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Optimization of first generation alcoholic fermentation process with *Saccharomyces cerevisiae*

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ABSTRACT. The influence of variables that affect the process of alcohol fermentation for the optimization of ethanol production is evaluated, with fermentation time, final substrate concentration, cells and ethanol as performance indexes. A statistical planning for process optimization was employed by analyzing three independent variables: temperature, pH and Brix and the influence they have on dependent variables. Brix and pH had a significant effect on fermentation time with a 77% rate by analysis of variance. In the case of concentration of substrate and product, only Brix had a significant effect, with regression above 75 and 87%, respectively. Since the two models are valid at 95% confidence interval since $F_{calculated}$ is greater than $F_{tabulated}$, they may be employed to estimate fermentation time and the concentration of substrate and ethanol.

Keywords: biofuel, ethanol, fermentation processes.

Otimização do processo de fermentação alcoólica de primeira geração utilizando *Saccharomyces cerevisiae*

RESUMO. O objetivo do trabalho foi avaliar a influência de variáveis que afetam o processo de fermentação alcoólica para a otimização da produção de etanol, tomando como índices de desempenho o tempo de fermentação, concentração final de substrato, células e etanol. Foi utilizado um planejamento estatístico para otimização do processo, analisando três variáveis independentes: temperatura, pH e Brix e qual a influência destas sobre as variáveis dependentes. Constatou-se que o pH e o Brix apresentaram efeito significativo sobre o tempo de fermentação com concordância de 77% por meio da análise de variância. Para concentração de substrato e produto, somente o Brix apresentou efeito significativo, com regressão superior a 75 e 87%, respectivamente. Ambos os modelos são válidos em um intervalo de confiança de 95%, pois o $F_{calculado}$ é maior que o $F_{tabelado}$, sendo estes passíveis de serem utilizados para estimar o tempo de fermentação, concentração de substrato e de etanol.

Palavras-chave: biocombustíveis, etanol, processos fermentativos.

Introduction

The uncertainty on the availability of fossil resources in the future and the geopolitical tensions in the oil-producing regions have triggered an increasing global demand for biofuel as a strategy for reducing greenhouse gas emissions (FERNANDES et al., 2014).

Biofuels are all fuels derived from biomass organic materials. When they are burned, they emit carbon dioxide, similar to fossil fuels; however, the released gas is consumed by its raw materials during its cultivation. Since there is a closing of the biological cycle, the use of renewable fuels does not increase the concentration of carbon dioxide in the atmosphere (LOFRANO et al., 2013; SALEMI, 2009).

Ethanol stands out among several renewable fuels as a substitute for fossil fuels since it has proved to be efficient in combating pollutant emissions. This biofuel may be produced from several biomass sources, such as saccharides (sugar cane, beetroot and saccharide sorghum), starch (corn, soybean and grains) and cellulose materials (wood and ligno-cellulose residues) (WU et al., 2010).

Ethanol production has several stages, ranging from the preparation of raw materials to the storage of ethanol. The main stages during its production include milling of the raw material, fermentation and distilling. The alcoholic fermentation of sugars for ethanol production is one of the most important stages in the category of energy consumption and

productivity in alcohol. At this stage, the raw sugars are converted into ethanol by microorganisms under anaerobic conditions (CYTED, 2011; COSTA et al., 2013; GOLDEMBERG, 2008).

Ethanol productivity, the most important product during alcohol fermentation by the microorganism *Saccharomyces cerevisiae*, is associated with cell growth and development. According to Silva et al. (1999), yeast cells are subjected to tensions inherent to the process, caused by environmental conditions and by physical and chemical factors in which the microorganisms are found, such as high or low temperature, salinity, pH and high concentrations of sugars and ethanol.

Pretorius (2001) reports that the microorganisms of *Saccharomyces cerevisiae* are of the yeast type, usually with an ellipsoidal shape. Under appropriate conditions, its biomass doubles every 90 minutes, reproducing asexually through sprouts (mitosis) or sexually by sporulation (meiosis) and crossing.

