0273-2289/03/110/0151/$25.00

Ethanol Production by *Saccharomyces cerevisiae* Grown in Sugarcane Blackstrap Molasses Through a Fed-Batch Process

Optimization by Response Surface Methodology

João C. M. Carvalho,* Michele Vitolo, Sunao Sato, and Eugênio Aquarone

Department of Biochemical and Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, University of São Paulo, Av. Prof. Lineu Prestes, 580, B-16, 05508-900 Brazil, E-mail: jcmdcarv@usp.br

Received April 2002; Revised August 2002; Accepted September 2002

Abstract

We studied the effect of reactor filling time (T) (3–5 h), initial mass of inoculum (M) (1000–2100 g), and exponential time decay constant for the substrate feed rate (K) (0.6–1.6 h^{-1}) on ethanol production by *Saccharomyces cerevisiae* grown in sugarcane blackstrap molasses through a fed-batch culture. The highest ethanol productivity (16.9 g/[L · h]) occurred at T = 3 h, K = 1.6 h^{-1}, and M = 1300 g. In addition, productivity was affected by both M (for T = 3 and 4 h) and K (for T = 3 h) and varied inversely with T under any value fixed for M and K. By the quadratic regression multivariable analysis method, equations were determined to estimate ethanol yield and productivity as function of the variables studied (T, K, and M).

Index Entries: Ethanol; *Saccharomyces cerevisiae*; fed-batch fermentation; response surface methodology.

Introduction

The petroleum crisis in the 1970s impelled countries depending on petroleum importation to focus their attention on biomass as an alternative for fuel production. In Brazil, the solution was to use sugarcane juice and

*Author to whom all correspondence and reprint requests should be addressed.

molasses as the main component of the culture medium for ethanol production by *Saccharomyces cerevisiae*. The alcohol-chemical industry is now well developed, and residual yeast and bagasse are valuable commercial commodities *(1)*. Yeast is used as an ingredient of the feedstock, as well as a source of enzymes, nucleic acids, and yeast extract. Bagasse, in turn, is used directly as a heat generator in distillery plants or as raw material for lignocellulosic hydrolysate preparation, which has been utilized as the mash for xylitol production *(2)*.

Of course, ethanol yield depends on the yeast strain, culture conditions, and type of fermentative process employed (batch, continuous, or fed batch). Undoubtedly, the fed-batch process is generally chosen, because it permits work with a noninhibitory concentration of substrate, controls the foam, avoids contamination, and couples the reactor filling time with the end of fermentation.

Because filling of the fermentor and fermentation of the mash are simultaneous, often the foamy phase of the process occurs when the reactor is about half full. Thus, the loss of fermenting medium through splashing is not a serious problem, and only a small addition of antifoam is needed.

Fed-batch culture can be conducted by adding the mash to the fermentor at either constant *(3,4)* or variable rates *(5–7)*. Thus, the growth and ethanol-producing capability of the yeast can be followed under a large interval of substrate concentration in the culture medium.

There is not much information in the literature about simultaneous optimization of the initial wet mass of inoculum (M), filling time (T), and feeding profile used in industrial-scale fed-batch ethanol fermentation, or about the interaction among these variables. The present study deals with the effect of the T (3–5 h), M (1000–2100 g), and experimental time decay constant for the substrate feed rate (K) (0.6–1.6 h^{-1}) on ethanol production by *S. cerevisiae* grown in sugarcane blackstrap molasses. The intervals for each parameter considered were set taking into account the values more commonly employed in Brazilian distilleries. We used the response surface methodology (RSM), described by Box et al. *(8)* and utilized for optimization in several studies of the fermentation process *(9–11)*.

Materials and Methods

The inocula were prepared by suspending 1000, 1300, 1600, or 2100 g of wet pressed yeast (*S. cerevisiae*) in distilled water (total inoculum volume = 3 L). This volume was sufficient to ensure a homogeneous cell suspension by means of a 6-flat-blade turbine (diameter = 7.5 cm). The pressed ferment had 29.6% dry matter (SD = 1.1%). The mash, prepared by diluting blackstrap molasses in distilled water, was clarified but not sterilized *(12)*, supplemented with urea (0.5 g/L) and penicillin V acid (500 IU/L). The pH of the mash was adjusted to 4.5–5.0, and the total reducing sugar concentration of the clarified mash was 213 g/L (SD = 10 g/L). The fermentation

experiments were carried out in a 14-L New Brunswick fermentor at (32 ± 1)°C; the impeller speed was 200 min^{-1} and no air was supplied. The pH of the medium was not controlled because it practically did not vary during the tests. After addition of the inoculum to the empty fermentor, impeller speed and temperature were adjusted and the mash was fed into the reactor from an initial volume of 3 L up to a total volume of 10 L with filling time (T) of 3, 4, or 5 h, at an exponential time decay constant (K) equal to 0.6, 0.8, 1.2, or 1.6 h^{-1}. The variable rates obeyed the following equation (for $K > 0$):

$$F = dV/dt = F_0 \cdot e^{-Kt} \quad (1)$$

Considering that when $t = 0$ the value of V is V_0 (3 L), and that when $t = T$ the value of V is V_T (10 L), Eq. 2, obtained by integration of Eq. 1, allows calculation of F_0 for each value of K and T:

$$F_0 = K \cdot (V_T - V_0)/(1 - e^{-K \cdot T}) \quad (2)$$

Equation 1 may also be integrated from $t = 0$ to t, leading to

$$V - V_0 = (F_0/K) \cdot (1 - e^{-K \cdot t}) \quad (3)$$

Combining Eqs. 2 and 3, we may write

$$V_m = V - V_0 = [(V_T - V_0)/(1 - e^{-K \cdot T})] \cdot (1 - e^{-K \cdot t}) \quad (4)$$

Equation 4 permits calculation of the volume of medium added to the fermentor from the beginning of the feeding phase until moment t.

