Appl Microbiol Biotechnol (2009) 83:627–637
DOI 10.1007/s00253-009-1885-z

Optimization of temperature, sugar concentration, and inoculum size to maximize ethanol production without significant decrease in yeast cell viability

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Received: 27 October 2008 / Revised: 16 January 2009 / Accepted: 19 January 2009 / Published online: 21 February 2009

Abstract Aiming to obtain rapid fermentations with high ethanol yields and a retention of high final viabilities (responses), a 2^3 full-factorial central composite design combined with response surface methodology was employed using inoculum size, sucrose concentration, and temperature as independent variables. From this statistical treatment, two well-fitted regression equations having coefficients significant at the 5% level were obtained to predict the viability and ethanol production responses. Three-dimensional response surfaces showed that increasing temperatures had greater negative effects on viability than on ethanol production. Increasing sucrose concentrations improved both ethanol production and viability. The interactions between the inoculum size and the sucrose concentrations had no significant effect on viability. Thus, the lowering of the process temperature is recommended in order to minimize cell mortality and maintain high levels of ethanol production when the temperature is on the increase in the industrial reactor. Optimized conditions (200 g/l initial sucrose, 40 g/l of dry cell mass, 30 °C) were experimentally confirmed and the optimal responses are 80.8±2.0 g/l of maximal ethanol plus a viability retention of 99.0±3.0% for a 4-h fermentation period. During consecutive fermentations with cell reuse, the yeast cell viability has to be kept at a high level in order to prevent the collapse of the process.

Keywords RSM · Viability · Ethanol production · Temperature · Sugar concentration · Inoculum size

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Introduction

High ethanol yields in a short fermentation time are an economically relevant factor in industrial ethanol production. However, this is dependent on the yeast strain, type of process (batch or fed-batch), cell density, temperature, and sugar concentration and enrichment of the medium with the proper nutrients, along with other factors that influence the microbial activity. Studies related to ethanol production have been carried out in complex and synthetic media. Although these have not yet being implemented on an industrial scale due to economical reasons, a synthetic medium exhibits favorable characteristics over the traditional complex or natural media since it is composed of pure chemicals in precisely known proportions (Zhang and Greasham 1999).

High sugar concentrations can inhibit both yeast growth and fermentative activities. As described in literature (Casey and Ingledew 1985), ethanol inhibition becomes significant in the concentration range of 15–25% sugar (w/v), while complete inhibition of the fermentation has been reported at 40% glucose (w/v) in batch cultures (Holcberg and Margalith 1981).

Typical yeast fermentations require temperatures between 30 and 35 °C to maximize ethanol production (Damore et al. 1989). Yeast strains used for the commercial production of ethanol usually produce lower levels of ethanol at high temperatures. Concentrations of ethanol above 3% (w/v) lead to decreases in the maximal temperature of growth (Casey and Ingledew 1986). Strains isolated from Brazilian alcohol plants have produced high levels of ethanol in batch cultures operating within the range of 35 to 40 °C in a rich medium containing sucrose, yeast extract, and peptone (Laluce et al. 1991), but losses in viability were greater at 40 °C.

Ethanol is well known as an inhibitor of microbial growth. The rate of ethanol production and its accumulation within cells of *Saccharomyces cerevisiae* in rapid fermentations leads to sharp drops in viability (Dasari et al. 1990). In addition, the loss in viability leads to decreases in the activity of the alcohol dehydrogenase due to high levels of internal ethanol (Nagodawithana et al. 1974). Rapid fermentation also enhances thermal death (Loureiro and van Uden 1986; Nagodawithana and Steinkraus 1976). Nevertheless, some strains of *S. cerevisiae* show tolerance to ethanol and can be adapted to high concentrations of alcohol (Alexandre et al. 1994). A tolerant strain of yeast isolated from a Brazilian alcohol plant was able to produce ethanol in batch cultures having up to 8% (v/v) ethanol added initially as described by Peres and Laluce (1998). Any natural strain of *S. cerevisiae* is able to tolerate up to 14–16% (v/v) of ethanol excreted into the medium (Casey et al. 1983) or even up to 21% (v/v), depending on the nutritional supplementation (Thomas and Ingledew 1992). Ethanol also induces cell lysis (Jones 1989) due to the formation of cross-linked peptidoglycan, which is aggravated by increasing the temperature to above 35 °C.

Statistical methods can either identify or quantify the various interactions occurring between the independent variables and the corresponding microbial responses. Mathematical models generated by the statistical methods allow the prediction of process responses such as ethanol production and viability. In the present work, experimental design and response surface methodology (RSM) were used to study the effects of increasing inoculum size, sucrose concentration, and temperatures on rapid fermentations with small variations in viability during ethanol production in synthetic medium (Thomas et al. 1998). Rapid fermentation was defined as a fermentation in which the ethanol level increases from zero to 9.5% (v/v) in 6-h or less (Nagodawithana et al. 1974).

Materials and methods

Microorganism

The hybrid strain 63M used in this study was constructed using genetic segregants derived from industrial isolates of *S. cerevisiae* (Laluce et al. 2002). This yeast was able to grow overnight on yeast–peptone–dextrose (YPD) plates at 40 °C (Souza et al. 2007). A stock culture was stored at 4 °C on solid YPD medium with transfers to fresh medium every 4 months.

