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Alcoholic fermentation with high sugar and cell concentration at moderate temperatures using flocculant yeasts

Ana Carolina Tolentino Brandão, Miriam Maria de Resende†, and Eloízio Júlio Ribeiro

Federal University of Uberlândia, Faculty of Chemical Engineering, Av. João Naves de Ávila, 2121, Bloco 1K, Campus Santa Mônica, Uberlândia - MG, 38.408-144, Brazil
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Abstract−This paper studied bioethanol production at very high gravity (VHG) conditions using flocculent *Saccharomyces cerevisiae*, evaluating the response yield, ethanol concentration, productivity, and residual sugar through a central composite design (CCD). This CCD was evaluated at 12 and 24 h fermentation times. In the CCD evaluated for 12 h of fermentation, the best condition for alcoholic fermentation was 27 °C, 260 g/L substrate concentration and a 30% v/v cell concentration; a maximum overall desirability of 0.937 was achieved. For CCD at 24 h of fermentation, the best condition was 27 °C, 300 g/L substrate concentration, and a 26% v/v cell concentration. The desirability achieved was 0.811. These conditions allowed us to verify, experimentally, that the CCD models described the fermentation behavior well. VHG alcoholic fermentation in fed-batch with the reuse of cells without chemical treatment was performed using the optimum conditions obtained from the desirability function (27 °C, 300 g/L, 26% v/v). This resulted in favorable alcohol content 132.90 g/L in comparison to the conventional fermentation process.

Keywords: Batch Fermentation, Ethanol, VHG Fermentation, Flocculating Yeast

INTRODUCTION

The United States of America and Brazil are the largest ethanol producers in the world. Together, both countries produce more than 94 billion liters of bioethanol per year, representing around 85% of world production [1]. Since the launch of the flex car in 2003, 535 million tons of CO_2eq are no longer released into the atmosphere due to the use of hydrous and anhydrous ethanol, which is added to gasoline at a proportion of 27% [2].

Of the technological advances developed in the production of ethanol, the introduction of very high gravity (VHG) technology, which involves preparation and fermentation with high concentrations of fermentable sugars, has aroused great interest. Typically, there are three classes of initial sugar concentrations employed in alcoholic fermentation: normal concentrations (normal gravity) up to 180 g/L, high concentrations (high gravity) in the range 180 to 240 g/L, and VHG above 250 g/L [3,4].

With high concentrations of sugar in worts, this process may impose stressful conditions on yeast cells, such as osmotic stress and stress related to high levels of ethanol at the end of fermentation. This induces loss of cell viability, reduced fermentation rates, and incomplete fermentations. In this scenario, the main challenge is the development of strains resistant to the multiple stresses present in the process. In addition, determining the temperature effect, as well as the impact of high substrate and ethanol concentrations on cell growth kinetics, is important, as these variables clearly affect the progress of ethanol production [4].

†To whom correspondence should be addressed.
E-mail: mresende@ufu.br

For VHG fermentation, the temperature effect on substrate consumption and ethanol production rates should be investigated quantitatively to determine the most appropriate operating conditions, as this process imposes stressful conditions on yeast cells. When using high concentrations of sugar in the process, it is recommended to lower the temperature, minimizing cell death and, consequently, avoiding decreases in cell viability due to the increased production of bioethanol, and to maintain high levels of ethanol production when the temperature is rising in the industrial reactor [5,6].

In the production of ethanol via fermentation, shortly after the end of fermentation, it is fundamental to separate the microorganisms (yeast) from the wine. The most conventional way to promote this separation is to use centrifuges, since natural sedimentation becomes unviable due to time constraints and cell deposition is 6,000 times faster by centrifugation than natural sedimentation [7,8].

Alternatively, to decrease the production costs of sugarcane plants installed in the country, researchers were able to successfully select *S. cerevisiae* yeast strains with flocculant capacity. The advantage of these yeasts over traditional yeasts is that they do not require centrifugation after fermentation. The elimination of this centrifugation step is expected to lower the processing cost, since it saves on the investment in and maintenance of centrifuges and decreases the energy consumption required for their operation. Thus, this adjustment is considered an environmentally friendly, environmentally correct operation, as well [9,10].

Therefore, to ensure clean and renewable production, technological innovations and cost reductions are required for this process. Given this context, we studied the influence of lower temperatures, compared to the classic, industrial-temperature processes in VHG fermentations. We employed high cell density flocculant yeasts, in a central composite design (CCD), to determine the best process-

ing conditions for the following variables: temperature and sugar and cell concentrations in the reactor. Then, these conditions were applied in batch fermentation fed with cell reuse.

MATERIAL AND METHODS

1. Microorganism

The *S. cerevisiae* strain with flocculant characteristics, termed C2/00, was donated by the Chemical, Biological and Agricultural Pluridisciplinary Research Center (CPQBA). The cells (two spatulas) were placed in 500 mL flasks, with useful volume of the culture medium of 200 mL and incubated in a shaker at room temperature (26±1 °C) under agitation (120 rpm) for 24 h. After cultivation time, the liquid phase was discarded. The decanted mass cells were transferred to an Erlenmeyer of 2 L with a new sterile culture medium. This cell suspension was kept refrigerated at 7±1 °C. To increase the stock of cells, they were repeated weekly. The medium consisted of sucrose (100 g/L), KH_2PO_4 (5 g/L), $MgSO_4 \cdot 7H_2O$ (1 g/L), $[NH_4]_2SO_4$ (2 g/L) and yeast extract (6 g/L) [11]. All of the reagents used were of analytical grade, with the exception of sucrose, which was replaced by commercial sugar. The yeast was grown prior to fermentation in Erlenmeyer flasks with a gradual increase in volume over time to obtain a decanted mass of cells for each assay.

2. Ethanol Production Medium

The production medium was similar to the culture medium described at item 2.1, with the exception of the concentration of sucrose used, which varied for each experiment. The initial pH of the medium was adjusted to 4.5 with 1 M HCL and/or 1 M NaOH for all experiments.