The feed was done intermittently *(13)*, with intervals of 10 min, according to Eq. 5:

$$V_{mad(t)} = V_{m(t+\theta)} - V_{m(t)}, \; t < T \quad (5)$$

in which $V_{mad(t)}$ is the volume of mash added at instant t; and $V_{m(t+\theta)}$ and $V_{m(t)}$ are the V_m calculated by Eq. 4 at $t = t + \theta$ and t, respectively, in which θ is the interval of feeding (in this case, 0.1667 h or 10 min). As an example, the $V_{mad(t)}$ values calculated for test 9 (T = 3 h and K = 1.2 h^{-1}; Table 1) through Eqs. 4 and 5 are presented in Table 2.

Each test condition shown in Table 1 was repeated three times. The variables T and K are closely related to fed-batch operation, while M (inoculum mass) is an important variable in industrial alcoholic fermentation, because it affects the productivity and yield of the process.

The volume of cells in the fermentor was evaluated by the method proposed by Borzani and Baralle *(14)*. Sugars and ethanol concentrations were measured in the supernatant liquid by the Somogyi *(15)* and dichromate *(16)* methods, respectively. Dry matter cell concentrations were measured by filtering a 5.0-mL sample through a Millipore membrane (pore diameter = 1.2 μm); after washing with 50 mL of distilled water, the cake was dried in order to ensure constant weight (105–110°C for 2 to 3 h). The fermentation was considered finished when the ethanol concentration became constant.

Table 1
Ethanol and Cells as Function of Fermentor Filling-Up Time (T), Exponential Time Decay Constant (K), and Wet Cell Mass of Inoculum (M)

Test	T (h)	K (h^{-1})	M (g)	T' (h)	η (%)	Pe (g/[L · h])	Y (g/kg)	Px (g/[L · h])
1	3	0.6	1000	4.5 ± 0.5	72.4 ± 0.8	12.4 ± 1.0	67.5 ± 6.3	2.3 ± 0.4
2	3	0.6	1300	3.5 ± 0	69.0 ± 2.9	14.5 ± 0.4	92.5 ± 5.5	3.8 ± 0.1
3	3	0.6	1600	3.2 ± 0.3	65.9 ± 2.6	16.3 ± 1.3	86.2 ± 2.1	4.2 ± 0.2
4	3	0.6	2100	3.0 ± 0	61.0 ± 4.5	16.6 ± 0.6	78.6 ± 10.1	4.2 ± 0.4
5	3	0.8	1000	4.2 ± 0.3	71.1 ± 3.0	12.7 ± 0.3	79.7 ± 15.9	2.8 ± 0.6
6	3	0.8	1300	3.3 ± 0.6	67.0 ± 2.9	15.6 ± 1.6	86.2 ± 10.3	3.9 ± 0.4
7	3	0.8	1600	3.0 ± 0	65.7 ± 2.8	16.8 ± 0.3	92.1 ± 13.1	4.6 ± 0.7
8	3	0.8	2100	3.0 ± 0	61.0 ± 3.9	16.6 ± 0.4	83.3 ± 10.5	4.4 ± 0.4
9	3	1.2	1000	4.2 ± 0.3	72.6 ± 2.4	12.9 ± 1.3	91.2 ± 7.8	3.2 ± 0.5
10	3	1.2	1300	3.3 ± 0.3	71.5 ± 1.6	15.7 ± 1.5	102.5 ± 10.0	4.4 ± 0.2
11	3	1.2	1600	3.0 ± 0	64.8 ± 0.9	16.5 ± 0.2	102.1 ± 4.9	5.1 ± 0.3
12	3	1.2	2100	3.0 ± 0	62.5 ± 0.6	16.0 ± 0.4	88.3 ± 28.9	4.4 ± 1.4
13	3	1.6	1000	3.8 ± 0.3	71.7 ± 2.4	13.9 ± 0.9	85.8 ± 6.0	3.3 ± 0.5
14	3	1.6	1300	3.0 ± 0	68.9 ± 3.5	16.9 ± 1.0	91.5 ± 3.2	4.4 ± 0.3
15	3	1.6	1600	3.0 ± 0	62.9 ± 1.1	16.0 ± 0.3	99.3 ± 13.7	4.9 ± 0.8
16	3	1.6	2100	3.0 ± 0	63.7 ± 1.2	16.2 ± 0.2	83.5 ± 22.0	4.2 ± 1.1
17	4	0.6	1000	4.0 ± 0	71.3 ± 2.5	13.5 ± 0.2	77.4 ± 6.0	2.9 ± 0.3
18	4	0.6	1300	4.0 ± 0	69.1 ± 2.4	13.1 ± 0.1	84.8 ± 4.5	3.2 ± 0.3
19	5	0.6	1000	5.2 ± 0.3	71.8 ± 1.1	10.9 ± 0.7	75.6 ± 17.8	2.2 ± 0.5
20	5	0.6	1300	5.0 ± 0	70.3 ± 2.3	10.8 ± 0.2	89.5 ± 7.5	2.7 ± 0.3
21	5	0.8	1000	5.0 ± 0	70.7 ± 1.4	11.0 ± 0.1	75.8 ± 12.6	2.3 ± 0.4
22	5	0.8	1300	5.0 ± 0	69.9 ± 2.0	10.9 ± 0.3	84.0 ± 10.5	2.6 ± 0.3