Inoculum propagation

For the inoculum propagation, the synthetic medium described by Thomas et al. (1998) was modified by replacing the glucose with sucrose (carbon source) and by adding 2% yeast extract to improve cell proliferation. Separate solutions containing salts, trace elements, vitamins, growth, and survival factors were prepared as described by the authors (Thomas et al. 1998) and then mixed to give the concentration of each ingredient as required for the final medium: $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $MgSO_4$, $CaCl_2$, NaCl, $ZnSO_4$, H_3BO_3, KI, $MnSO_4$, $CuSO_4$, Na_2MoO_4, $CoCl_2$, $FeCl_3$, biotin, calcium pantothenate, folic acid, *myo*-inositol, niacin, *p*-aminobenzoic acid, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, ergosterol, and Tween 80. The propagation was carried out in 250-ml Erlenmeyer flasks, initially containing 50 ml of synthetic medium (Thomas et al. 1998), which were inoculated with fresh culture to start the propagation with an initial cell density of 0.85 g/l. After 16-h propagation at 30 °C in a rotary shaker operating at 125 rpm, cells were harvested by centrifugation at 5,000×*g* for 2 min at 4 °C. The harvested cells were resuspended in sterilized water and the washed cell pellet was separated by centrifugation. In the next step, the washed cells were again resuspended in sterilized water, resulting in a highly concentrated yeast cream (160 g/l in dry weight or 48%, v/v), which was used to start the high cell density fermentations.

Fermentation procedures

The synthetic medium (Thomas et al. 1998) containing ammonium sulfate (nitrogen source) and other ingredients as described above, except glucose (carbon source replaced with sucrose), was used to study the effects of the independent variables (sucrose, temperature, and inoculum) on viability and ethanol formation during the fermentation.

Yeast extract was not added to this medium. A solution twice as concentrated (50 ml) containing the different medium ingredients was prepared and sterilized as described by the authors (Thomas et al. 1998) and then mixed before adding different amounts of sucrose or inoculum as follows: sucrose concentration varying from 100 to 200 g/l in the final medium, inoculum amounts varying from 30 g/l (around 9%, v/v) to 40 g/l (dry weight), and fermentation temperature varying from 30 to 40 °C. The pH of the medium was also adjusted to 4.5 prior to inoculation. Erlenmeyer flasks were sealed closed using perforated rubber stoppers to which a glass tube was inserted to allow the fermentation gas to escape from the 100 ml of final medium. A second glass tube was inserted to the rubber stopper to collect samples from the bottom of the Erlenmeyer flasks during the fermentation. The flasks were then transferred to a rotary shaker operating at 100 rpm for the duration of the fermentation period.

Analytical assays

The determinations were cell viability using the methylene-blue method (Lee et al. 1981); total reducing sugar in acid hydrolysates (1.2 M HCl for 10 min at 60 °C), using the 3,5-dinitrosalicylic acid method (Miller 1959); and ethanol concentration using a gas chromatograph (model CG-37; Instrumentos Científicos, São Paulo, Brazil). For the biomass assays, cells were washed by vacuum filtration and dried at 105 °C until constant weight and expressed as grams per liter in the final medium.

Experimental design

The RSM is a technique (Box and Wilson 1951) that consists of the following: (1) the designing of experiments that will yield adequate and reliable measurements of the response of interest, (2) the determining of a mathematical model that best fits the data obtained from the design, and (3) the determining of optimal values for the experimental factors that will give maximal or minimal values for the responses. In the present study, the dependent variables were ethanol production (Y_1) and viability (Y_2), which were assayed after 4-h fermentation periods. The independent variables were temperature (X_1), sucrose concentration (X_2), and inoculum size (X_3), as shown in Table 1. This table also shows that 35 °C, 150 g/l sucrose, and 35 g/l inoculum were adopted as central points to predict the dependent variables. A 2^3 full-central-composite design with replication at the central point and having six axial points (n=6) was used for the optimization, with the data being obtained from a total of 20 experiments carried out in triplicate. Data analysis was performed using the MINITAB statistical software package (version 14.0) with a level of significance of 5% and a confidence level of 95% (p=0.05).

Statistical analysis of data by RSM

RSM is a sequential procedure with the initial aim of allowing the researcher to rapidly and efficiently obtain data near optimum values. It includes a full factorial central composite design and regression analysis. In the present work, response surface models were fitted to the ethanol production and viability using the MINITAB software package (version 14.0). The experimental results of the RSM were fitted via the response surface regression procedure using the following second-order polynomial equation:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3, \quad (1)$$

where the following can be found: Y_i is the predicted response; X_1, X_2, and X_3 are the independent variables; b_o is the intercept term; b_1, b_2, and b_3 are the linear effects; b_{11}, b_{22}, and b_{23} the square effects; and b_{12}, b_{23}, and b_{13} are the interaction terms. Y represents viability (Y_1, percent) or ethanol production (Y_2, grams per liter), while X_1 (temperature), X_2 (sucrose concentration), and X_3 (inoculum concentration) were independent values. This equation represents an empirical model, in which the response functions allow the estimation of responses due to changes in the dependent variables. This model was regressed given two-fitted model equations, one for viability response (Eq. 2) and the other for the ethanol production (Eq. 3) as described in Table 3 ("Results" section).