According to Lima et al. (2001), there are several factors that affect the efficiency of the conversion of sugars into ethanol, ranging between physical (temperature, osmotic pressure), chemical (pH, oxygen, mineral and organic nutrients, inhibitors) and microbiological (species, strain and concentration of yeast, bacterial contamination). These factors may influence the efficiency of alcoholic fermentation and, thus, the efficacy of the conversion of sugar into ethanol.

Since current assay seeks the optimization of bioethanol production based on variables that affect the fermentation process, it evaluates the best time required for the fermentation process, the final substrate concentration (S), cell concentration (X) and the concentration of ethanol (P) during alcoholic fermentation, at different pH, temperature and concentration of sugars, following the statistical design of the Rotational Central Composite Design (RCCD).

Material and methods

Figure 1 shows the procedures of sugar cane harvest, preparation, juice and correction up to the beginning of alcohol fermentation.

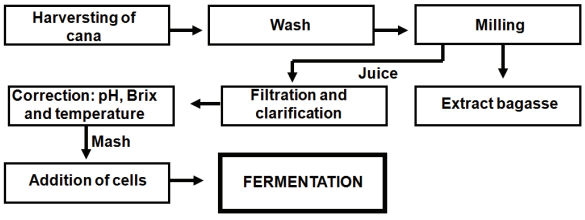

Figure 1. Flowchart of the fermentation process.

After extraction and juice clarification, the sugarcane pH, °Brix and temperature were modified according to the experimental design described in the following sections.

Input variables: concentration of soluble compounds, pH and temperature

The experiments had differentiated °Brix rates (related to concentration of soluble compounds), pH and temperature, and varied according to the statistical design to optimize alcohol fermentation.

The initial amount of soluble compounds was measured by a refractometer and, by diluting with distilled water, the must was adjusted to 8; 9.62; 12; 14.38 and 16 °Brix, through dilution calculation (Equation 1):

$$C_1 \times V_1 = C_2 \times V_2 \quad (1)$$

where C_1 is the initial concentration of soluble solids; V_1 is the initial volume of juice; C_2 is the desired concentration; V_2 is the desired volume.

The pH adjustment to a requested rate was performed after the dilution of the juice, using the solution H_2SO_4 0.1 mol L^{-1} for acidification, or the solution NaOH 0.1 mol L^{-1} for basification. The rates of the initial pH tested in the experiments were 3; 3.81; 5; 6.19 and 7, as established in the experimental design.

Assays were performed in a water bath (SOLAB, SL155/22) and temperature was controlled at 28; 29.62; 32; 34.38 and 36°C.

Process and analyses

After the correction of the juice (must) according to the parameters defined for each assay, 100 mL of adjusted-juice were added in an Erlenmeyer flask and placed in a water bath so that microorganisms would be added later on. The microorganisms were *Saccharomyces cerevisiae* at 1 g L^{-1} concentration.

The monitoring of sugar consumption, ethanol production and cell concentration was performed during the first 6 hours of the experiment, at 2-hour intervals and subsequently at 4-hour intervals, until the attenuation of the fermentation process was achieved.

Determination of the concentration of cells, substrate and product

To determine the concentration of cells, 10 mL of juice were dried in a sterile membrane (diameter = 47 mm and pore size = 0.45 μm) connected to a vacuum filtration system. After drying, the membrane was placed in an electric kiln at 105°C for 24 hours and, later on, in a glass

desiccator for 2 hours. It was weighed on an analytical balance to measure cell mass, following Equations 2, 3, 4 and 5.

Determination of the mass of solids in the microorganism-free sample (Equation 2):

$$m_{white\ solids} = m_{white} - m_{membrane} \quad (2)$$

Determination of the mass of solids in samples with microorganisms and mass of microorganisms in the sample (Equations 3 and 4):

$$m_{total\ solids} = m_{weighted} - m_{membrane} \quad (3)$$

$$m_x = m_{total\ solids} - m_{white\ solid} \quad (4)$$

Once the mass of cells (m_x) has been determined, the following calculation was carried out for its conversion into g L^{-1} (Equation 5):

$$X(g\ L^{-1}) = \frac{m_x}{10\ (mL)} x1000 \quad (5)$$

where: X = concentration of cells; m_x = mass of cells.