3. CCD

Temperature, initial cell concentration, and sugar concentration have a great effect on the ethanolic fermentation process [6,12]. Thus, these variables were selected as the most appropriate to perform a 2^3 CCD, with six axial points and three repetitions at the central point, resulting in 17 runs (orthogonality alpha of 1.35313). These runs were carried out in triplicate. All the experiments were completed in 250 mL Erlenmeyer flasks containing 70 mL of fermentation medium and inoculum with variable cell concentrations. Fermentations were conducted in an incubator (New Ethics) under agitation at 120 rpm and varying temperatures for each run over a 24-hour period. For the preparation of the variable cell concentration inoculum, where the final volume and concentration were known, the inoculum concentration required to produce the desired volume of decanted cells was obtained. The pH was adjusted to 4.5, and the samples were collected at 1.5 h time intervals. The conditions for the studied variables, temperature (X_1), initial total reducing sugar concentration (X_2), and initial cell concentration (X_3) are presented in Table 1. The response variables analyzed were yield (Y_1), ethanol concentration (Y_2), productivity (Y_3), and residual sugar (Y_4). For CCD analysis, optimizations were completed at 12 and 24 h for the same experiment, i.e., in the middle and at the end of the experiment. Assays were carried out in random order to avoid false positive results.

Table 1. Values used for the three independent variables

	$-\alpha$ (−1.35313)	−1	0	+1	$+\alpha$ (1.35313)
X_1 (°C)	21	22	25	28	29
X_2 (g/L)	240	250	280	310	320
X_3 (%v/v)	12	14	21	28	30

For greater reliability in the analysis of response surfaces, the desirability function was used, present in Software Statistica 7.0 and proposed by Derringer and Suich in 1980. This is a simultaneous optimization technique that consists of finding the values of independent variables (factors) that optimize one or more responses, or at least keeps them in desirable ranges, using nonlinear programming methods. Initially, this function converts each response (yi)

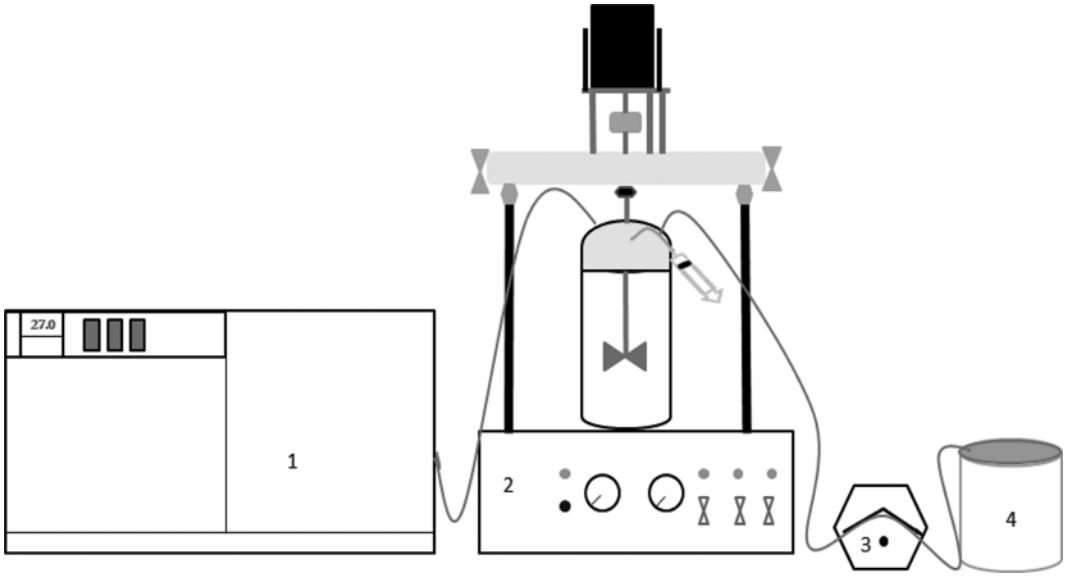

Fig. 1. Schematic representation of the work unit in fed-batch.
1. Thermostatic bath 2. New Brunswick Multigen fermenter 3. Peristaltic pump 4. Feed tank

into an individual desirability function (di) ranging from 0≤di≤1. If the answer is the most desirable value, di=1, otherwise, di=0, and the response is outside the acceptable region. Thus, independent variables are chosen to maximize the overall desirability, which is given by the geometric mean of individual desirabilities (di). This overall desirability can range from 0 to 1, and when D is closer to unity the reliability of the method is greater [13].

4. VHG Fermentation in fed-batch with Cell Recycling

The assays were carried out in a bioreactor (New Brunswick Multigen), as shown schematically in Fig. 1. This fermenter has a volume of 2 L, as well as agitation and temperature control. Assays used the fermenter in fed-batch mode with a useful volume of 1.5 L. The inoculum volume for all fermentations was 30% of the bioreactor's useful volume (0.45 L). In the beginning, 1.05 L of fermentation broth was fed to the reactor by a peristaltic pump (Masterflex® 7553-76). In this study, two fermentative cycles were performed, each consisting of three fed-batch fermentations with cell recycling and without chemical treatment. The experiments were conducted at 27 °C, with a pH regulated to 4.5, medium was fed for 4 h, initial reducing sugar concentrations (C_{TRSi}) of 300 g/L, and initial cell concentrations of 26% of cell in volume in the bioreactor for 24 h. Samples were withdrawn at 1 h intervals.

5. Analytical Methods

Sucrose, glucose, fructose, and ethanol were measured using a high-performance liquid chromatograph (HPLC) - Prominence Shimadzu LC-20A. Samples were diluted, filtered, and injected into the chromatograph. A supelcogel™ C610H column and refractive index detector were used. The mobile phase was H_3PO_4 (0.1%). The measurement conditions were as follows: 0.5 mL/min pump flow, 32 °C oven temperature, and 20 μL injection volume.