Table 1 Levels of real and codified values of independent variables utilized in the 2^3 full central composite design

Independent variables	Symbols	Range of natural levels				
		−1.682	−1.000	0.000	+1.000	+1.682
Temperature (°C)	X_1	30	32	35	38	40
Initial sucrose concentration (g/l)	X_2	100	120	150	180	200
Yeast inoculum sizes (g/l)	X_3	30	32	35	38	40

Application variance analysis to the fitted models

The adequacy of the fitted model equations was evaluated by application variance analysis (ANOVA), using the MINITAB software package (version 14). If the model is not satisfactory, a more complex model with a better fit is required, and this is indicated by the analysis of variance. In this work, the F test for regression was taken as significant at a significance level of 5% or a 95% confidence level (p=0.05) for both ethanol production and viability responses. If the F test is significant for its lack of fit, then a more complicated model is needed. Both the t test (measuring how large the coefficient is in relation to its standard error) and p values (reflecting the chance of getting a larger t value and also indicating the patterns of the interaction among the variables) were used to confirm the significance factor of the model equations.

Surface and contour plots

The response surface was plotted to understand the interactions between variables and to determine each variable's optimum response level. In the present work, surface and contour plots of key variables were derived from linear (plain surface graphs) and quadratic (curved surfaces) models, fitting experimental data to calculate optimal responses for ethanol production and viability. The plots were obtained with the aid of the STATISTICA software package (version 7.0). The simultaneous interactive effects of the independent variables are shown by the three figures described in the "Results" section.

Optimization of response and model validation

The "Response Optimizer" option of the MINITAB software package (version 14) was used to search for a combination of the factors involved that jointly optimize ethanol production with the retention of a high viability. The range of viability used for optimization was between 80% and 100% and for ethanol between 68 and 100 g/l. Desirability is a measure of how well the optimal solution satisfies the aim of the responses. A desirability of one indicates complete satisfaction, while a desirability of zero indicates that the response is not acceptable. In order to validate the optimized conditions (40 g/l biomass in dry weight, 30 °C, 200 g/l sucrose) derived from the use of the "Response Optimizer", experiments were carried out in triplicate to obtain time curves for viability, ethanol production, biomass, and total residual sugar.

Results

Using factorial design and RSM, variations in viability and ethanol production were predicted as functions of the variations in inoculum size, sucrose concentration, and temperatures.

Factorial planning

Table 2 shows the predicted and experimental data related to both the ethanol production and the viability responses, which were obtained using a factorial design. Twenty experiments were carried out in 4-h fermentation periods using different combinations of the independent variables. The highest predicted values of ethanol (77.2 g/l) and viability (87.5%) were observed in run 10, in which real values of viability and ethanol were 87.2% and 77.0 g/l, respectively. High real values of viability (around 92%) were also observed in runs 14 and 19, but the real levels of ethanol were much lower (54.6 g/l in run 14 and 68.7 g/l in run 19). Real and predicted data obtained can be low, as follows: 65.9% viability and 55.2 g/l ethanol in run 7 and 59.1% viability and 60.1 g/l ethanol in run 8, as shown in Table 2. High temperatures (38 °C in run 7 and 40 °C in run 8, as shown in Table 2) inhibited the ethanol production and killed the cells, as is indicated by the low values of viability.

Model fitting using RSM

Using the data shown in Table 2, the proposed polynomial experimental model (Eq. 1, "Materials and methods") was regressed, resulting in two expanded equations or fitted models, which are shown in Table 3, and these exhibit maximal viability (Eq. 2 or Y_1 model) and ethanol production (Eq. 3 or Y_2 model). The R-squared value (R^2, coefficient of correlation resulting from the regression of the model equation) provides a way to evaluate how much the measured variability could be explained by the experimental factors and their interactions in the observed responses. The matching quality of data, provided by the model equations (Eq. 2 for viability and Eq. 3 for ethanol production as shown in Table 3), indicates that 98.7% of the variability (R^2=0.987) in the viability response and 98.6% (R^2=0.986) in the ethanol production response can be explained by the models. Regression also provides a way to evaluate the nature and the degree of correlation between dependent and independent variables. The closer the R^2 value is to 1.00, the stronger the model and the better the response predictions (Haaland 1989). The R^2 value is always between zero and one. For the ethanol production model, a R^2=0.986 was obtained, indicating the adequacy (or a high probability) of this model. The adjusted R^2 (adj. R^2), which was derived from the sample size and from the number of terms in the model equation, corrects the predicted R^2 value. In the present case, the differences between values of R^2 and adj. R^2 are small, and thus, they are in reasonable agreement.

Table 2 Experimental and predicted values of ethanol and viability resulting from the application of the 2^3 full-central-composite design

Runs	Independent variables (real values)			Yeast cell viability (%)		Ethanol (g/l)	
	Temperature (°C)	Sucrose (g/l)	Inoculum (g/l)	Experimental	Predicted	Experimental	Predicted
1	35	200	35	79.9	79.8	73.6	73.6
2	35	150	30	79.6	81.0	64.4	65.5
3	35	100	35	75.8	77.3	45.8	45.6
4	38	180	32	70.5	69.6	68.7	68.1
5	35	150	35	78.4	78.9	67.8	67.2
6	30	150	35	96.3	96.3	62.8	63.4
7	38	120	32	65.9	64.6	55.2	55.5
8	40	150	35	59.1	60.4	60.1	59.3
9	35	150	40	81.0	81.0	70.7	69.4
10	32	180	38	87.2	87.5	77.2	77.0
11	38	120	38	69.9	69.0	51.0	51.5
12	35	150	35	80.1	78.9	69.0	67.2
13	35	150	35	77.4	78.9	65.7	67.2
14	32	120	32	92.2	91.2	54.6	53.6
15	35	150	35	77.1	78.9	65.7	67.2
16	32	120	38	89.5	89.3	55.6	56.3
17	35	150	35	80.5	78.9	67.7	67.2
18	35	150	35	80.1	78.9	67.3	67.2
19	32	180	32	92.0	91.9	68.7	68.3
20	35	180	38	71.4	71.4	68.9	70.1