Table 2
Volume of Mash Added to Fermentor until t ($V_{m(t)}$; Eq. 4) and Volume of Mash Added at Instant t ($V_{mad(t)}$; Eq. 5) to Test 9 (T = 3 h; K = 1.2 h^{-1})

t (h)	$V_{m(t)}$ (L)	$V_{m(t+\theta)}$ (L)	$V_{mad(t)}$ (L)
0.000	0.000	1.304	1.304
0.167	1.304	2.372	1.068
0.333	2.372	3.247	0.875
0.500	3.247	3.963	0.716
0.667	3.963	4.549	0.586
0.833	4.549	5.029	0.480
1.000	5.029	5.422	0.393
1.167	5.422	5.743	0.321
1.333	5.743	6.007	0.264
1.500	6.007	6.223	0.216
1.667	6.223	6.399	0.176
1.833	6.399	6.544	0.145
2.000	6.544	6.662	0.118
2.167	6.662	6.759	0.097
2.333	6.759	6.838	0.079
2.500	6.838	6.903	0.065
2.667	6.903	6.956	0.053
2.833	6.956	7.000	0.044
3.000	7.000	—	—

The models that fitted the centered data on the responses of ethanol productivity, ethanol yield, substrate-cell conversion factor, and cell productivity had the form

$$Z = b_0 \sum_i b_i X_i + \sum_{i \leq j} \sum b_{ij} X_i X_j + e \quad (6)$$

in which Z is the predicted response; subscripts i and j take values from 1 to the number of the variables; b_0 is the intercept term; b_i values are the linear coefficients; b_{ij} are quadratic coefficients; X_i and X_j are the levels of the independent variables; and e is the error term *(17)*. The fitted model (Eq. 6) was evaluated for each dependent variable based on the test of the coefficients above ($p \leq 0.1$) and analysis of variance (ANOVA) for the regression.

Results and Discussion

The volume of mash added to the fermentor was calculated through Eq. 4 by using T (reactor filling-up time) and K (exponential time decay constant for the substrate feed rate). The values of these variables are given in Table 1. T values were set taking into account those normally employed in distilleries, and K was fixed according to the desired pattern of decreasing exponential addition of substrate at the same filling time. Equation 2 shows that many combinations of the variables K and T could be used. Certainly,

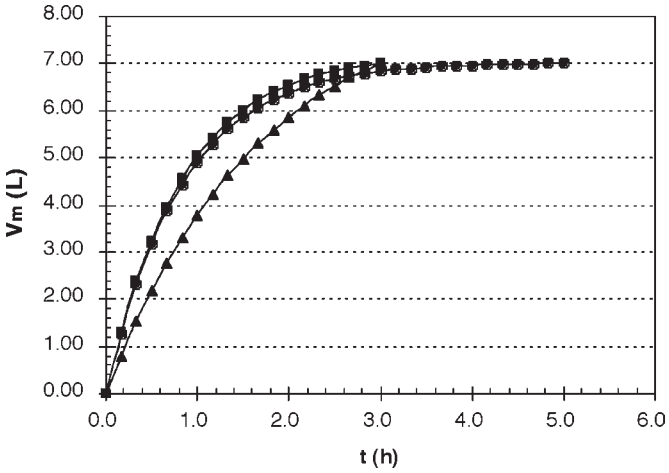

Fig. 1. Volume of mash added for filling times of 3 (K = 0.6 h^{-1} [▲] and K = 1.2 h^{-1} [■]) and 5 h (K = 1.2 h^{-1} [●]).

several *K*-*T* pairs would lead to similar feeding rate patterns, which would be meaningless if we consider the probable metabolic variability presented by the yeast growing in crude blackstrap molasses. Through theoretical simulations, *K*-*T* pairs were screened in order to eliminate those leading to similar feeding rate profiles. As shown in Fig. 1, e.g., the feeding rate patterns related to K = 1.2 h^{-1} and filling times of 3 and 5 h are very similar. Thus, for K = 1.2 h^{-1} the option was T = 3 h; otherwise, the overall fermentation time would be unnecessarily high. For the same reason, for T = 4 h only tests at K = 0.6 h^{-1} were conducted. However, Fig. 1 shows also that it is possible at the same filling-up time (T) to attain different patterns of mash addition in a decreasing exponential manner depending on the value of K fixed (0.6 or 1.2 h^{-1}).