6. Cell Viability

The cell viability was determined by the Methylene Blue technique as described by [14]. The samples were taken from the reactor and diluted with 5 mM EDTA in citrate buffer at pH 3 to the cells break the flakes and allow them to be counted, then mixed in a 1 : 1 ratio with methylene blue. The dye methylene blue can be taken up by dead or severely damaged cells, but not by living cells. After counting the number of live and dead cells in a Neubauer camera under an optical microscope (Olympus), viability was calculated according to Eq. (1).

$$\text{Viability} = \frac{n_{viable\ cells}}{n_{total\ cells}} \times 100 \quad (1)$$

7. Calculation of Yields and Productivity

The ethanol yield was calculated using a theoretical yield of 0.511 $g_{ethanol}/gC_{TRSi}$ (100%) using Eq. (2).

$$\text{Yield} = \frac{C_{ethanol}}{0.511 * C_{TRSi}} \times 100 \quad (2)$$

Productivity was determined by Eq. (3), based on the ethanol concentration at the end of fermentation and the time of fermentation.

$$\text{Pr} = \frac{C_{ethanol}}{t} \quad (3)$$

RESULTS AND DISCUSSION

1. CCD for 12 h Fermentation

In this experimental design, we studied how the yield (Y_1), calculated by Eq. (2); measured ethanol concentration (Y_2); productivity (Y_3), as defined by Eq. (3); and the amount of residual sugar (Y_4) are affected by temperature (X_1), C_{TRSi} (X_2), and initial cell concentration (X_3). The results are shown in Table 2

After 12 h of fermentation, the yield ranged from 54.71 to 82.58%, ethanol concentration from 79.31 to 121.7 g/L, residual sugar from

Table 2. Coded and nominal values and their responses in 12 hours of fermentation

Experiments	Nominal value (encoded value)			Y_1	Y_2	Y_3	Y_4
	X_1 (°C)	X_2 (g/L)	X_3 (% v/v)	(%)	(g/L)	($g_{ethanol}$/L·h)	(g/L)
1	22 (−1)	250 (−1)	14 (−1)	72.91	96.82	8.10	24.52
2	22 (−1)	250 (−1)	28 (+1)	81.45	105.88	8.82	0.434
3	22 (−1)	310 (+1)	14 (−1)	51.38	80.89	6.70	84.42
4	22 (−1)	310 (+1)	28 (+1)	62.39	100.58	8.4	70.33
5	28 (+1)	250 (−1)	14 (−1)	76.13	97.14	8.10	19.11
6	28 (+1)	250 (−1)	28 (+1)	82.58	114.67	9.6	2.68
7	28 (+1)	310 (+1)	14 (−1)	64.37	102.08	8.5	61.16
8	28 (+1)	310 (+1)	28 (+1)	70.42	114.74	9.6	47.02
9	21 ($-\alpha$)	280 (0)	21 (0)	54.71	79.31	6.6	82.78
10	29 ($+\alpha$)	280 (0)	21 (0)	81.60	121.7	10.1	2.82
11	25 (0)	240 ($-\alpha$)	21 (0)	81.07	104.67	8.7	0.88
12	25 (0)	320 ($+\alpha$)	21 (0)	58.89	103.7	8.6	82.02
13	25 (0)	280 (0)	12 ($-\alpha$)	65.45	95.12	7.9	65.83
14	25 (0)	280 (0)	30 ($+\alpha$)	75.18	110.77	9.2	21.26
15	25 (0)	280 (0)	21 (0)	77.63	112.72	9.4	30.62
16	25 (0)	280 (0)	21 (0)	78.42	111.26	9.27	28.58
17	25 (0)	280 (0)	21 (0)	77.44	110.68	9.22	30.23

0.434 to 84.42 g/L, and productivity from 6.6 to 10.1 $g_{ethanol}$/L·h. In addition, the central points presented a small variation for all responses, indicating good process repeatability. All parameters were statistically significant at the level of 90% ($p<0.1$). The responses to coded variables for yield (Y_1), ethanol concentration (Y_2), productivity (Y_3), and total residual sugar (Y_4) after 12 h of the fermentation process are described in Eqs. (4)-(7), respectively.

$$Y_1=75.77+5.29X_1-2.95X_1^2-8.14X_2-1.92X_2^2 +3.94X_3-1.76X_3^2+2.08X_1X_2-0.88X_1X_3 \quad (4)$$

$$Y_2=109.85+8.74X_1-4.15X_1^2-1.51X_2-2.08X_2^2 +6.99X_3-2.90X_3^2+3.28X_1X_2 \quad (5)$$

$$Y_3=9.14+0.73X_1-0.33X_1^2-0.13X_2-0.17X_2^2 +0.59X_3-0.23X_3^2+0.28X_1X_2 \quad (6)$$

$$Y_4=33.84-13.53X_1+2.43X_1^2+28.07X_2-1.67X_2^2 -11.15X_3+2.85X_3^2-5.43X_1X_2+1.54X_2X_3 \quad (7)$$

Determination coefficients, after fitting of the model to the experimental data, were 0.89, 0.83, 0.83, and 0.97 for yield, ethanol concentration, productivity, and residual sugar, respectively.

Eqs. (4)-(7) show that in Eq. (4), the coefficient of sugar concentration (X_2) indicates that increasing sugar concentration negatively affects the total yield. On the other hand, an increase in temperature (X_1) and cell concentration (X_3) positively affects the yield. Guidini et al. [15] studied the initial concentration of substrate, cell concentration in the inoculum, and the reactor filling time in fed-batch. They found that high concentrations of sucrose and cell concentrations contribute, respectively, to a decrease and increase in the yield response. Thus, as predicted by Eq. (4) of the present study.

The responses of ethanol concentration and productivity are strongly affected by temperature (X_1) and cell concentration (X_3). The increase in substrate concentration has a negative effect on the responses, confirming the data in Eqs. (5) and (6). Cruz et al. [6] evaluated the combined effects of temperature, cell concentration, and initial sucrose variables on fermentation and also found that cell concentration positively influences the ethanol concentration and productivity responses, as shown in this paper.

The coefficient in Eq. (7) related to substrate concentration has a high value. Thus, an increase in substrate concentration (X_2) implies a higher residual sugar concentration, and an increase in cell concentration (X_3) implies a decrease of residual sugar. Santos et al. [16] analyzed the combined effect of temperature, substrate, and cell concentrations and, in analyzing the coefficients of the model equation for the substrate concentration response, found that the variable that most affected the residual sugar increase was the substrate concentration, as found in the present study by Eq. (7).