Positive signs in terms of the fitted equations (Eqs. 2 and 3 in Table 3) represent synergistic effects, while negative signs indicate antagonistic effects. Interactive, linear, and squared effects can be observed among independent variables. The linear equation model (Eq. 2, Table 3) shows the linear and negative effects of the temperature (X_1) and inoculum (X_3), as well as the positive effect of the interaction between temperature (X_1) and inoculum size (X_3). Effects of independent variables on ethanol production are related to a greater number of terms, as shown in Eq. 3 (Table 3). This equation shows the linear and positive effects of sucrose concentration (X_2) and temperature (X_1) on ethanol production. In addition, Eq. 3 (Table 3) shows a negative interaction between temperature (X_1) and inoculum size (X_3), but a positive interaction between sucrose concentration (X_2) and inoculum size (X_3) are also shown. Negative and quadratic effects of temperature $\left(X_1^2\right)$ and sucrose concentration $\left(X_2^2\right)$ can also be observed in Eq. 3 (Table 3).

Analysis of variance (ANOVA) for the fitted models

The two fitted equations (Table 3), resulting from the analysis of variance, were a linear equation (Eq. 2) for viability and a quadratic equation (Eq. 3) for ethanol production. Table 4 describes the F values (statistical significance of the model) and p values for the viability (Eq. 2) and the ethanol production (Eq. 3) models. The regression F values were high enough to indicate statistical significance and that most of the variations in the response variables can be explained by the regression equations. Concerning the R^2 value (Table 3), both Eqs. 2 and 3 were highly significant and adequate to represent the true relationship between the three independent variables.

Table 3 Best-fit equations for viability and ethanol production responses resulting from the complete 2^3 factorial design

Responses	Best-fit equations	Regression	R^2	adj. R^2
Viability (Y_1)	$Y_1 = 509.379 - 10.00X_1 - 10.723X_3 + 0.172X_1.X_3$	Linear	0.987	0.975
Etanol (Y_2)	$Y_2 = -481.391 + 23.050X_1 + 0.798X_2 - 0.229X_1^2 - 0.003X_2^2 - 0.187X_1.X_3 + 0.017X_2.X_3$	Quadratic	0.986	0.974

X_1 temperature, X_2 initial sucrose concentration, X_3 inoculum, F Fisher test for regression, R^2 coefficient of determination, *adj.* R^2 adjusted R^2

Table 4 Analysis of variance (ANOVA) for the linear model of viability and the squared model of ethanol production using strain 63M of *S. cerevisiae* and 4-h fermentation periods

Responses	Sources of variations	Seq sum of squares	Adj sum of squares	Degrees of freedom	Adj mean of squares	*F* ratio	*p* Values
Viability (fitted Eq. 2)							
	Regression	1,604.98	1,604.98	9	178.33	83.60	0.000
	Linear	1,563.83	25.03	3	8.34	3.91	0.044
	Square	9.30	9.30	3	3.10	1.45	0.285
	Interaction	31.84	31.85	3	10.62	4.98	0.023
	Residual error	21.33	21.33	10	2.13	–	–
	Lack-of-fit	10.16	10.16	5	2.03	0.91	0.540
	Pure error	11.17	11.17	5	2.24	–	–
	Total model	1,626.31	–	19	–	–	–
Ethanol (fitted Eq. 3)							
	Regression	1,185.92	1,185.92	9	131.77	80.43	0.000
	Linear	988.78	91.79	3	30.60	18.68	0.000
	Square	154.33	154.33	3	51.44	31.40	0.000
	Interaction	42.82	42.82	3	14.27	8.71	0.004
	Residual error	16.38	16.38	10	1.64	–	–
	Lack-of-fit	7.85	7.85	5	1.57	0.92	0.535
	Pure error	8.53	8.53	5	1.71	–	–
	Total model	1,202.31	–	19		–	–

Seq sequential, *Adj* adjusted

Concerning viability (Eq. 2), the regression *F* test was highly significant (p=0.000), as shown in Table 4. As the significance of the regression *F* test shows a value of 5% (p=0.05), the *F* test was significant for the linear regression (p<0.05), but not for the lack of fit and the square (p>0.05) of the linear model for viability (Table 4). However, the value of adj. R^2=0.975 obtained for the viability indicates a well-fitted model, as shown in Table 3.

Concerning ethanol production (Eq. 3 in Table 3), the *F* test for the regression (Table 4) was also highly significant (p=0.000). As the significance of the regression *F* test shows a level of 5% (p=0.05), the *F* test was significant for both the linear and square regressions (p<0.05), but not for the lack of fit (p>0.05). Thus, a well-fitted model was obtained for the equation of ethanol production, as indicated by the adj. R^2 value of 0.974 shown in Table 3. In addition, the value of adj. R^2=0.974 for ethanol production also indicates a well-fitted model in Table 3.

Table 5 shows the regression coefficients, standard errors of coefficients, *t* values, and *p* values for the models representing viability (Eq. 2) and ethanol production (Eq. 3). Concerning viability, Table 5 shows that the linear effects of temperature (X_1) and inoculum size (X_3) on viability (Eq. 2) were negative and significant at a 5% probability level (p value<0.05). The linear effect of sucrose concentration (X_2) on viability was negative but not significant (p>0.05). Concerning interactions between variables, positive but not significant interactions (p value> 0.05) were noted between temperature (X_1) and sucrose concentration (X_2, p>0.05), so the coefficient $X_1.X_2$ was omitted from Eq. 2 as described in Table 3 for viability. On the other hand, the interactions between sucrose concentration (X_2) and inoculum size (X_3) were negative but not significant, so that $X_2.X_3$ was also omitted from Eq. 2.

