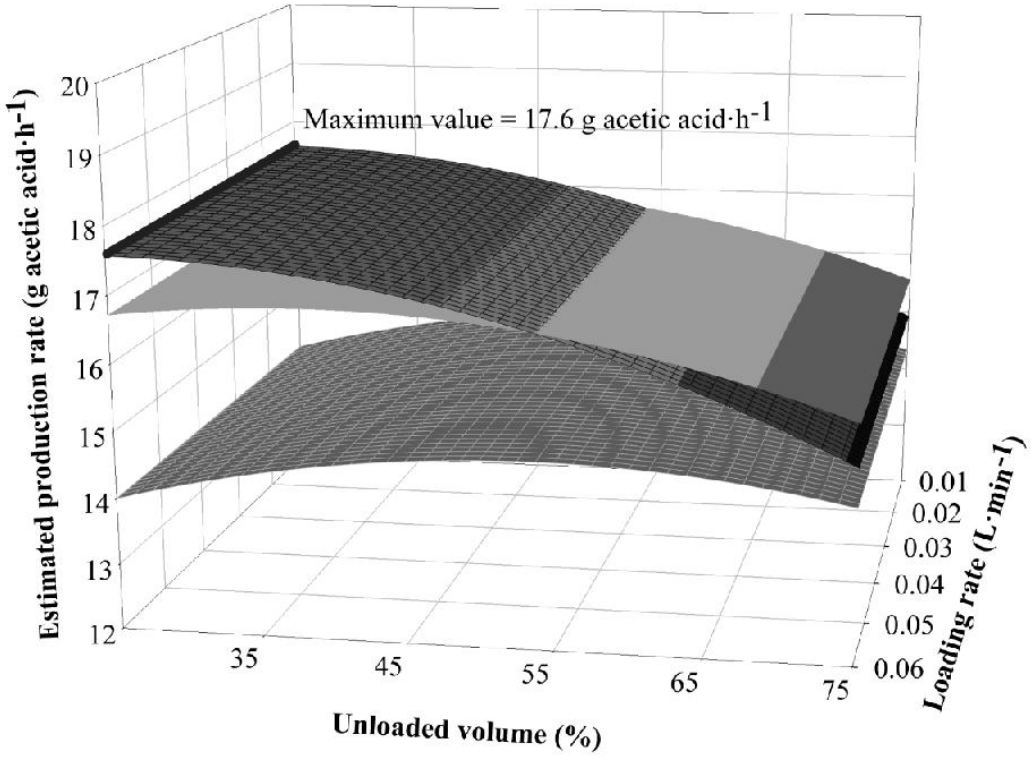
Maximum value = 17.6 g acetic acid·h-1
Estimated production rate (g acetic acid·h-1)
20
19
18
17
16
15
14
13
12
35
45
55
65
75
Unloaded volume (%)
0.01
0.02
0.03
0.04
0.05
0.06
Loading rate (L·min-1)

Fig.6