In a general way, it is clear how T affects a fed-batch process: when T increases, the rate of substrate addition decreases, which could affect the overall fed-batch process. Nonetheless, the role of K in the fed-batch process can be understood from Eq. 1 at the condition $t = T$. Thus, this equation becomes

$$K = \ln(F_0/F)/T \tag{7}$$

Therefore, we can conclude that high K values are attained at high F_0/F ratio. In other words, K is a typical exponential time decay constant for the substrate feed rate.

On the other hand, the mass of the inoculum (M) was chosen near as possible to those employed in Brazilian distilleries, but, when necessary, a high amount of inoculum was employed to confirm tendencies of ethanol productivity curves, as shown in Fig. 2.

Table 1 shows that at filling times of 3 and 4 h the mass of the inoculum affected ethanol productivity (Pe), whereas K only affected Pe at T = 3 h.

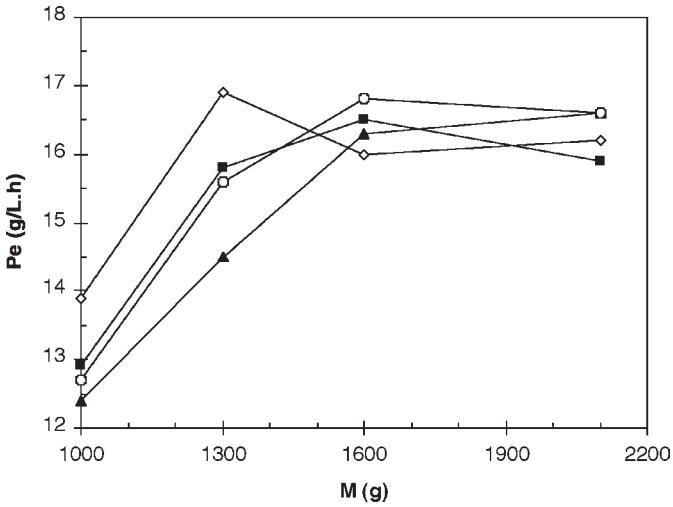

Fig. 2. Ethanol productivity for filling time of 3 h as function of inoculum (wet mass) at different *K* values: (▲) $K = 0.6\ h^{-1}$; (○) $K = 0.8\ h^{-1}$; (■) $K = 1.2\ h^{-1}$; (◇) $K = 1.6\ h^{-1}$.

Through multiple variable regression, the variables studied are correlated as follows:

$$Pe\hat{} = -34.31 + 12.93 \cdot T + 3.613 \cdot K + 3.706 \cdot 10^{-2} \cdot M - 4.836 \cdot 10^{-3} \cdot T \cdot M - 1.992 \cdot 10^{-3} \cdot K \cdot M - 1.102 \cdot T^2 - 5.672 \cdot 10^{-6} M^2 \quad (8)$$

By regression ANOVA, a good data adjustment was verified ($p < 0.0001$), with $R^2 = 0.89$. The average variation in the absolute differences of mean ethanol productivity (Table 1) and estimated values (by Eq. 8) was 2.3% (SD = 1.8%). Thus, this result agrees very well with that attained from the regression variance analysis, as can be seen from Fig. 3.

From Eq. 8 we can estimate *Pe* for any *K*-*T* combination within the interval studied. Naturally, the value calculated could be used as a point of reference for projecting ethanol production on an industrial scale. For instance, substituting $T = 3.5$ h, $M = 1500$ g, and $K = 1.35\ h^{-1}$ in Eq. 8, we have $Pe\hat{} = 15.7\ g/(L \cdot h)$. Graphics presenting level curves, such as exemplified in Fig. 4, can also be employed in estimating *Pe*.

Ethanol productivity decreased with *T*, mainly at high mass of inoculum (*M*). For instance, Table 1 shows that the ethanol productivity at $K = 0.6\ h^{-1}$ and $M = 1300$ g for $T = 3$, 4, and 5 h was 14.5, 13.1, and 10.8 g/(L · h), respectively. Thus, an increase of about 33% on *Pe* occurred when *T* diminished. However, for the same filling times and *K* but $M = 1000$ g, the *Pe* values are 12.4, 13.5, and 11.0 g/(L · h), which represent an increase of 13%. The interaction effect of *M* and *T* on *Pe* is illustrated in Fig. 4. As can be clearly seen, *Pe* increased with *M* for $T = 3$ h but decreased for $T = 5$ h.

From Fig. 2 it can be seen that for $T = 3$ h the highest *Pe* (about 17 g/[L · h]) occurred at M = 1300 g at $K = 1.6\ h^{-1}$, whereas for $K < 1.6\ h^{-1}$ the maximum *Pe* value occurred at $M = 1600$ g. This result is in accordance with