In relation to ethanol production, Table 5 shows that the quadratic effects (Eq. 3 in Table 3) of temperature (X_1) and sucrose concentration (X_2) were significant (p<0.05), while the effect of inoculum size (X_3) was positive, although not significant (p>0.05). Positive and significant interactions were observed between sucrose concentration (X_2) and inoculum size (X_3), while a negative but significant interaction was observed between temperature (X_1) and inoculum size (X_3). Despite the negative coefficient, the interactive effects between temperature (X_1) and sucrose concentration (X_2) were not significant (p value>0.05), as shown in Table 5.

Three-dimensional surface and contour plots for ethanol production and viability

Figures 1, 2, and 3 show the three-dimensional response surfaces resulting from the fitted equations to investigate

Table 5 Regression coefficients, standard errors, *t* test, and significance level for the models representing ethanol production responses and viability responses as a function of variations in the independent variables in 2^3 full-central-composite design

Terms of model equations	Regression coefficient	Standard error	*t* Test	*p* Value
Viability				
Constant	509.379	114.244	4.459	0.001
X_1 (temperature)	−10.000	3.707	−2.697	0.022
X_2 (sucrose)	−0.098	0.312	−0.315	0.759
X_3 (inoculum)	−10.723	3.707	−2.892	0.016
$X_1.X_1$	−0.020	0.043	−0.460	0.655
$X_2.X_2$	−0.000	0.000	−0.322	0.754
$X_3.X_3$	0.082	0.043	1.929	0.083
$X_1.X_2$	0.012	0.006	2.082	0.064
$X_1.X_3$	0.172	0.057	3.002	0.013
$X_2.X_3$	−0.007	0.006	−1.259	0.237
Ethanol production				
Constant	−481.391	100.116	−4.808	0.001
X_1 (temperature)	23.050	3.249	7.094	0.000
X_2 (sucrose)	0.798	0.273	2.918	0.015
X_3 (inoculum)	3.709	3.249	1.142	0.280
$X_1.X_1$	−0.229	0.037	−6.112	0.000
$X_2.X_2$	−0.003	0.000	−7.947	0.000
$X_3.X_3$	0.010	0.037	0.280	0.785
$X_1.X_2$	−0006	0.005	−1.182	0.264
$X_1.X_3$	−0.187	0.050	−3.718	0.004
$X_2.X_3$	0.017	0.005	3.304	0.008

Significance at 5% probability level; R^2 of 0.987 for ethanol production and 0.986 for viability; R^2 adjusted for ethanol production was 97.4% and 94.5% for viability

Coef. coefficients

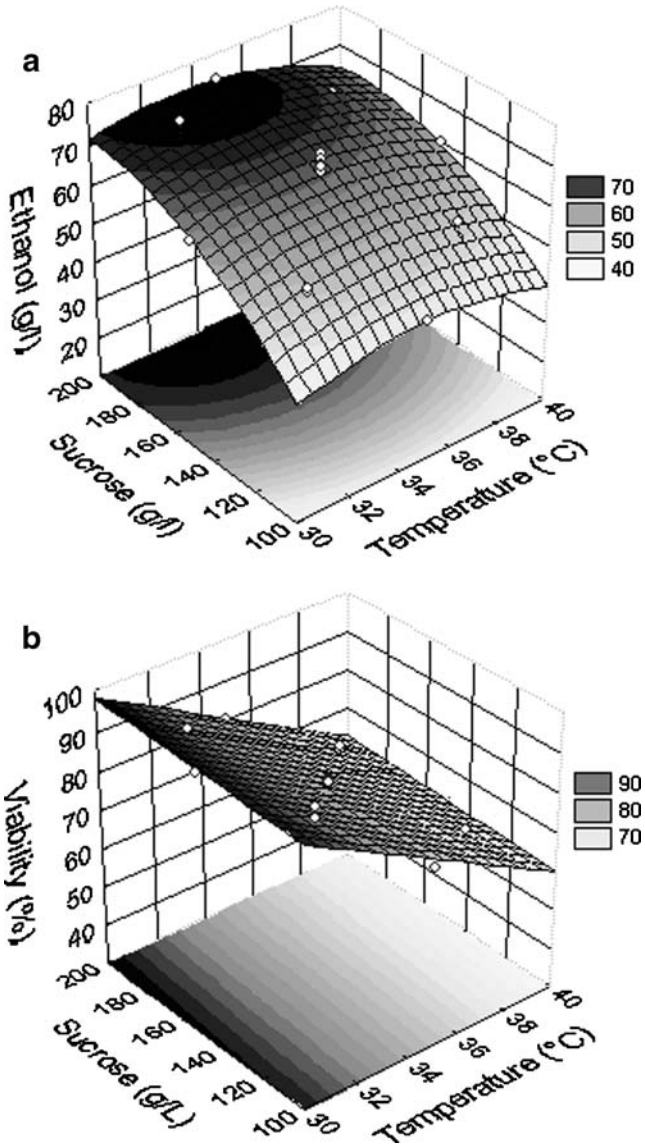

Fig. 1 Response surface curves and contour plot lines showing the variations in ethanol production (**a** quadratic model) and viability (**b** linear model) as functions of the interactive effects between temperature (X_1) and sucrose concentration (X_2) when 35 g/l inoculum is used as the central point

the interactions between variables and to determine the optimal values of each factor for maximal retention of viability (linear model in Eq. 2) and ethanol production (quadratic model in Eq. 3).