The contour curves shown in Fig. 2 were constructed with yield, ethanol concentration, productivity, and residual sugar concentration results obtained from the CCD experiments. The joint evaluation of the proposed variables in agreement with the studied answers is important. This evaluation is to select the operational regions where the best results are obtained. Thus, the contour curves of the four responses obtained by CCD will be presented, fixing one of the variables and analyzing the other two together as shown in Fig. 2.

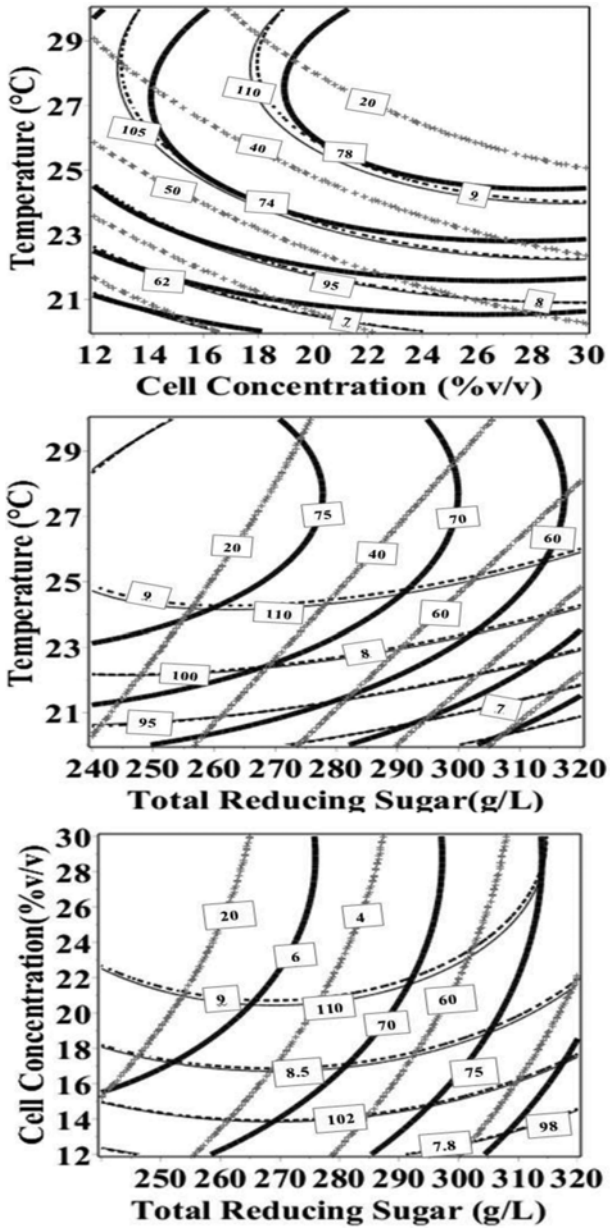

Fig. 2. Contour curves for temperature, cell concentration and substrate concentration of the optimal region for the responses ethanol concentration —, productivity ····, yield — and residual sugar +.

Fig. 2 shows that the selected region for maximizing the response yield, ethanol concentration, and productivity and minimizing residual sugar includes substrate concentration from 260 to 310 g/L, temperature from 25 to 29 °C and cell concentration from 21 to 30% v/v. To these variables' conditions, the range evaluated to the ethanol yield was 80.34-70.94%, ethanol concentration 109.93-119.10 g/L, productivity 9.15-9.98 $g_{ethanol}$/L·h, and residual sugar 14.37-31.64 g/L.

For greater reliability of the studied ranges, the desirability function (Statistica® 7.0) was used to obtain the optimal values of the investigated parameters. Fig. 3 shows the individual and global de-