Stoichiometric calculations were performed to determine the substrate concentration in g L^{-1}, since degrees Brix represent the percentage of soluble compounds, in mass.

The ethanol content in ºGL (Gay-Lussac) was calculated by Equation 6 from rates obtained by refractometer (CARVALHO et al., 2008).

$$E = \frac{(B_i - B_f) \times 4}{7.4} \quad (6)$$

where: E is the alcohol content in ºGL; B_i is the Initial Brix; B_f is the Final Brix.

Since the degrees Gay-Lussac represented the volume percentage of ethanol in an alcohol/water mixture, necessary stoichiometric calculations were performed for conversion into g L^{-1}.

Rotational central composite design

The influences of three independent variables, temperature, pH and Brix, in the alcohol fermentation process were assessed. In the case of these variables, RCCD was carried out, with a 2^3 full factorial, in eight assays at levels +1 and -1; six assays at levels +1.68 and -1.68, plus a triplicate at the central point 0, totaling 17 experiments, performed at random. The Statistica program (version 10) provided the development of a mathematical model to obtain the response surface, determining optimal conditions, and analysis of variance (ANOVA) to verify the quality of the model adjustment (RODRIGUES; IEMMA 2009).

Results and discussion

The analysis of the different variables identified the best conditions for the fermentation process by varying pH, temperature and concentration of the soluble compounds. Table 1 is a planning matrix, applying the Rotational Central Composite Design (RCCD) where, through the actual and coded data involved in several experiments, the response surface with regard to the specific variables was obtained.

Fermentation time

Data in Table 1 generated the Pareto chart (Figure 2a) to evaluate the influence of temperature, pH and Brix on fermentation time.

Actually Brix and pH had significant effect on total fermentation time, at 5% level of significance (Figure 2a). Thus, a quadratic model for fermentation time was generated, represented by Equation 7, with a concordance greater than 77% (Table 2) by the analysis of variance (ANOVA).

$$t_{fermentation} = 42.3968 - 2.2167 * pH\text{^}2 + 6.1884 * Brix \quad (7)$$

Table 1. Planning matrix (RCCD) with factors (coded and actual) and results for the fermentation time (tf), cell concentration (X), substrate concentration (S) and concentration of product (P).

Number of Assays	Coded Rates			Actual Rates			Response Variables			
	T	pH	Brix	T (ºC)	pH	Brix	Tf (h)	X (g L^{-1})	S (g L^{-1})	P (g L^{-1})
1	-1	-1	-1	29.62	3.81	9.62	32	1.98	31	24.49
2	1	-1	-1	34.38	3.81	9.62	37	0.97	28	28.75
3	-1	1	-1	29.62	6.19	9.62	34	1.26	26	29.60
4	1	1	-1	34.38	6.19	9.62	31	3.82	31	27.49
5	-1	1	1	29.62	6.19	14.38	47	3.97	44	42,08
6	1	-1	1	34.38	3.81	14.38	45.5	3.15	48	42.92
7	-1	1	1	29.62	6.19	14.38	46	3.98	43	42.49
8	1	1	1	34.38	6.19	14.38	45.5	3.49	46	41.23
9	-1.68	0	0	28	5	12	43	3.7	39	31.20
10	+1.68	0	0	36	5	12	37	2.43	48	30.35
11	0	-1.68	0	32	3	12	30.5	4.07	34	36.26
12	0	+1.68	0	32	7	12	43	1.72	47	29.98
13	0	0	-1.68	32	5	8	30.5	3.27	23	25.71
14	0	0	+1.68	32	5	16	51	3.95	51	45.95
15	0	0	0	32	5	12	45	3.41	39	34.15
16	0	0	0	32	5	12	46.5	1.19	46	31.20
17	0	0	0	32	5	12	46	4.05	37	34.99

