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Study of alcoholic fermentation of an aqueous extract of butiá palm (*Butia eriospatha* (*Martius*) *Beccari*) pulp: Optimization by response surface methodology

Estudo da fermentação alcoólica do extrato aquoso da polpa do butiá (*Butia eriospatha* (*Martius*) *Beccari*): Otimização pela metodologia de superfície de resposta

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Abstract

The fermentation conditions were established and an incomplete Box-Behnken factorial design was applied. The independent variables used were yeast extract, yeast and $NH_4H_2PO_4$, and the alcohol yield obtained was optimized. The validity of the model for predictive purposes was confirmed by analysis of variance; being significant at the 5% level with a coefficient of determination (R^2) of 0.82 and non-significant lack of fit (p = 0.0715). The optimization indicated a formulation containing 3.0 g L^{-1} yeast extract, 6.0 g L^{-1} yeast and 0.30 g L^{-1} $NH_4H_2PO_4$ for an optimum ethanol yield of 77.95%. To validate the predictive equation the experiment was repeated in triplicate under optimum conditions, resulting in an average yield of 78.00%.

Keywords: Butiá. Box-Behnken design. Ethanol.

Resumo

As condições de fermentação foram estabelecidas e um delineamento fatorial incompleto de Box-Behnken foi aplicado. Como variáveis independentes foram utilizadas o extrato de levedura, levedura e $NH_4H_2PO_4$ e o rendimento de álcool obtido foi otimizado. A validade do modelo para fins preditivos foi confirmada pela análise de variância para o qual se mostrou significativo em nível de 5 % apresentando um coeficiente de determinação (R^2) experimental de 82,03 % e desvio da regressão (p=0,0715) não significativo. A otimização indicou uma formulação contendo 3,0 g L^{-1} de extrato de levedura, 6,0 g L^{-1} de levedura e 0,30 g L^{-1} de $NH_4H_2PO_4$ para um rendimento ótimo de produção de etanol de 77,95 %. Para a validação da equação preditiva o experimento foi repetido em triplicata, nas condições ótimas estabelecidas, obtendo-se um valor médio de 78,00 % de rendimento.

Paravras chave: Butiá. Box-Behnken. Etanol

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Introduction

In order to reduce emissions of greenhouse gases and partially replace petroleum, biofuels are increasingly coming into use worldwide, with 50 billion litres of ethanol and 5 billion litres of biodiesel being sold per year (LEITE; LEAL, 2007). This is especially so in Brazil, which is considered a pioneer in production and produces 40 % of the world's ethanol, made almost exclusively from fermenting wine, sugar cane juice or molasses (SILVA et al., 2008).

There is a wide range of different raw materials in addition to sugarcane from which to produce ethanol. Among the alternatives available on a large scale are palm trees that have sugars in their fruits, an essential element in the fermentation process. Palm trees represent one of the largest plant families, occupying almost all habitats. In Brazil, around 119 species are distributed belonging to 39 different genera (DONATTI, 2004).

Among many species, Butia eriospatha (Martius) Beccari, belonging to the family Arecaceae (Palmae), is native to South America (HENDERSON; GALEANO; BERNAL, 1995) and popularly named butiá or butiá-da-serra (REITZ, 1974). Its fruits are globular or oblong, yellow or purplish, 1.8 to 4.2 cm in length, mesocarp fleshy and sweet (NOBLICK, 2010).

According to Franco (1999), 100 g of butiá pulp contain about 11.4 g of carbohydrate, 1.8 g of protein, 1.5 g of fat, 23 mg of calcium, 24 mg of phosphorus, and 40 mg of thiamine and riboflavin. In addition, it is a good source of vitamin C, with 33 mg of this vitamin per 100 g. Maturation generally occurs from November to May, peaking in the summer (ROSA; CASTELLANI; REIS, 1998). Therefore, the concentrated juice is frozen and stored so it can be commercialized throughout the entire year until the next harvest (BÜTTOW; BARBIERI; HEIDEN, 2009).