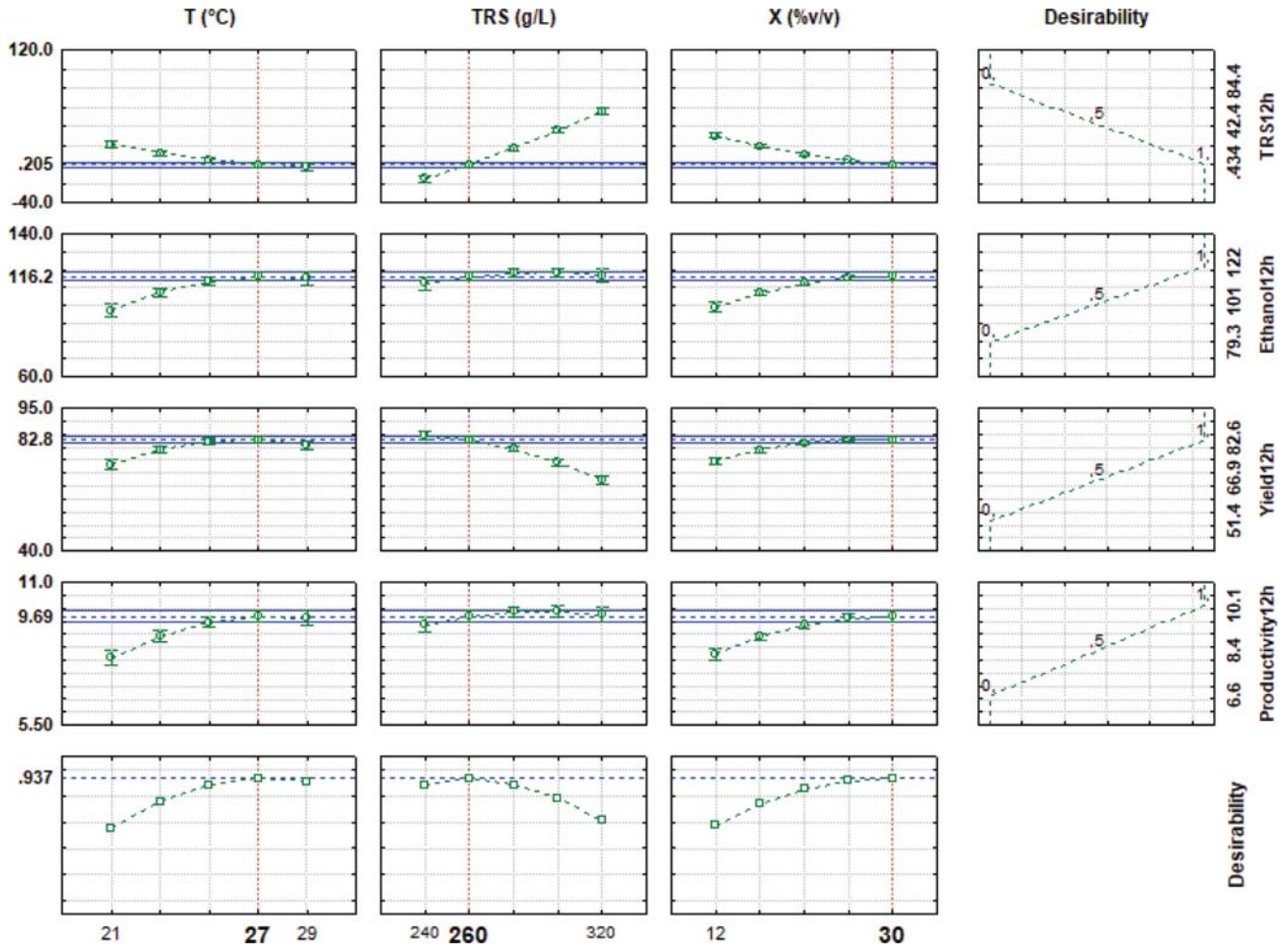

Fig. 3. Desirability function profiles for 12 h of fermentation.

Table 3. Coded and nominal values and their responses in 24 hours of fermentation

Experiments	Nominal value (encoded value)			Y_1 (%)	Y_2 (g/L)	Y_3 ($g_{ethanol}$/L·h)	Y_4 (g/L)
	X_1 (°C)	X_2 (g/L)	X_3 (% v/v)				
1	22 (−1)	250 (−1)	14 (−1)	80.58	102.94	4.29	1.76
2	22 (−1)	250 (−1)	28 (+1)	82.97	106.00	4.42	0.10
3	22 (−1)	310 (+1)	14 (−1)	74.47	117.97	4.92	10.72
4	22 (−1)	310 (+1)	28 (+1)	83.10	131.65	5.49	8.06
5	28 (+1)	250 (−1)	14 (−1)	82.59	105.51	4.40	1.39
6	28 (+1)	250 (−1)	28 (+1)	90.21	115.24	4.80	0
7	28 (+1)	310 (+1)	14 (−1)	80.11	126.91	5.29	9.98
8	28 (+1)	310 (+1)	28 (+1)	84.35	133.62	5.57	4.88
9	21 ($-\alpha$)	280 (0)	21 (0)	81.71	116.91	4.87	8.07
10	29 ($+\alpha$)	280 (0)	21 (0)	85.06	121.7	5.07	2.98
11	25 (0)	240 ($-\alpha$)	21 (0)	84.86	104.07	4.34	0.65
12	25 (0)	320 ($+\alpha$)	21 (0)	83.99	137.4	5.72	5.35
13	25 (0)	280 (0)	12 ($-\alpha$)	83.54	119.5	4.98	6.75
14	25 (0)	280 (0)	30 ($+\alpha$)	84.46	120.9	5.04	1.11
15	25 (0)	280 (0)	21 (0)	87.09	125.04	5.22	0.89
16	25 (0)	280 (0)	21 (0)	87.35	124.98	5.21	0.85
17	25 (0)	280 (0)	21 (0)	87.85	125.68	5.24	0.87

sirability profiles under the conditions established for this analysis. The optimized variables were temperature, initial substrate concentration, and initial cell concentration, while the individual desirability functions utilized were aimed at minimizing the residual sugar and maximizing productivity, yield, and ethanol concentration.

According to Fig. 3, for 12 h of fermentation, the best conditions for alcoholic fermentation are 27 °C, 260 g/L substrate concentration, and 30% cell volume. To this condition the ethanol yield was 83.04%, ethanol concentration 116.65 g/L, productivity 9.74 $g_{ethanol}$/L·h, and residual sugar 0.0 g/L. The maximum overall desirability achieved was 0.937. Therefore, the conditions for the completed validation experiments were chosen based on the desirability results and technical and economic criteria.

2. CCD for 24 h Fermentation

Table 3 shows the coded and nominal values of the study variables and the responses yield (Y_1), ethanol concentration (Y_2), productivity (Y_3) and residual sugar (Y_4). After 24 h of fermentation, the yield varied from 74.47 to 90.21%, ethanol concentration from 102.94 to 137.4 g/L, productivity from 4.29 to 5.72 $g_{ethanol}$/L·h, and residual sugar from 0.0 to 10.72 g/L. The central points presented a small variation for all responses, indicating good process repeatability. The runs were statistically analyzed using the Statistic® 7.0 software with parameters at the significance level of 90% ($p<0.1$). Furthermore, it was observed that in 24 h of fermentation, the yield and ethanol concentration were high compared to the 12 h fermentation, and due to this, there were still many experiments with high amounts of residual sugars, requiring more time for conversion to ethanol.

The coded variables for yield, ethanol concentration, productivity, and residual sugar at 24 h of production are shown in Eqs. (8)-(11), respectively, with determination coefficients of 0.82, 0.96, 0.96, and 0.77, respectively.

$$Y_1=87.15+1.78X_1-1.94X_1^2-1.34X_2-1.35X_2^2+2.13X_3-1.69X_3^2 \quad (8)$$

$$Y_2=124.9+2.52X_1-2.90X_1^2+10.80X_2-2.11X_2^2 +3.09X_3-2.57X_3^2+0.95X_2X_3 \quad (9)$$

$$Y_3=5.21+0.10X_1-0.12X_1^2+0.45X_2-0.09X_2^2 +0.13X_3-0.11X_3^2+0.04X_2X_3 \quad (10)$$

$$Y_4=1.44-0.98X_1+1.88X_1^2+3.17X_2+0.51X_2^2-1.59X_3 +1.07X_3^2-0.43X_1X_2-0.27X_1X_3-0.59X_2X_3 \quad (11)$$

Eq. (8 shows that the coefficient of substrate concentration, X_2, indicates a reduction in yield. On the other hand, temperature and cell concentration contribute to an increased yield response. Pacheco et al. [11] worked on a tower-type reactor system operating with upward flow in recirculation. They used self-flocculating yeast and found that cell concentration in the inoculum was the variable that most influenced the yield response. A growth of cells in the inoculum causes an increase in yield and decreases in residual sucrose levels.