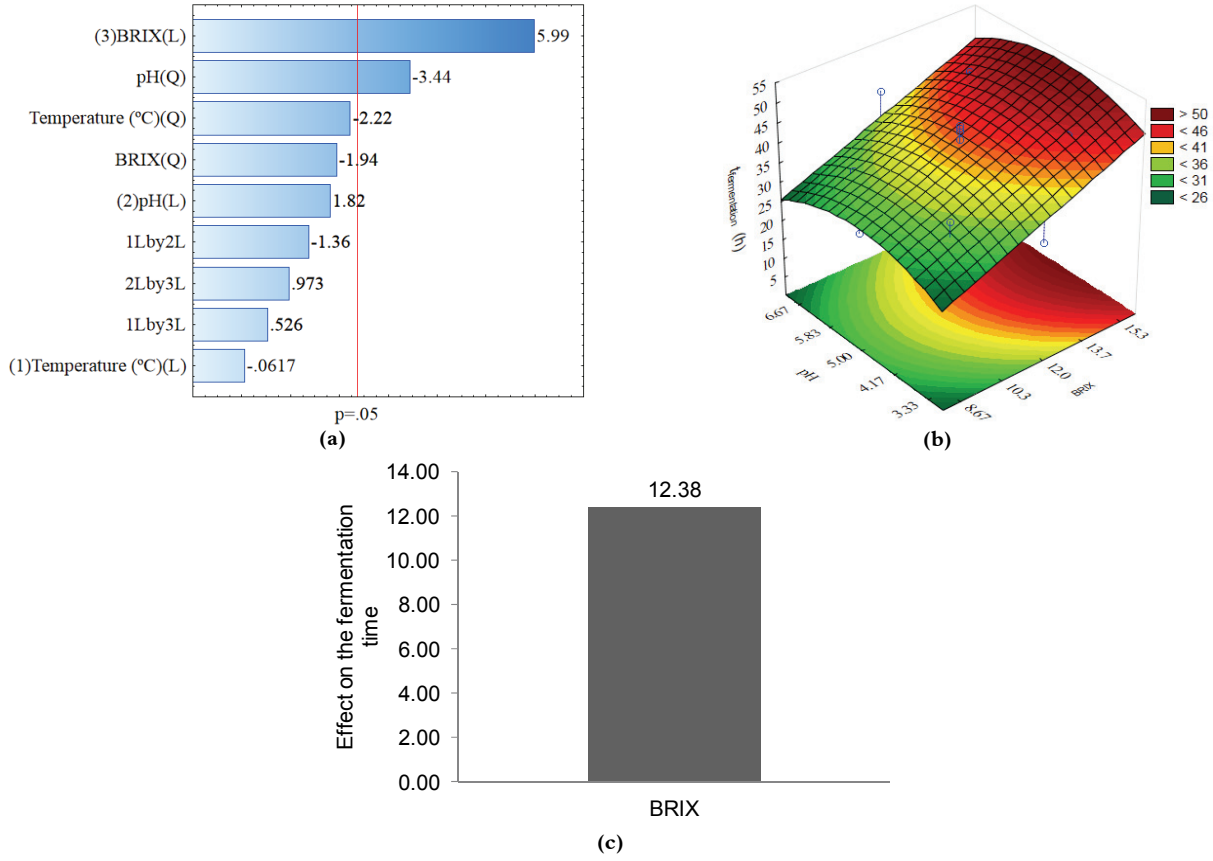

Figure 2. Pareto chart, response surface and analysis of the effect of Brix on the alcohol fermentation time. a) Pareto chart for the alcoholic fermentation time; b) Response surface for the alcoholic fermentation time; c) Analysis of the effect of Brix on the alcohol fermentation time.

Table 2. ANOVA of the quadratic model for the alcoholic fermentation time.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	$F_{calculated}$	$F_{tabulated}$	R^2
Regression (Model)	2	586.3342	293.1671	23.5299	3.7388	0.7707
Residue	14	174.4306	12.4593			
Total	16	760.7647				

In fact, the model is valid at 95% confidence interval, considering that $F_{calculated}$ is greater than $F_{tabulated}$, which makes the model liable to estimate total fermentation time from the response surface. Figure 2b is the response surface obtained for alcohol fermentation time.

According to the response surface, when the fermentation is conducted with 8 °Brix and pH < 4, the fermentation time decreases to less than 26 hours. However, in rates higher than 15 °Brix, at the same pH, process time increases to 46 hours or more. Therefore, increase of Brix of the must to be fermented also causes an increase in fermentation time (Figure 2b).