The interactive effects between temperatures (X_1) and sucrose concentration (X_2) on viability and ethanol production, using 35 g/l inoculum as the central point, are shown in Fig. 1. The two response surface graphs show that increases in temperature had greater negative effects on viability (Fig. 1b) than on ethanol production (Fig. 1a). Increasing sucrose concentrations (X_2) improved ethanol production (Fig. 1a) with small effects on viability (Fig. 1b). The best value of viability (Fig. 1b) was 90% at 32 °C, while the corresponding value of ethanol produced was around 74 g/l. At 30 °C, the amount of ethanol produced was lower.

The interactive effects between temperatures (X_1) and inoculum size (X_3) are shown in Fig. 2, using 150 g/l sucrose as the central point. The two response surface graphs show that the increases in temperature (X_1) had a greater negative effect on viability (Fig. 2b) than on ethanol production (Fig. 2a). It was observed that increasing the inoculum size (X_3) improved ethanol production (Fig. 2a) with little or no effect on viability (Fig. 2b). The maximal value of ethanol was seen to around 32 °C (around 73 g/l ethanol), while the corresponding viability was 90%. The maximal viability was obtained with 40 g/l inoculum at 30 °C, but the ethanol accumulated was lower. Thus, increases in temperature improved ethanol production (Fig. 2a) up to a threshold temperature of 32 °C, and then

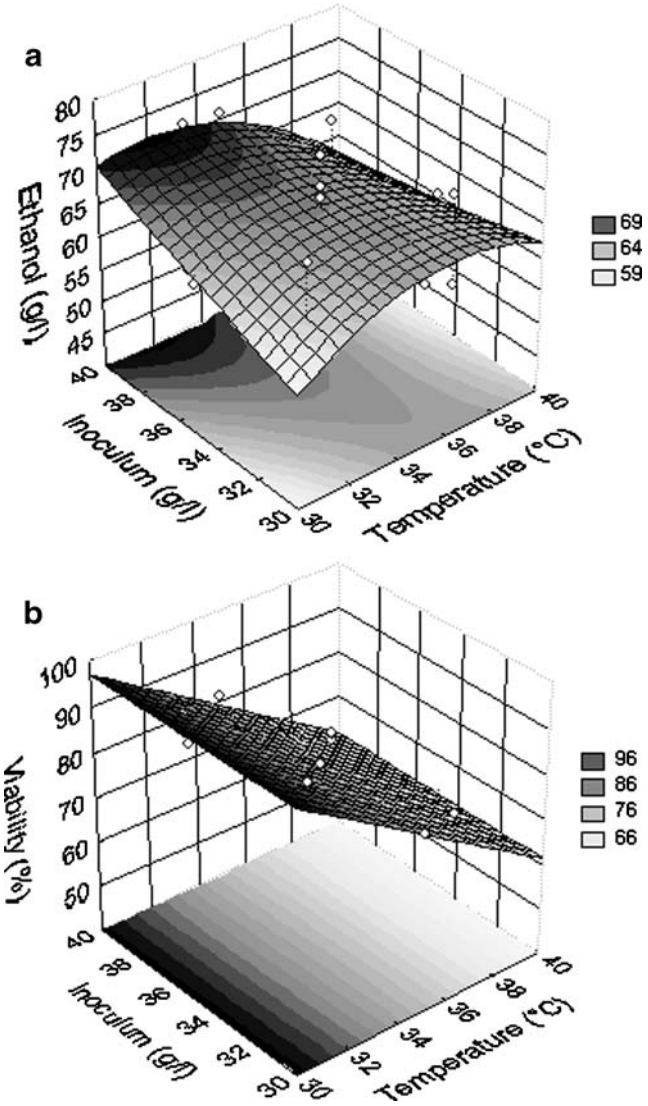

Fig. 2 Response surface curves and contour plot lines showing the variations in ethanol production (**a** quadratic model) and viability (**b** linear model) as functions of the interactive effects between sucrose concentration (X_2) and inoculum sizes (X_3) when 150 g/l sucrose is used as the central point

ethanol levels decreased above this temperature showing negative effects on viability (Fig. 2b). The viability (Fig. 2b) did not increase with the inoculum size (X_3).

The interactive effects between amounts of sucrose (X_2) and inoculum (X_3) are shown in Fig. 3, using 35 °C as the central point. The two response surface graphs show that increases in sucrose concentration (X_2) had a greater positive effect on ethanol production (Fig. 3a) than on viability (Fig. 3b). In addition, sucrose concentration (X_2) and inoculum size (X_3) did not have impacting effects on viability (Fig. 3b), as is also shown in Fig. 2. The maximal value of ethanol produced was around 80 g/l, while the corresponding viability was around 80%.

Optimization and experimental validation of the models

The determination of the optimal values of the factors that affected the ethanol production and viability (dependent variables) was attempted using the Response Optimizer of the MINITAB software package (version 14). Based on the Response Optimizer, the predicted optimal fermentation conditions were as follows (Table 6): 200 g/l sucrose, 30 °C, and 40 g/l inoculum.