The inspection of Eqs. (9) and (10) shows that the coefficients of variables X_1, X_2, and X_3 positively affect productivity and ethanol production. In agreement with Wheals et al. [17], the use of high temperature and high cell concentration contributes to the increase of productivity and bioethanol concentration. The CCD experiment corroborates, for the highest temperature (29 °C) studied, an increase in yield (10.1 $g_{ethanol}$/L·h) and bioethanol concentration (121.7 g/L).

The coefficient related to substrate concentration (X_2) in Eq. (11) indicates that an increase of substrate implies a higher residual sugar concentration, and an increase in the cell concentration implies a decrease of residual sugar. Guidini et al. [15] employed a flocculant *S. cerevisiae* strain and found that an increase in substrate implied a higher residual sugar concentration and an increase

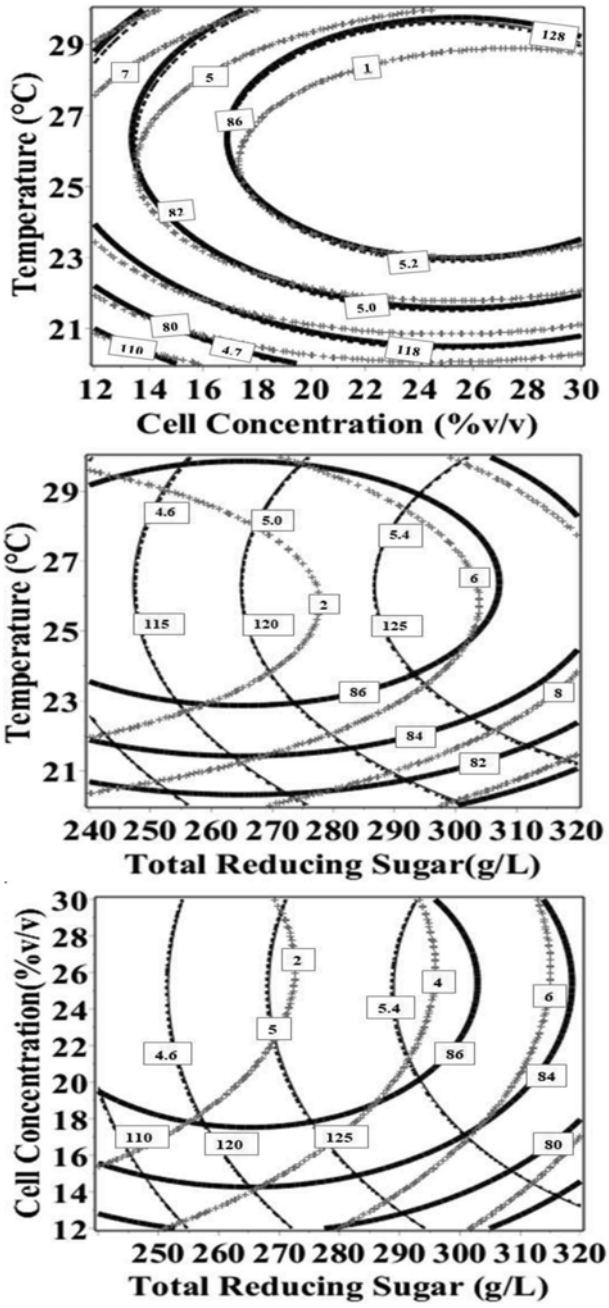

Fig. 4. Contour curves for temperature, cell concentration and substrate concentration of the optimal region for the responses ethanol concentration —, productivity ····, yield — and residual sugar +.