In ethanol production, yeasts such as Saccharomyces cerevisiae transform the main sugars present in extracted butiá pulp, namely sucrose, fructose and glucose, into ethanol, i.e. they carry out alcoholic fermentation. This process requires some favourable conditions such as optimum temperature and pH (STUPIELLO; HORII, 1981).

The appropriate concentration of nutrients such as nitrogen, phosphorus and magnesium and the amount of yeast added are very important for the production of ethanol. Either insufficient or exaggerated quantities may reflect negatively on the fermentation process and affect the yield (CESAR et al., 1987). To increase efficiency in determining the optimal solution to this problem, various statistical techniques have been proposed, particularly planning and multifactorial approaches; among these, response surface methodology (RSM) has distinguished itself by enabling satisfactory data to be obtained with fewer experiments (ROSA et al., 2011; BOTARO; BORSATO; BATISTUTI, 2011, EGEA et al., 2012). RSM is an efficient tool to optimize the properties of products and is based on simultaneous variations in several factors (independent variables) selected for their influence on the properties of the process (dependent variables or responses). Using mathematical and statistical techniques, the experimental results indicate a combination of factor levels within an optimal region (GIESE et al., 2005; BARBOSA et al., 2010).

The aim was to study the influence of the variables yeast extract, yeast and $NH_4H_2PO_4$ supplementation, in order to optimize ethanol production by discontinuous fermentation of an aqueous extract of butiá pulp using a 3^3 incomplete Box-Behnken factorial.

Materials and Methods

Fruits

The fruits were harvested from native Butia eriospatha (Martius) Beccari plants in January in

the city of Laguna, situated in the Southern part of Santa Catarina State (Brazil). The harvested fruits were frozen and transported to the chemistry laboratory of the State University of Londrina.

Substrate

An aqueous extract of butiá pulp, with a pH of 3.5, was filtered through a cotton cloth filter and its pH adjusted with 1 mol L^{-1} sodium hydroxide solution to 4.5.

Chromatography

Chromatographic analyses were performed with HPLC (high performance liquid chromatography) using a Shimadzu unit, LC-10AD pump, CTO-10A oven, RIF-10A refractive index detector and C-R6A integrator at a flow of 0.6 ml.min^{-1}, oven temperature 353 K and 48 atm. An AMINEX HPX 87C (300 mm x 7.8 mm) carbohydrate column with a mobile phase of ultra pure MILLI-Q water was used. Solutions of glucose, sucrose, fructose and fructooligosaccharides (FOS) were employed as standards.

Sugar determinations

Total sugar was quantified by the phenol sulphuric acid test (DUBOIS et al., 1956) and total reducing sugars by the Nelson - Somogy method (NELSON, 1944).

Yeast

Commercial yeast blocks of *Saccharomyces cerevisiae* (brand Itaiquara) were left to equilibrate at ambient temperature for one hour. The external layer of the blocks was discarded to avoid possible contamination (JONES; PAMMENT; GREENFIELD, 1981). The stabilized yeast was added to the aqueous extract in quantities corresponding to the tests laid down by the RSM.

Nutrient supplementation

Yeast extract was used, with $NH_4H_2PO_4$ as the nitrogen and phosphorus source and $MgSO_4$.7H2O (0.25 g L^{-1}) and $ZnSO_4$ (0.2 g L^{-1}) added to the medium as magnesium and zinc sources, respectively (CORDENUNSI et al., 1985; SILVA, 2006; ZIMMERMAN, 1970).

Experimental Design

The experimental region for each independent variable was chosen through preliminary tests and the specialized literature (SILVA et al., 2008; CORDENUNSI et al., 1985). These were transformed into coded variables (x_1, x_2 and x_3), respectively, in three levels of variation, with the restrictions presented in Table 1.

Table 1 - Independent variables and levels of variation.