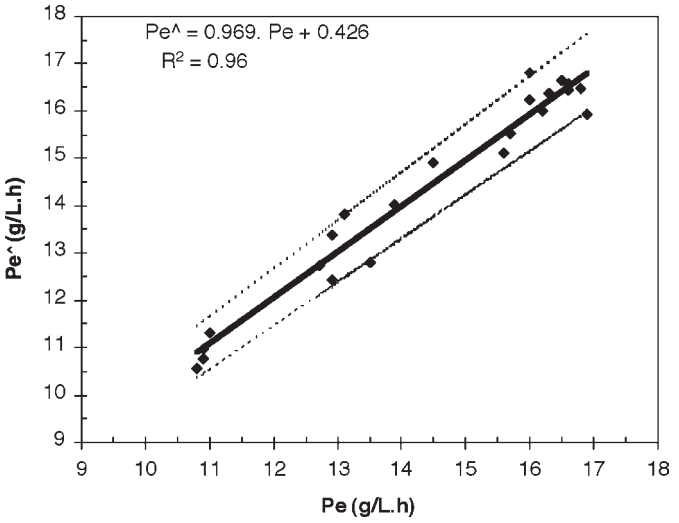

Fig. 3. Estimated ethanol productivity (*Pe*^) vs mean ethanol productivity (*Pe*). The values on the straight line had an SD of ± 5% (dashed lines).

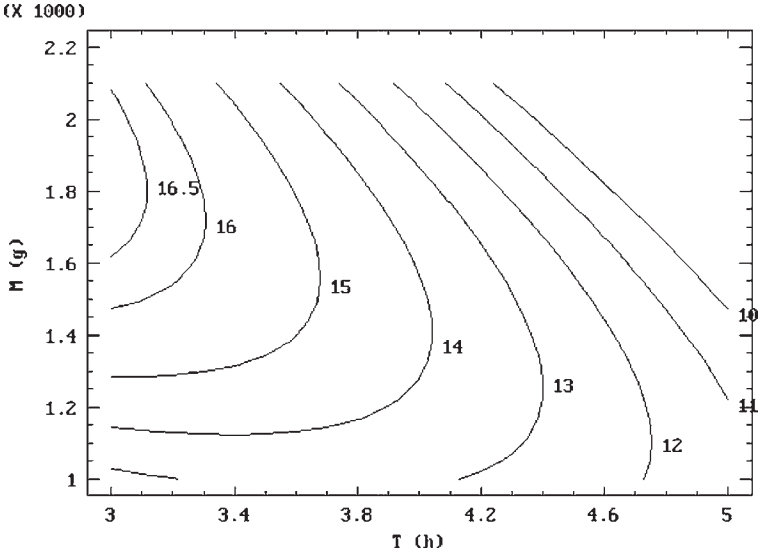

Fig. 4. Levels curves related to *Pe* (g/[L · h]) as function of *T* and *M*, for $K = 0.8\ h^{-1}$.

the findings of Chen *(9)* and Koshimizu et al. *(3)*, who observed a similar behavior by yeast grown in batch and fed-batch ($K = 0$) processes, respectively. If we consider $K = 0$, as in the case studied by Koshimizu et al. *(3)*, by applying Eq. 8, for $T = 3$ h, we conclude that the M needed for $Pe = 16$ g/(L · h) would be about 1600 g. From Table 1 we observe that $T' = T$ for $M = 2100$ g independently of K. The increase in M leads to the diminution of T' until $T' = T$, and, as consequence Pe increases. Since $Pe = Me / T' \cdot V$, in which

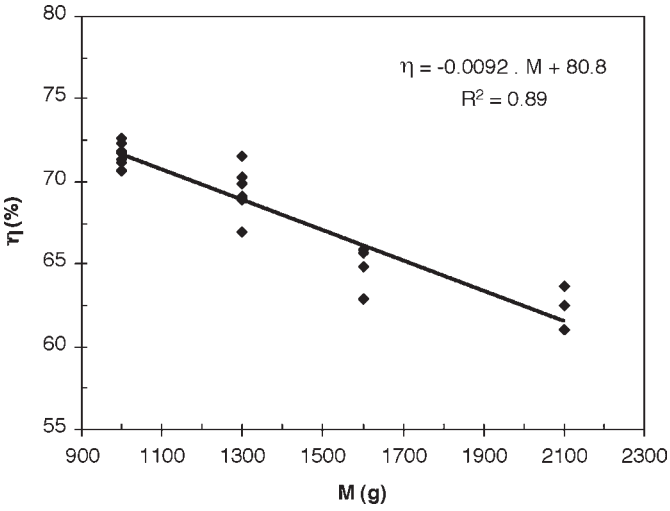

Fig. 5. Ethanol yield as function of initial inoculum cell mass.

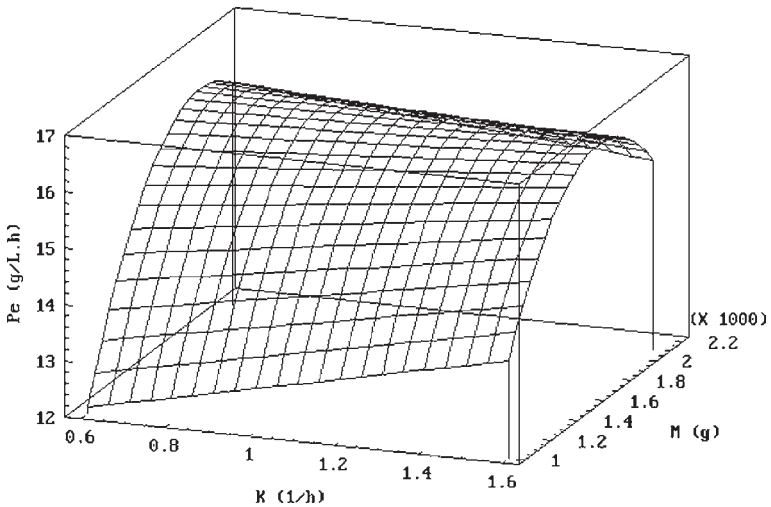

Fig. 6. Ethanol productivity as function of *K* and *M*, for *T* = 3 h.