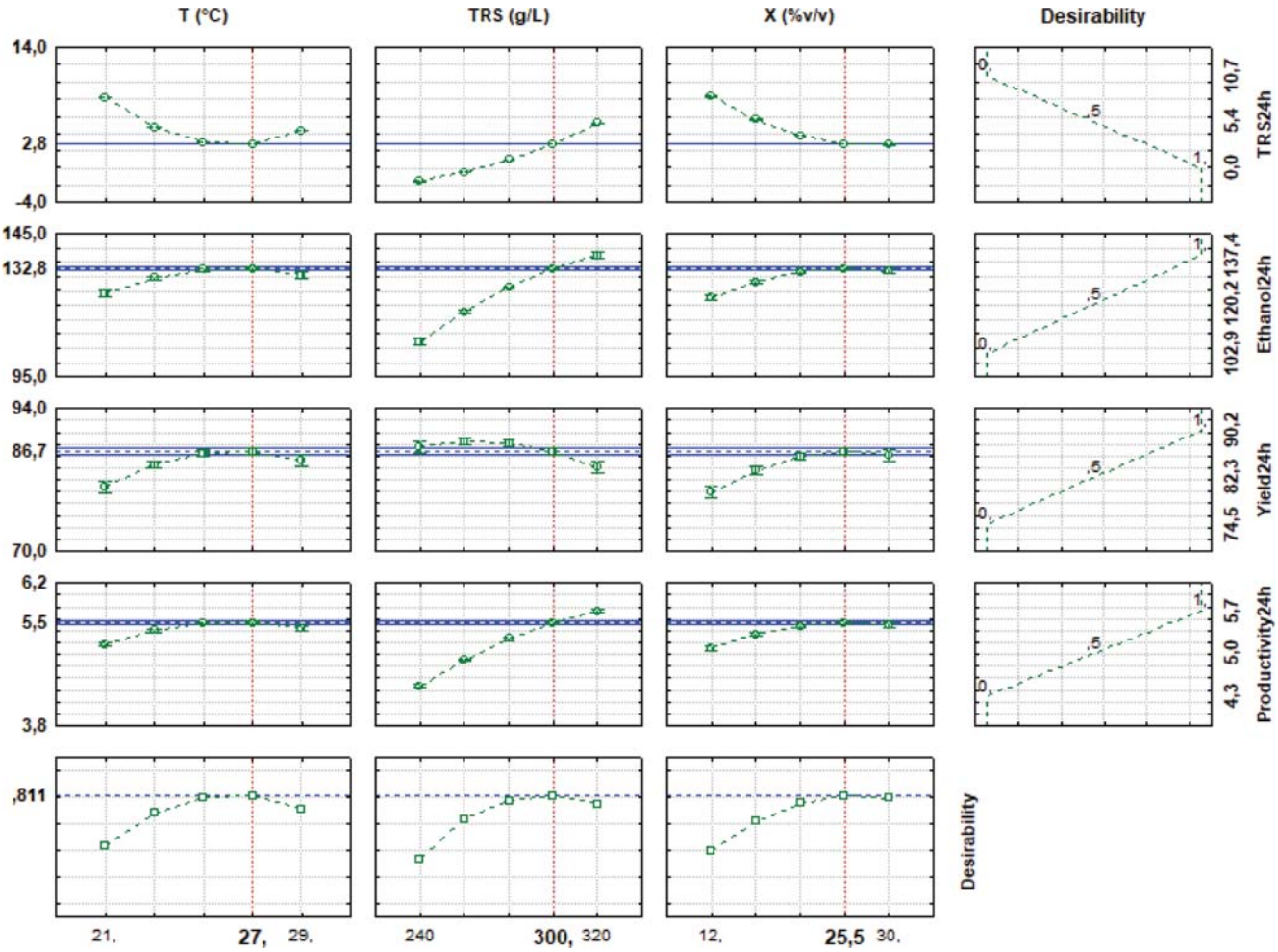

Fig. 5. Desirability function profiles for 24 h of fermentation.

in cell concentration decreased the residual sugar. This expected behavior was also observed in this work.

Fig. 4 illustrates the contour curves where the three process variables are presented graphically, two by two, along with all the evaluated answers, and it shows that the selected region for the best values of yield, ethanol concentration, productivity, and residual sugar includes substrate concentrations from 280 to 320 g/L, temperatures from 24 to 30 °C, and cell concentrations from 20 to 30% v/v.

For greater reliability of the ranges, the desirability function (Statistica® 7.0) was used to obtain the optimal values of the investigated parameters. Fig. 5 shows the individual and global desirability profiles for 24 h fermentation. The best conditions for the fermentation occur at 27 °C, a substrate concentration of 300 g/L, and a cell concentration of 26% v/v. To this condition the ethanol yield was 86.63%, ethanol concentration 132.90 g/L, productivity 5.54 $g_{ethanol}$/L·h, and residual sugar 2.77 g/L. The overall desirability achieved was 0.811.

3. Validation of the Optimized Conditions for 12 and 24 h Fermentation

At this step, the main purpose was to determine the experimental model reproducibility results at the optimal point from the contour curves. The conditions used experimentally were defined by the results provided from the contour curves and desirability function (Table 4).

Table 5 shows the experimental and predicted results obtained

Table 4. Experimental conditions for 12 and 24 h validations

	Temperature (°C)	Conc. substrate (g/L)	Conc. cell (%v/v)
12 h	27	260	30
24 h	27	300	26

Table 5. Experimental and predicted values for 12 h validations

Conditions	Experimental	Predicted	Deviation (%)
(27 °C; 260 g/L; 30%v/v)			
Y_1 12 h (%)	87.25	83.04	4.82
Y_2 12 h (g/L)	117.28	116.65	0.54
Y_3 12 h (g/L·h)	9.77	9.74	0.31
Y_4 12 h (g/L)	0.102	0.0	10.2

Table 6. Experimental and predicted values for 24 h validations

Conditions	Experimental	Predicted	Deviation (%)
(27 °C; 300 g/L; 26%v/v)			
Y_1 24 h (%)	90.30	86.64	4.05
Y_2 24 h (g/L)	135.63	132.90	2.01
Y_3 24 h (g/L·h)	5.65	5.54	1.65
Y_4 24 h (g/L)	2.58	2.77	7.36

by the experimental validation tests at 12 hours. The optimization equations provided by the CCD well describe the behavior of the ethanol production, indicating a good agreement between the experimental values and those indicated by the model. The experimental and predicted results obtained in the validation tests for the 12 h fermentation time were satisfactory, validating it in this way with a deviation of up to 10.2%.

Table 6 shows the experimental and predicted results obtained by the experimental validation tests at 24 hours. For this validation experiment, the initial cell viability was 91.45% and after 24 hours 81.53% were obtained. The experimental results are similar to the predicted results, and it can be considered that the results obtained in the validation tests were satisfactory with a deviation of up to 7.36%. These results show that the use of the CCD promotes the realization of experimental conditions, where it is possible to optimize more than one response at the same time, establishing conditions that guarantee optimal conditions for the product.

4. Prediction of VHG Fed-batch Experiments with Cell Recycle

Based on the optimal conditions obtained by the desirability function for the 24 h time, three fermentations were performed at 27 °C, with an initial TRS concentration of 300 g/L, an initial cell concentration of 26% (% cell in volume) and medium fed for 4 h. Fig. 6(a) shows the substrate consumption and ethanol production profiles obtained during the ethanol production for the first fed-batch fermentation. The sugar was not fully consumed in the 24 h period of fermentation. A yield of 80.48%, productivity of 5.14 $g_{ethanol}$/L·h, alcohol concentration of 125.12 g/L, residual sugar concentration of 60.96 g/L, and cell viability of 85.58% were obtained.

Reusing the yeast from the first fermentation, a second fermentation was performed with the same experimental conditions as the first. The results are shown in Fig. 6(b). After 24 h, the residual sugar concentration was 157.27 g/L, alcohol content 80.98 g/L, yield 52.73%, and productivity 3.37 g/L. Cell viability was 82.35%, practically the same as the first fermentation. However, there was a drop in ethanol concentration and a higher amount of residual sugar.