Independent variables	Coded variables		
	-1	0	1
X_1=Yeast Extract (g L^{-1})	2.0000	3.0000	4.0000
X_2 = Yeast (g L^{-1})	2.000	6.000	10.000
X_3 = $NH_4H_2PO_4$ (g L^{-1})	0.2000	0.3000	0.4000

Source: author

Fermentation

Pre-sterilized 125 mL Erlenmeyer flasks were used containing the supplemented aqueous extract and yeast at concentrations defined by the mixture design. The flasks were closed with hydrophobic cotton and incubated for eight hours at 303 K in a stabilized oven. After centrifugation and interruption of fermentation the alcohol content was measured.

Determination of alcohol

The alcohol content in g L^{-1} was determined by the Zimmerman method (ZIMMERMAN, 1970) and yield was calculated based on the maximum alcohol content in g L^{-1} that could be obtained from the initial total sugar content.

Mathematical Model

The function used was:

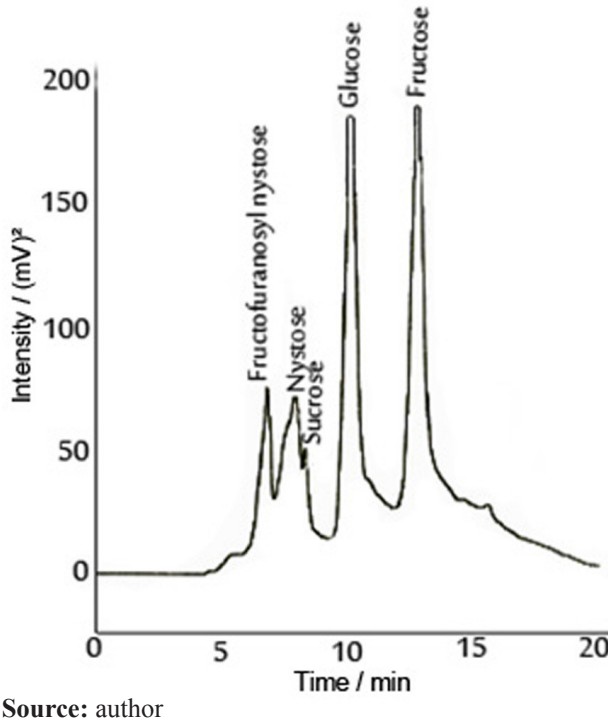
$$Y_n = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \qquad (1)$$

Where Yn represents the response function of the experimental data, x_1, x_2 and x_3 are the coded independent variables corresponding to concentrations of yeast extract, yeast and $NH_4H_2PO_4$, respectively, β are the estimated parameters and ε is the observed error (BREITKREITZ; JARSIM; BRUNS, 2009; STATISTICA…, 2009).

Results and discussion

The pH of the aqueous butia extract was adjusted to 4.5 since, according to Novaes (NOVAES et al., 1974), a pH range between 4.5 and 5.5 is required for optimal yeast growth. Additionally, the total sugar concentration determined by the phenol-sulphuric acid method was 91.2 g L^{-1}. Chromatographic analysis (Figure 1) indicated that the initial extract contained 6.82 g L^{-1} (7.48%) of fructofuranosyl nystose, 11.14 g L^{-1} (12.21%) of nystose, 3.43 g L^{-1} (3.76%) of sucrose, 29.97 g L^{-1} (32.86%) of glucose and 39.84 g L^{-1} (43.69%) of fructose as a carbohydrate source.