T' and *V* are constant after that point, *Pe* is a function only of the amount of ethanol mass dissolved in the medium (*Me*). This result is clearly seen from Fig. 5, in which the yield of ethanol diminishes as the mass of inoculum increases.

Figure 6 shows the interaction effect of *K* and *M* on *Pe*. It is clear that for low *M* an increase in *K* leads to an increase in *Pe*; meanwhile, for high *M* the inverse occurs.

Undoubtedly, increasing *K* (exponential time decay constant) leads to high initial and low final substrate feeding rates, as noted earlier. Of course,

a tradeoff between *K* and the inoculum concentration is expected, because high or low feeding rate means more or less nutrient available to the cells. For lower *M*, a high *K* should be preferred, unless the microorganism is inhibited by substrate at a concentration above 100 g/L *(18,19)*. At the beginning of fermentation, the more the substrate is available, the more cell growth is favored, as well as ethanol formation. At the end of the fermentation, when cell growth and uptake substrate rate diminish owing to the ethanol formed, a slowdown on substrate addition, resulting from high *K*, allows the cells to consume it completely, with additional ethanol formation. However, this fact is not so relevant when high inoculum mass is employed, because the diminution of the final substrate uptake rate is compensated by the huge amount of cells in the fermentor. The interaction of *K* and *M* is represented in Eq. 8. Accordingly, the exponential time decay constant and inoculum concentration pair must be adjusted so that *T* and *T'* are as close as possible.

Ethanol yield (η) was significantly influenced by the cell concentration of the inoculum, as shown in Fig. 5. Since the η values were calculated taking into account the difference between the volume of the mash and the volume occupied by the cells, as proposed by Borzani and Baralle *(14)*, an inverse correlation between η and *M* was observed. This is an important consideration, because the ethanol to be distilled is present in the liquid-fermented medium. On the other hand, if η were calculated as usual, not considering the biomass volume in the reactor, the ethanol yield would not be affected by inoculum mass.

Because residual cells are an important byproduct of ethanol fermentation *(20)*, we discuss next the values of productivity and the substrate/cell conversion factor (*Y*). By applying RSM, we observed that *Y* depended on the mass of the inoculum and exponential time decay constant (*K*) as follows:

$$Y\hat{} = -47.74 + 65.37 \cdot K + 1.346 \cdot 10^{-1} \cdot M - 25.17 \cdot K^2 - 4.300 \cdot 10^{-5} \cdot M^2 \quad (9)$$

Variance analysis of the regression indicated a good adjustment ($p < 0.0001$). This was confirmed by comparing the mean values of the experimental results (Table 1) with those estimated by Eq. 9, which led to an average difference of 3.8% (SD = 2.6%), although this regression presented the smallest determination coefficient ($R^2 = 0.30$).

Figure 7 shows that the increase in the cell mass in the inoculum favors cell growth (*Y* increases) up to about *M* = 1500 g. After that *Y* diminishes, as predicted by the quadratic model (Eq. 9). Queiroz et al. *(21)* gathered similar data through batch fermentation processes, although the values that they found were about 700 g. This difference could be owing to the yeast strain and raw material used in the fermentation process *(21)*, dissolved oxygen in the medium *(22)*, the mode of substrate addition in the fed batch *(23,24)*, temperature *(25)*, among others.

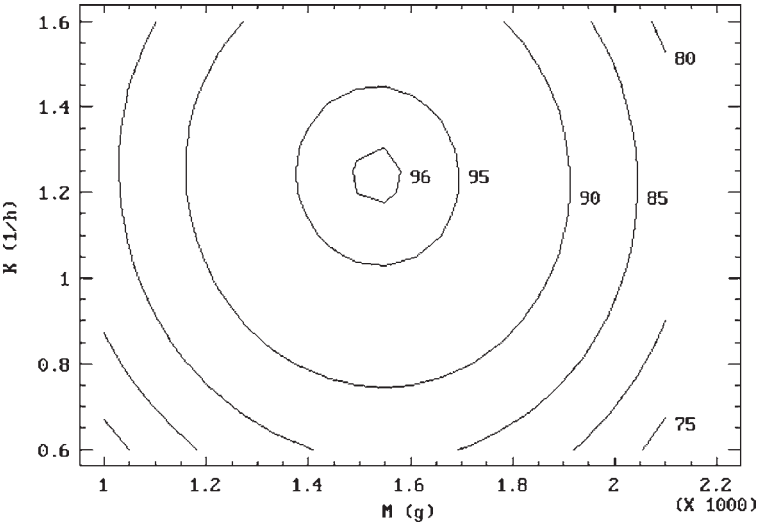

Fig. 7. Levels curves related to *Y* (g/kg) as function of *M* and *K*.