According to Paschoalini and Alcarde (2009), alcohol fermentation time, alcohol, toxic metabolism, temperature, contaminant bacteria, pH, sugar concentration, type of process and yeast affect the yield of alcohol fermentation.

According to Laluce et al. (2009), maximum production of ethanol in less time proved to be economically relevant to the ethanol industries. However, the above depends on the type of yeast, number of cells, temperature, pH, sugar concentration, nutrient concentration and other factors that influence microbiological activity.

The chart in Figure 2c was constructed to evaluate the effect of Brix changing (independent variable) on the fermentation time and to show this relationship.

Figure 2c reveals that an increase of Brix causes increase of fermentation time. In fact, highly diluted musts ferment quickly (LIMA et al., 2001) and, when highly concentrated, inhibit yeast growth and fermentation activity (LALUCE et al., 2009).

Although the fermentation time could have been minimized by using greater quantities of cells, as reported by Laluce et al. (2009), the

conditions for the metabolism and growth of microorganisms were less favorable at higher cell densities, since the access to nutrients was difficult, with space limitations and cellular interactions (JARZEBSKI et al., 1989).

Concentration of cells

By using the data in Table 1, the Pareto chart evaluated the influence of temperature, pH and Brix on cell concentration (Figure 3). The analysis of this variable is of great importance, since microorganisms cause the oxidation process of sugars and ethanol excretion.

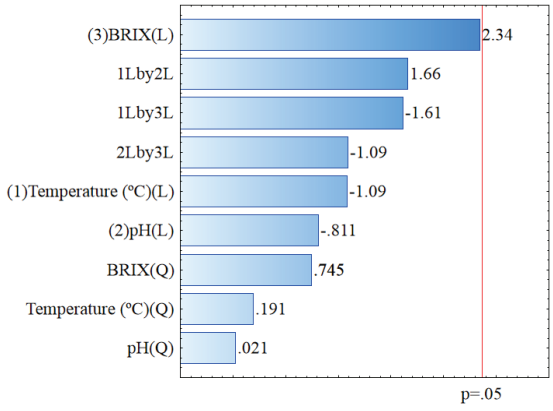

Figure 3. Pareto chart for the concentration of cells.

As Figure 3 shows, there was no significance of tested temperature, pH and Brix ranges on the concentration of cells, since all the initial conditions (Table 1) were close to or at a range tolerated by microorganisms: pH between 4 and 5. It is a characteristic of industrial yeast to tolerate pH close to 7. Further, there is also a reduction in alcohol yield; temperature from 26 to 35°C; Brix from 10 to 18 (LIMA et al., 2001; NAVES et al., 2010).

Concentration of the substrate

Data from Table 1 were basic to generate the Pareto chart (Figure 4a) to evaluate the influence of the independent variables on the concentration of the substrate.

Only Brix had a significant effect on the concentration of the substrate during alcohol fermentation (Figure 4a). Thus, a linear model was generated for the response variable, represented by Equation 8, at 5% significance level (Table 3).

$$= 38.8823 + 8.2112 * \text{Brix} \quad (8)$$

By the analysis of variance, presented in Table 3, the model showed linear regression rates greater than 75%.

Figure 4b reveals the response surface obtained for the concentration of the substrate. The linear model is valid at 95% confidence interval, since $F_{calculated}$ is greater than $F_{tabulated}$ and thus the model may estimate the rate of the concentration of the substrate from the generated response surface.

According to Figure 4b, since the temperature did not influence the concentration of the substrate (Figure 4a), the lower the degrees Brix used in the experiments, the lower was the final substrate concentration, or rather, there was a higher consumption of sugars by microorganisms. The medium with the lowest final substrate concentration (25 g L^{-1}), as observed at the response surface, was assay 13, which initially had 8 °Brix.

The increase of sugar concentration enhances fermentation speed and provides loss of sugar transport activity, with less alcohol. The stress induced by the increase of this variable reduces growth and loss of viability of yeast cells due to disturbances in the osmotic gradient through the plasma membrane (SOUZA et al., 2007). The evaluation of the effect of the independent variable on the substrate concentration (g L-1) in fermentation is given in Figure 4c.