The experimental validation of these three optimal conditions (predicted values) was carried out in shaken flasks, and the corresponding time curves of the fermentation process are shown in Fig. 4. The experimental responses (Fig. 4 and Table 6) were: 99.0±3.0% viability and 80.8±2.0 g/l ethanol

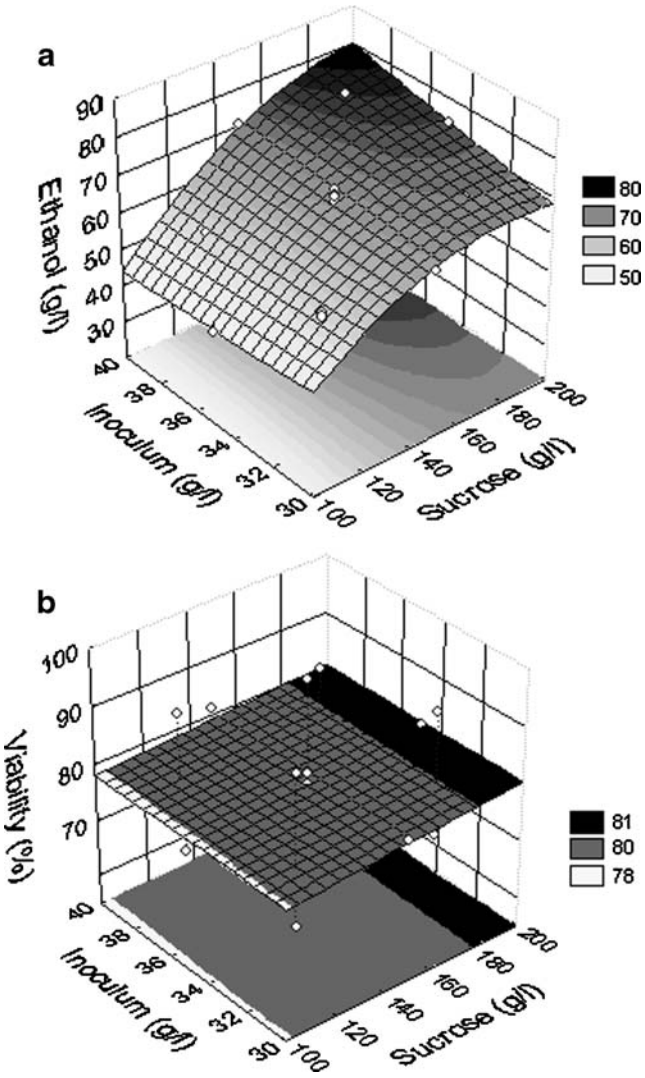

Fig. 3 Response surface curves and contour plot lines showing the variations in ethanol production (**a** quadratic model) and viability (**b** linear model) as functions of the interactive effects between the inoculum size (X_3) and sucrose concentration (X_2) when 35 °C is used as the central point

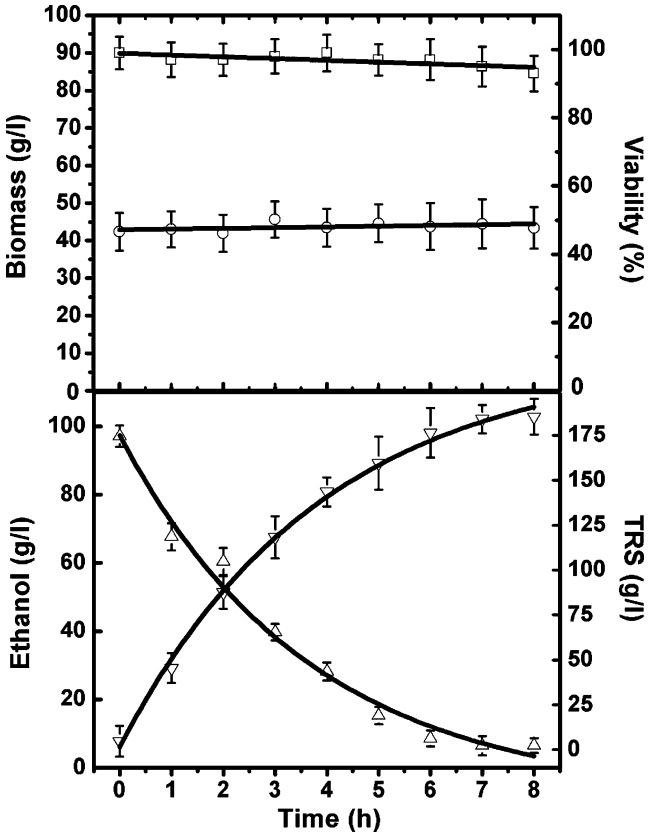

Fig. 4 Kinetics of growth and fermentation by strain 63M under the best conditions as indicated by the MINITAB's Response Optimizer (software package, version 14): biomass (*circles*); viability (*squares*); ethanol (*upside-down triangles*); total reducing sugar or TRS (*right-side-up triangles*)

in a 4-h fermentation. In a 7-h fermentation, the responses were (Fig. 4): 102.1±4.0 g/l ethanol and 95.0±2.2% viability. The initial biomass (Fig. 4) was 42.4±2.0 g/l and the final biomasses were 43.4±2.2 g/l in 4 h and 44.5±2.8 g/l in 7 h of fermentation.

Discussion

An economically relevant factor associated with industrial ethanol production is to obtain high ethanol yields over a succession of fast fermentation cycles, in which cells from one cycle are used as inoculum of the next fermentation cycle. The retention of high viabilities during the fermentation cycles is a prerequisite to carry out a long-lasting succession of fermentation cycles. In the present study, short fermentation times were obtained by using high amounts of inoculum to start simple batch fermentations at high cell density. However, conditions for growth and metabolism at high cell densities are less favorable due to hindered access to nutrients, space limitations, and cell interactions (Jarzebski et al. 1989). In addition, variation in temperature often occurs in the summertime due to fluctuations in the temperature of the cooling water of the bioreactors mainly in tropical climates. To this end, the optimization of independent variables (temperature, sucrose concentration, and inoculum size) and the corresponding responses (ethanol produced and viability retention) were obtained in the present work using a 2^3 full-central-composite design.