Lastly, a third fermentation was performed at the same experimental conditions with cell reuse (Fig. 6(c)). With a fermentation time of 24 h, the alcohol concentration reached 99.46 g/L, the yield 64.80%, productivity 4.14 $g_{ethanol}$/L·h, and the residual sugar 121.26 g/L. At the end of fermentation, cell viability decreased to 77.78%. Cell viability is strongly correlated with the amount of ethanol, since the higher the bioethanol concentration, the lower the viability, and the lower the ethanol concentration, the higher the viability. This drop in cell viability with the reuse of cells could be due to the lack of chemical treatment of yeasts during recycling, which is

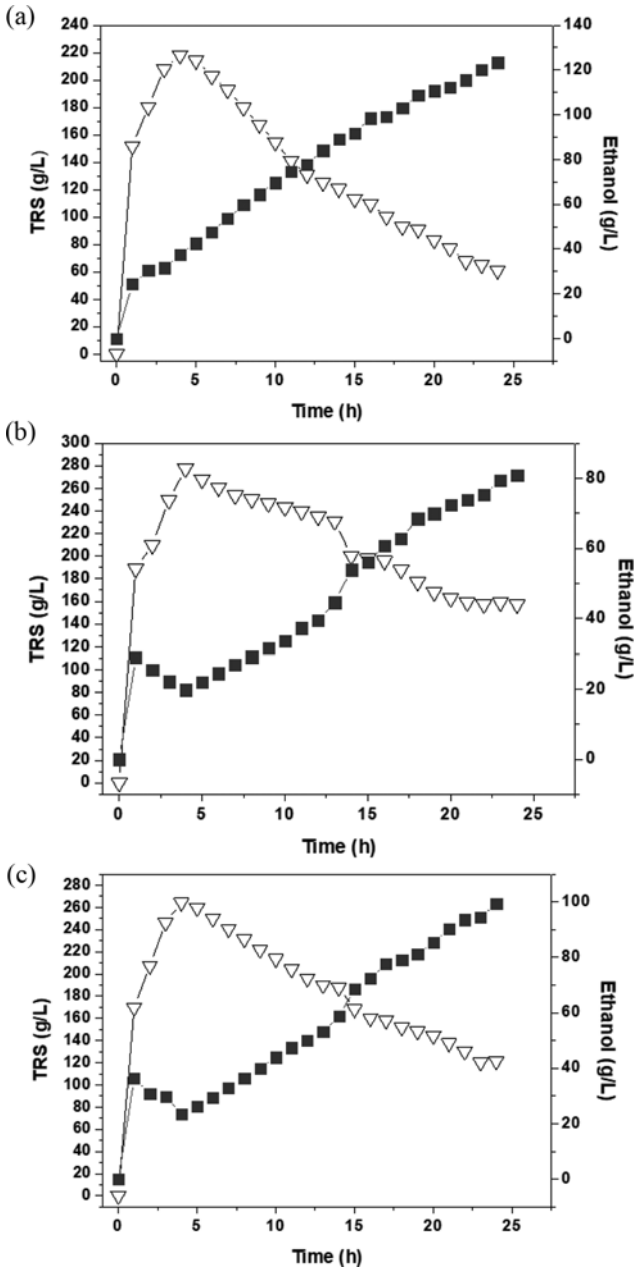

Fig. 6. Sugar concentration (▽) and ethanol concentration (■) profiles as a function of time. (a) 1st fermentation in fed-batch; (b) 2nd fermentation in fed-batch; (c) 3rd fermentation in fed-batch reuse experiments.

the mode of operation used by conventional distilleries. Failure to do so may expose the yeast to a buildup of the product, and this causes a toxic effect on the yeast that involves the inhibition of its growth, enzymatic inactivation, decreased cell viability, and inhibition of various transport systems, such as the amino acid permease and hexoses [18,19].

Phukoetphim et al. [20] used *S. cerevisiae* NP01 yeast in repeated batch fermentations (290 g/L) for five successive cycles. The ethanol concentration was 112 g/L (14.2 °GL), productivity 1.55 g/L·h, and yield 86.10%. Monteiro et al. [21] used *S. cerevisiae* CAT-1 yeast in VHG (30 °Brix) fermentations in fed-batch for six consec-

utive cycles. The alcohol content was 12.02 °GL, productivity was 4.26 g/L·h and yield was 79.76%. Yamakawa et al. [22] used the continuous process in multistage reactors for VHG fermentation with an acid treatment, and cell recycling was quantified for five successive cycles, yielding an alcohol content of 14.6 °GL, yield of 89%, and viability of 67%.

In this present work, for all fermentations, C2/00 yeast was used, which is different from the yeast used in the other works and was without acid treatment. However, Phukoetphim et al. [20] and Yamakama et al. [22] used acid treatment in the cell recycling stage, and this difference probably contributed to the results obtained in this work. The main fact to be highlighted, in relation to the results obtained by the present work and other authors, is the possibility of recycling yeast even with alcohol content above 79 g/L (10 °GL) in VHG fermentations.

CONCLUSIONS

The CCD results indicated that, in a batch process, the maximum ethanol concentration, productivity, and yields are achieved by the optimal values of initial C_{TRSi} at 300 g/L, cell concentration at 26% v/v, and temperature at 27 °C. In addition, the obtained CCD models adequately described the behavior of the alcoholic production. The application of the optimized condition in the fed-batch process showed that high viability of yeast cells is vital for the VHG process to work efficiently. The yeasts used during several fermentation cycles must be intercalated with acid washing for bacterial disinfection.

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SYMBOLS USED

$n_{viablecells}$: number of viable cells [-]
$n_{totalcells}$: total number cells [-]
TRS : total reducing sugar concentration [g/L]
$C_{ethanol}$: fermentation final ethanol concentration [g/L]
C_{TRSi} : initial total reducing sugar concentration [g/L]
Pr : ethanol productivity [g/L·h]
X_1 : temperature [°C]
X_2 : initial concentration of total reducing sugar [g/L]
X_3 : initial reactor cell concentration [%v/v]
Y_1 : ethanol yield [%]
Y_2 : ethanol concentration produced [g/L]
Y_3 : ethanol productivity [g/L·h]
Y_4 : residual sugar concentration [g/L]
t : final fermentation time [h]

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