The higher the Brix is, the higher the final substrate concentration (Figure 4c). Osmotic stress on the microorganisms is caused by concentrated musts and loss of unfermented sugars (LIMA et al., 2001).

Concentration of product

Data obtained by the performance of fermentation processes (Table 1) helped generate the Pareto chart to evaluate the influence of temperature, pH and Brix in the final concentration of product (Figure 5a).

Brix showed a significant effect on the concentration of product in the various tests at 5% significance level (Figure 5a). The linear model generated for the response variable, represented by Equation 9, had an 87% regression coefficient by ANOVA (Table 4).

Table 3. ANOVA of linear model of the concentration of the substrate.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	$F_{calculated}$	$F_{tabulated}$	R^2
Regression (Model)	1	919.9813	919.9813	47.2944	4.5431	0.7592
Residue	15	291.7833	19.4522			
Total	16	1211.7647				

Table 4. ANOVA of linear model of concentration of product.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	$F_{calculated}$	$F_{tabulated}$	R^2
Regression (Model)	1	625.7244	625.7244	108.9985	4.5431	0.8790
Residue	15	86.1101	5.7406			
Total	16	711.8344				

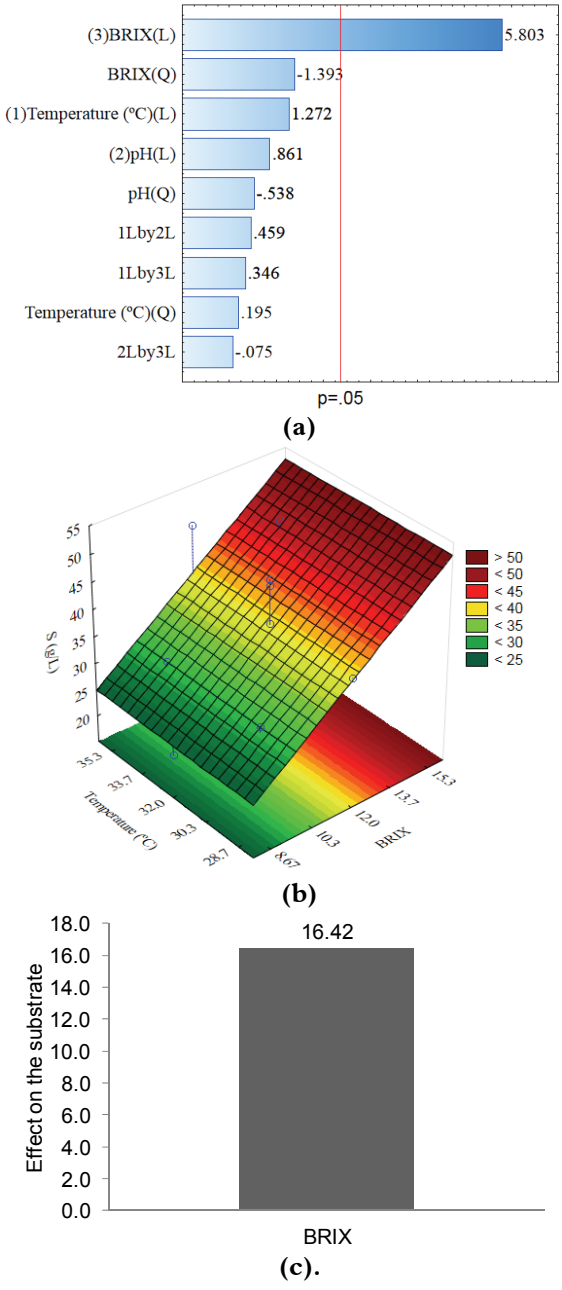

Figure 4. Pareto chart, response surface and analysis of the effect of Brix on the concentration of the substrate. a) Pareto chart for the concentration of the substrate; b) esponse surface for the concentration of the substrate.; c) Analysis of the effects of Brix on the concentration of the substrate