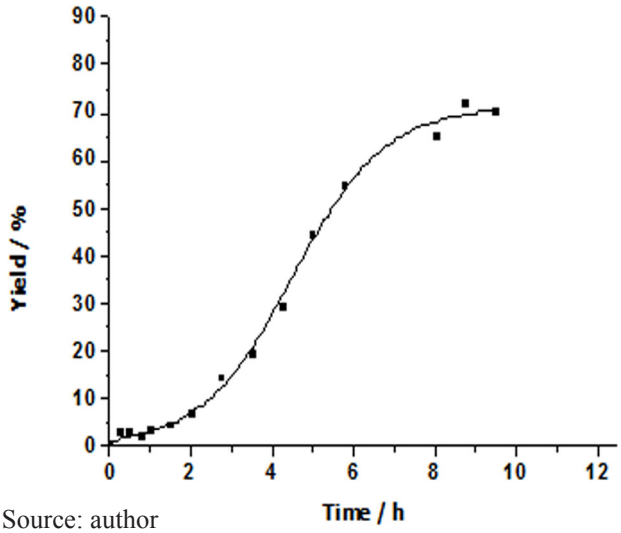
Figure 1 - Chromatogram of aqueous extract of butiá pulp.



Source: author

To determine the fermentation time, preliminary tests with the butiá pulp extract containing 3.0 g L^{-1} of yeast extract, 6.0 g L-1 of yeast, 0.30 g L^{-1} of $NH_4H_2PO_4$, all values at the centre point of the experimental design (Table 1), plus $MgSO_4.7H_2O$ (0.25 g L^{-1}) and ZnSO4 (0.2 g L^{-1}) were performed at 303 K. According to Lima, Aquarone e Borzani (1975), yeast performs over a wide temperature range, but the optimum range for growth is between 299 and 308 K. Figure 2 shows a progressive increase in the ethanol production yield and stabilisation of the response values after 8 hours of fermentation. Assays were performed in a temperature-controlled oven. Significant variations in pH were not observed throughout the whole fermentation process.

Figure 2 - Ethanol production yield, in %, as a function of fermentation time. The points represent the experimental data and the line shows the adjusted data.



Source: author

To optimize the fermentation of aqueous butiá pulp extract, a 3^3 incomplete Box-Behnken factorial totalling 13 experiments was used, with two replicates of the centre point to estimate the error variance (CALADO; MONTGOMERY, 2003).

Table 2 shows the coded independent variables and responses output in g L^{-1} and the yield in

%, expressed as mean values of two replicate fermentation tests employed as well as the centre point replicates.

Table 2 - Coded independent variables, ethanol production (g L^{-1}) and fermentation process yields (%).

Experiments	Coded variables			Ethanol Production (g L^{-1})	Yield (%)
	x_1	x_2	x_3		
1	-1	-1	0	34.39	73.79
2	1	-1	0	25.53	54.795
3	-1	1	0	27.87	59.805
4	1	1	0	30.02	64.42
5	-1	0	-1	28.55	61.275
6	1	0	-1	32.36	69.445
7	-1	0	1	32.16	69.01
8	1	0	1	32.75	70.27
9	0	-1	-1	24.16	51.855
10	0	1	-1	28.85	61.91
11	0	-1	1	24.56	52.705
12	0	1	1	32.45	69.645
13	0	0	0	36.27	77.84
14	0	0	0	37.38	80.22
15	0	0	0	35.32	75.79

Source: author

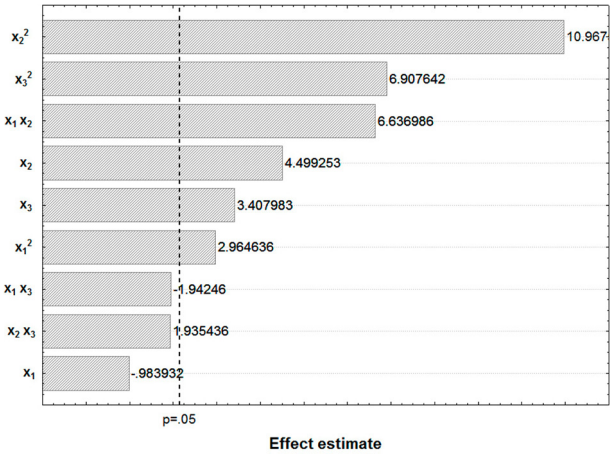
The quadratic model adjusted to alcoholic fermentation yield containing the coded independent variables is represented by equation 2, where regression coefficients were obtained for $\beta = (A'A)^{-1} A'B$, and A is the design matrix containing linear, quadratic and interaction terms while β is the response vector.