Regarding the exponential time decay constant (*K*), cell growth was favored as *K* increased. This was probably owing to the low ethanol concentration *(26)* and/or high amount of dissolved oxygen (not measured) *(22)* in the fermentor at the beginning of the culture resulting from the addition of the mash. As already mentioned, high *K* values lead to high mash feeding rate at the beginning of culture, which leads to dilution of ethanol formed and introduction of oxygen, which was not removed of the mash. The results obtained in our study confirm the data attained in a study by Carvalho et al. *(23)*, in which ethanol fermentation was carried out with a low initial mass of inoculum and high filling time. Table 1 shows that ethanol yield was not affected by the increase in *Y*, an indication that a small fraction of the substrate could be diverted for byproduct formation *(19,22)*.

For cell productivity the adjusted equation was as follows:

$$Px\text{^} = -10.06 + 1.370 \cdot T + 5.191 \cdot 10^{-1} \cdot K + 1.641 \cdot 10^{-2} \cdot M - 3.384 \cdot 10^{-6} \cdot M^2 - 1.562 \cdot 10^{-3} \cdot T \cdot M \quad (10)$$

In this case, the regression variance analysis was also satisfactory ($p < 0.0001$), with $R^2 = 0.76$, indicating the coherence of the statistical model applied. Equation 10 shows that the filling time had an effect on cell productivity, which is not surprising since the whole fermentation time is affected by the filling time (Table 1). Since the fermentation time depends on the *M* and *K* values, it is clear from Fig. 8 that the cell productivity had a tendency to reach a maximum followed by a smooth drop compared with *Y* (Fig. 7).

Figure 9 shows that ethanol and cell productivity were linearly correlated, confirming the data in the literature attained under different culture conditions.

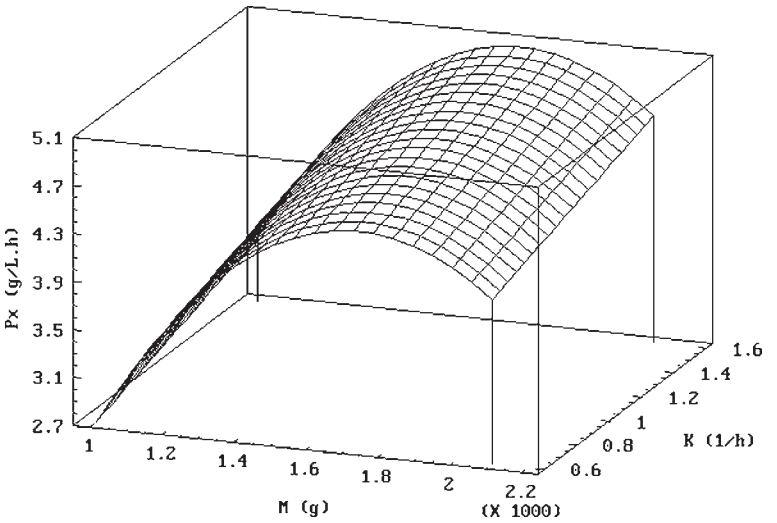

Fig. 8. Cell productivity as function of *M* and *K*, for *T* = 3 h.

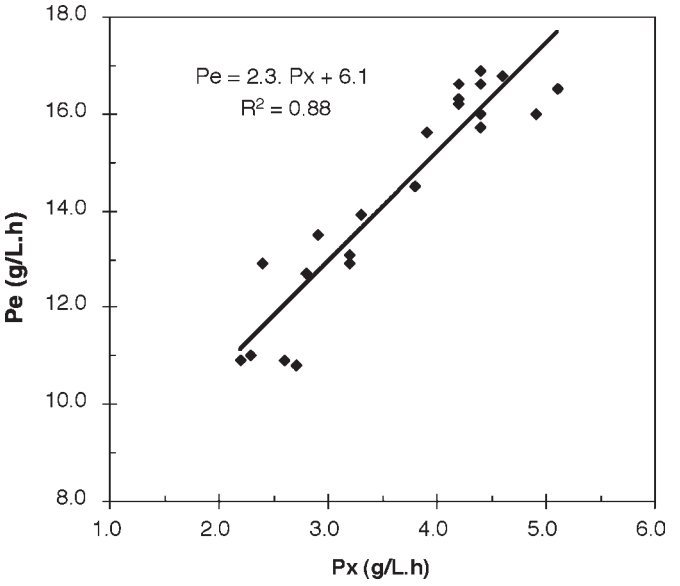

Fig. 9. Correlation between ethanol and cell productivities.

Conclusion

The data presented point to the possibility of applying the surface response statistical model to improve estimation of parameters related to ethanol production in distilleries. Although other parameters must also be evaluated, such as the initial substrate concentration and the percentage of viable cells in the inoculum, the data attained in this work indicate that *T*

= 3 h, M = 1300 g, and K = 1.6 h^{-1} are the most suitable culture conditions for ethanol production through a fed-batch process.