A factorial design (Table 2) involving predicted and experimental data indicated that the variations in viability at the end of the fermentation did not necessarily reflect the variations in ethanol production. High levels of viability were obtained, but the corresponding levels of ethanol can be much lower than expected. This was due to the kind of interactive effects between the independent variable. In literature (Dasari et al. 1990), increases in viability have been described as related to decreases in ethanol yields due to the inhibitory effects of ethanol.

In the present work, two fitted equations (Eqs. 2 and 3) were obtained, and they were highly significant and adequate to represent the true relationship between the

Table 6 Response optimization using the Response Optimizer of the MINITAB software package (version 14)

Parameters	Goal	Lower	Target	Upper	Weight
Viability (%)	Maximal	80	90	100	1
Ethanol (g/l)	Maximal	68	83	100	1
Global solution					
Temperature=30 °C					
[Sucrose]=200 g/l					
[Inoculum]=40 g/l					
Predicted responses					
Viabillity=90.00%, desirability=1.000					
Ethanol=82.65 g/l, desirability=0.9770, composite desirability=0.9884					
Experimental responses					
Viability=99.0±5.4%					
Ethanol=80.8±4.3 g/l					

three independent variables, as shown by the R^2 values, as shown for ethanol production and viability. The interactive effects between the process variables can be synergistic or antagonistic, as shown by the regression coefficients (Table 5) and responses surface graphs.

Concerning the interaction between ethanol accumulation and inoculum size, the response surface graph showed that increasing ethanol yields can be obtained using increasing inoculum sizes. As also described in literature (D'Amore et al. 1989), the rate and level of ethanol produced increased with the increases in inoculum sizes. In addition, Vega et al. (1987) proposed a mathematical model, which showed that increasing amounts of inoculum decreased the severity of ethanol inhibition. However, a high level of inoculum leads to rapid fermentation that may not always be favorable to the process depending on the strain and levels of ethanol produced. In the present work, the interaction between sugar concentration and inoculum sizes led to increases in the levels of the ethanol produced due to a strong and positive interaction between sugar concentration and inoculum size in high-cell-density cultures.

As observed in this work, interactions between sucrose concentration and inoculum size did not affect the final viability of the process in high-cell-density cultures. However, growth inhibition and cell mortality can be observed due to the increases in ethanol toxicity in media with high sugar concentration, and this toxicity is aggravated at high temperatures (Grubb and Mawson 1993; present work). In addition, correlations between the drops in viability and a massive leakage of intracellular metabolites, which were particularly severe above 10% ethanol in the media, have been described (Cot et al. 2007). If consecutive and/or abrupt drops in viability frequently occur, the fate of the fermentation process is its own collapse. When this happens, the entire process has to be restarted, incurring great economic losses for distilleries.

The experimental assays confirmed the reliability of the two mathematical models proposed to predict the variations in viability and ethanol production during fermentation in the present work. Despite the very low biomass accumulated at the end of the fermentation processes in the validation experiments (Fig. 4), the value of the final viability was kept at a high value (95.0±2.2%) for the 7-h fermentation, indicating that the cell population was alive in a quasistationary phase. A succession of fermentation cycles with cell reuse (cells from one cycle used to inoculate the next cycle) is not possible when significant drops in cell mass or viability are observed during the fermentation cycles. Nevertheless, a succession of fermentation cycles can be carried out over months at the industrial scale when cell proliferation is observed, and this indicates that the yeast cell population has been kept robust and healthy during the fermentation processes.

Values of optimized responses (viability and ethanol production) depend on the type of process (batch or continuous process), yeast strain, and other independent variables (nutrients and their concentrations, aeration, agitation, and so on), and these were not involved in this optimization study. Using a batch culture, kinetics of ethanol production from molasses was optimized by other authors (Rivera et al. 2006), showing that the maximal value of biomass (X_{max}) was obtained at 28 °C and P_{max} (ethanol) at 31 °C. In the present work, the experimental validation of the statistical data carried out in batch cultures (Fig. 4) showed a maximal ethanol production (102.1± 4.0 g/l) from 200 g/l sucrose after 7 h of fermentation at 30 °C without drops in viability (Fig. 4), but variations in biomass were not observed due to the high number of cells used as inoculum.

As indicated in the present work, it is seems feasible to predict the effects of sugar concentration, temperature, and inoculum size on viability and ethanol production for the operation of alcohol plants using statistical methodologies. When the sugar concentration increases, the process temperature has to be decreased in order to prevent drops in viability due to the ethanol-induced lethality of increasing amounts of ethanol produced by the yeast cells. Thus, the lowering of the process temperature is recommended in order to minimize cell mortality and maintain high levels of ethanol production when the temperature is increasing in the industrial reactor.

The batch cultures used in the present work have some limitations, mainly related to high sugar concentrations and ethanol yields. To overcome these problems, the fed-batch culture techniques have often been employed. In a pulse fed-batch culture using the same yeast strain as was used in the present work, high ethanol yields were obtained without significant variations in viability at temperatures as high as 37 °C (Souza et al. 2007).

Acknowledgments We are grateful to FAPESP for research grant no. 2005/02840-0.

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