In the resulting equation containing only terms significant at the 5% level, Y is the estimated yield, in the coded form, x_1 represents the yeast extract concentration, x_2, the yeast concentration and x_3, the $NH_4H_2PO_4$ concentration. In the complete model, only the linear term of the yeast extract ($p = 0.3418$) and the binary interactions between x_1 and x_3 ($p = 0.07$) and x_2 and x_3 ($p = 0.07$) were insignificant at the 5% level and thus removed from the equation.

$$Y = 77.95 + 2.83\ x_2 + 2.1431x_3 - 3.1381x_1^2 - 11.61\ x_2^2 - 7.31x_3^2 + 5.90\ x_1x_2 \quad (2)$$

The Pareto chart containing all terms (Figure 3) shows the most significant variables and their relative importance in the predictive model obtained. The numbers in front of the rectangles represent the values from the t-test.

Figure 3 - Pareto chart of the variables' significance.



Source: Statistica (2009)

Analysis of variance (Table 3) indicated that the proposed model is significant at the 5% level, and the lack of fit is not significant at the same level of variation.

Table 3 - Analysis of variance in fermentation reaction yield.

	G.L.	S.Q.	Q.M.	F_{calc}	F_{tab}
Regression	9	929.586	103.287	21.01*	19.38
Lack of fit	3	193.757	64.586	13.14(NS)	19.25
Pure error	2	9.831	4.916		
Total	14	1133.174			

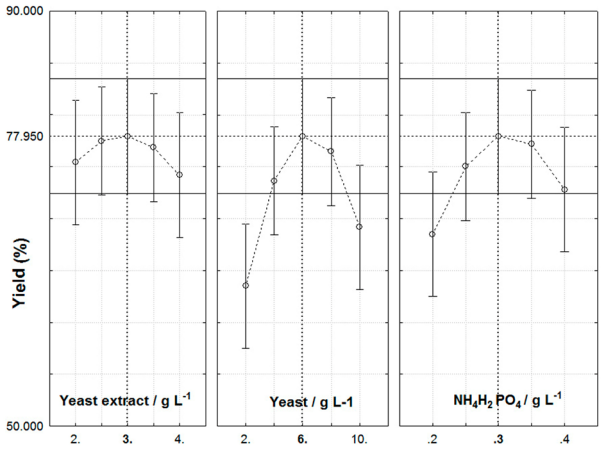
*Significant at the 5% level, (NS) not significant at the 5% level.
Source: author

The determination coefficient observed (R^2) was 82%, which can be considered appropriate. According to Joglekar and May (1987), to obtain a good model fit to the experimental data, the value of R^2 must be higher than 80%. Therefore, there was no significant lack of fit and the R^2 value showed that the equation so obtained,

without no significant terms, may be used for predictive purposes and is suitable for optimization procedures.

Figure 4 shows the variable optimization by Statistica 9.0 (STATISTICA…, 2009). The yield of 77.95% can be obtained using 3.0 g L^{-1} of yeast extract, 6.0 g L^{-1} of yeast and 0.3 g L^{-1} of $NH_4H_2PO_4$.

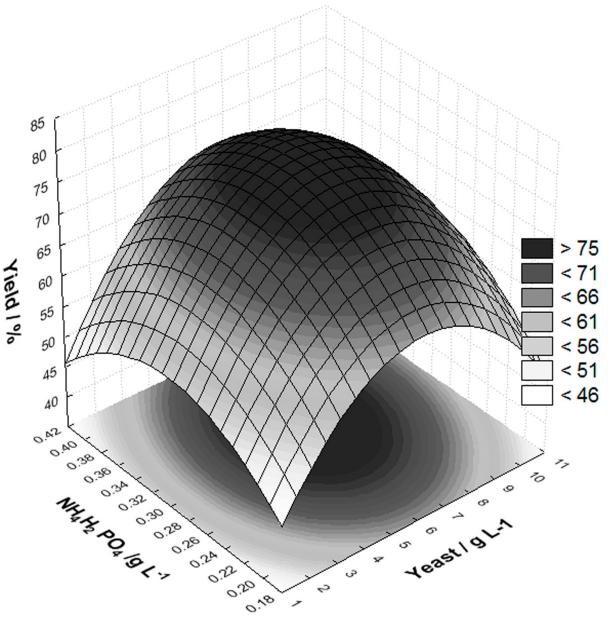
Figure 4 - Optimization of dependent and independent variables by Statistica 9.0.