Nomenclature

F = reactor feeding rate (L/h)
F_0 = initial reactor feeding rate (L/h)
K = exponential time decay constant for the substrate feed rate (h^{-1})
M = wet yeast mass in the inoculum (g)
p = significance level
Pe = ethanol productivity (g/[L · h]): mean value of three experiments
Pe^ = estimated ethanol productivity (g/[L · h])
Px = cell productivity- dry mass (g/[L · h]): mean value of three experiments
Px^ = estimated cell productivity (g/[L · h])
r = correlation coefficient
R^2 = determination coefficient
t = time (h)
T = fermentor filling-up time (h)
T' = fermentation time (h)
V = volume in fermentor at any time (L)
V_0 = initial volume in fermentor, which corresponds exactly to inoculum volume (L)
$V_m = V - V_0$ = volume of mash added in fermentor until t (L)
$V_{mad(t)}$ = volume of mash added at instant t (L)
V_T = volume of fermented mash at t = T (L)
Y(substrate-cell conversion factor) = ratio between cells formed (dry mass) and substrate added in fermentor (g/kg)
Y^ = estimated substrate-cell conversion factor (g/kg)
η^ = estimated ethanol yield (%)
η = ethanol yield (percentage of theoretical value): mean value of three experiments
θ = interval of mash additions (h)

Acknowledgments

We acknowledge the suggestions of Prof. Dr. Walter Borzani and the assistance of Kleber A. Almeida, Nilton M. Bloise, Dante A. Moraes, Eliana M. Yamaguchi, and Maria C. Pedrazzi in the experimental work.

References

1. Echegaray, O. F., Aquarone, E., Carvalho, J. C. M., Fernandes, A. N. R., Sato, S., and Vitolo, M. (2000), *Biomass Bioenergy* **19,** 39–50.
2. Sene, L., Vitolo, M., Felipe, M. G. A., and Silva, S. S. (2000), *Appl. Biochem. Biotechnol.* **84/86,** 371–380.

3. Koshimizu, L. H., Gomes, E. I. V., Netto, C. L. B., Cruz, M. R. M., Vairo, M. L. R., and Borzani, W. (1984), *J. Ferment. Technol.* **62,** 205–210.
4. Roukas, T. (1994), *Biotechnol. Bioeng.* **43,** 189–194.
5. Carvalho, J. C. M., Aquarone, E., Sato, S., Brazzach, M. L., Almeida, K. A., and Borzani W. (1990), *Biotechnol. Lett.* **12,** 777–782.
6. Krauter, M., Aquarone, E., Sato, S., Perego, L., Jr., and Borzani, W. (1987), *Biotechnol. Lett.* **9,** 647–650.
7. Vasconcelos, J. N. and Valdman, B. (1988), *Brasil Açucareiro* **106,** 38–48.
8. Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978), *Statistics for Experimenters*, John Wiley & Sons, New York.
9. Chen, S. L. (1981), *Biotechnol. Bioeng.* **23,** 1827–1836.
10. Escamilla, E. M. S., Dendooven, L., Magaña, I. P., Parra, R. S., and De la Torre, M. (2000), *J. Biotechnol.* **76,** 147–155.
11. Viswanathan, P. and Kulkani, P. R. (1995), *Bioresour. Technol.* **54,** 117–121.
12. Borzani, W., Gregori, R. E., and Vairo, M. L. R. (1977), *Biotechnol. Bioeng.* **29,** 844–849.
13. Borzani, W., Krauter, M., Aquarone, E., Sato, E., and Perego, L., Jr. (1988), *Arch. Biol. Technol.* **31,** 571–575.
14. Borzani, W. and Baralle, S. B. (1983), *Biotechnol. Bioeng.* **25,** 3201–3206.
15. Somogyi, M. (1952), *J. Biol. Chem.* **195,** 19–23.
16. Joslyn, M. A. (1970), in *Methods in Food Analysis*, Joslyn, M. A., ed., Academic, New York, pp. 447–474.
17. Prapulla, S. G., Jacob, Z., Chand, N., Rajalakshmi, D., and Karanth, N. G. (1992), *Biotechnol. Bioeng.* **40,** 965–970.
18. Ciftci, T., Constantinides, A., and Wang, S. S. (1983), *Biotechnol. Bioeng.* **25,** 2007–2023.
19. Converti, A., Perego, P., Lodi, A., Parisi, F., and Borghi, M. A. (1985), *Biotechnol. Bioeng.* **27,** 1108–1114.
20. Vitolo, M., Carvalho, J. C. M., Duranti, M. A., and Breda, M. (1991), *Biomass Bioenergy* **1,** 301–304.
21. Queiroz, M. F. V., Vairo, M. L. R., and Borzani, W. (1983), *J. Ferment. Technol.* **61,** 215–218.
22. Ryu, D. D. Y., Kim, Y. J., and Kim, J. H. (1984), *Biotechnol. Bioeng.* **26,** 12–16.
23. Carvalho, J. C. M., Aquarone, E., Sato, S., Brazzach, M. L., Moraes, D. A., and Borzani, W. (1993), *Appl. Microbiol. Biotechnol.* **38,** 596–598.
24. Taherzadeh, M. J., Niklasson, C., and Lidén, G. (1999), *Bioresour. Technol.* **69,** 59–66.
25. Nanba, A., Nishizawa, Y., Tsuchiya, Y., and Nagai, S. (1987), *J. Ferment. Technol.* **65,** 277–283.
26. Thatipamala, R., Rohani, S., and Hill, G. A. (1992), *Biotechnol. Bioeng.* **40,** 289–297.