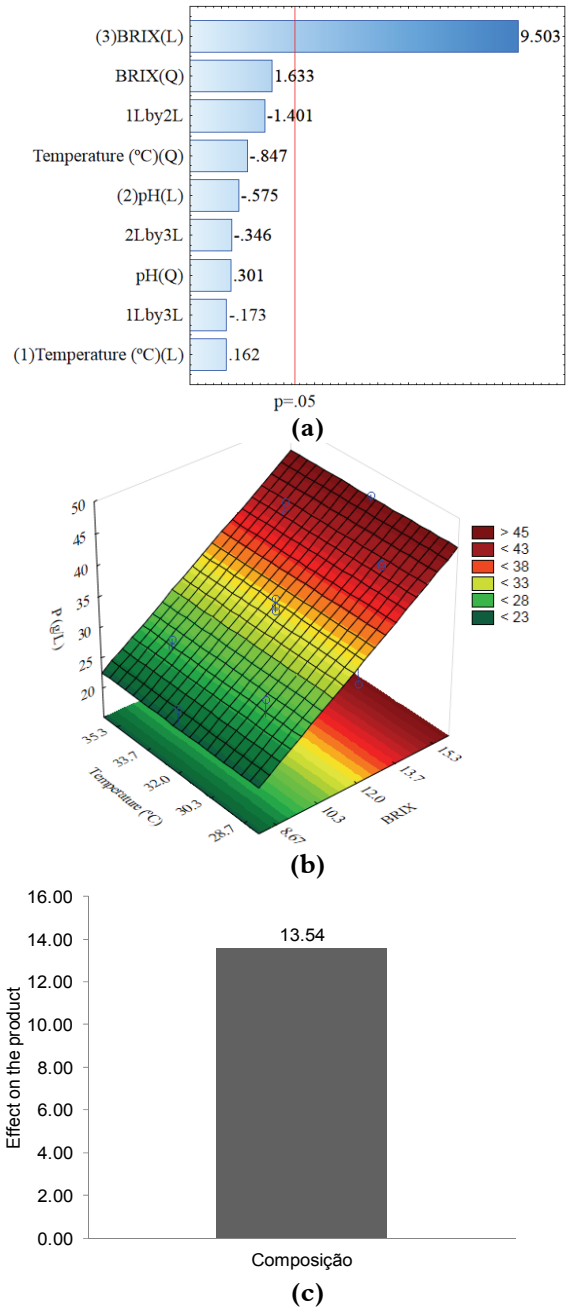

Figure 5. Pareto chart, response surface and analysis of the effect of Brix on the concentration of product. a) Pareto chart for the concentration of product; b) Response surface for the concentration of product; c) Analysis of the effect on the concentration of Brix product.

It has also been observed that the linear model is valid at 95% confidence interval since $F_{calculated}$ is greater than $F_{tabulated}$. Figure 5b shows the response surface obtained for the concentration of product.

Figure 5a reveals that only the degrees Brix were significant on the concentration of product. The concentration of ethanol was greater than 43 and less

than 45 g L^{-1} between 13 and 15 °Brix (Figure 5b). If the goal is to maximize the production of ethanol, the system must operate at higher initial concentrations of sugars, even though care should be taken with regard to the toxicity of the medium to organisms at higher alcohol concentrations. The evaluation of the effect of the independent variable on the concentration of product is shown in Figure 5c.

According to Naves et al.(2010) and Moreira et al. (2008), ethanol acts as an alcohol fermentation inhibitor since it slows the growth of the yeast and reduces the viability and ability of fermentation. The inhibitory effect of the product produced by *S. cerevisiae* microorganisms in the fermentation process is complex and results in the main factor that triggers incomplete fermentation and reduction in process yield.

Figure 5.c demonstrates that when the independent variable is greater, the concentration of product, a dependent variable, will also be higher, since the microorganisms metabolize all the sugar of the medium.

Conclusion

The methodology used for the optimization and monitoring of the kinetics of the alcohol fermentation process was suitable. It was thus possible to determine which was the best experiment performed from the results of each test and statistical analysis. Brix and pH were significant for the fermentation time, while Brix was relevant for the concentration of substrate and product.

For shorter alcohol fermentation time, the best assays were those performed at pH 3 and 5; Brix at 12 and 8; temperature at 32°C (assays 11 and 13) which stabilized the production of ethanol in 30.5 hours.

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