Source: Statistica (2009)

The response surface of the binary combination region between the original variables $NH_4H_2PO_4$ (g L^{-1}) and yeast content (g L^{-1}) can be observed by the level curves presented in Figure 5. The figure shows the response surface boundary regions for the dependent variable, alcohol yield, obtained from the mathematical model, fixing the variable X_1 at 3.0 g L^{-1} (Figure 3). Based on Figure 5, the optimal region for efficient ethanol production is located near the centre of the experimental design, providing an estimated optimum ethanol yield of about 75%.

Figure 5 - Response surface for alcoholic fermentation yield, in %, by fixing the concentration of the yeast extract at 3.0 g L^{-1}.

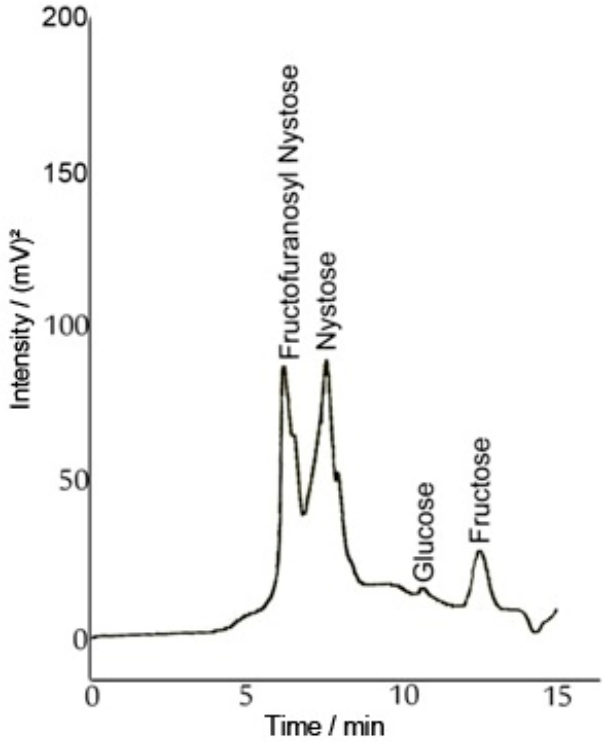


Source: Statistica (2009)

The predictive equation was validated by fermenting the aqueous butiá extract under optimal conditions. The average yield, in triplicate, was 78%. Applying the t-test it was found that there was no significant difference at the 5% level between this average value and that obtained in the optimization 77.95%.

Chromatographic analysis of the fermented product (Figure 6) under optimum conditions showed that FOS present in the extract did not ferment and also detected the presence of fructose (0.75 g L^{-1}), traces of glucose and no sucrose.

Figure 6 - Chromatogram of aqueous extract after fermentation under optimum conditions



Source: author

Conclusions

The aqueous extract of butiá pulp at the concentration utilized was adequate for ethanol production due to its carbohydrate content, with optimal performance in ethanol production reaching a yield of 78%. It can therefore be used as an alternate substrate for ethanol production when applied at a temperature of 303 K, 3.0 g L^{-1} of yeast extract, 6.0 g L^{-1} of yeast and 0.30 g L^{-1} of $NH_4H_2PO_4$.

Modelling using the response surface methodology was efficient and relatively simple as an optimization strategy, which can make it useful in researching and developing fermentation processes.

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