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Optimisation of the alcoholic fermentation of aqueous jerivá pulp extract

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ABSTRACT. The objective of this research is to determinate the optimum conditions for the alcoholic fermentation process of aqueous jerivá pulp extract using the response surface methodology and simplex optimisation technique. The incomplete factorial design 3^3 was applied with the yeast extract, $NH_4H_2PO_4$ and yeast as the independent variables and the alcohol production yield as the response. The regression analysis indicated that the model is predictive, and the simplex optimisation generated a formulation containing 0.35 g L^{-1} yeast extract, 6.33 g L^{-1} yeast and 0.30 g L^{-1} $NH_4H_2PO_4$ for an optimum yield of 85.40% ethanol. To validate the predictive equation, the experiment was carried out in triplicate under optimum conditions, and an average yield of 87.15% was obtained. According to a t-test, no significant difference was observed (on the order of 5%) between the average value obtained and the value indicated by the simplex optimisation technique.

Keywords: simplex optimisation, experimental design, yeast.

Otimização da fermentação alcoólica de extrato aquoso da polpa de jerivá

RESUMO. O objetivo deste trabalho foi determinar as melhores condições do processo de fermentação alcoólica do extrato aquoso da polpa de jerivá, utilizando a metodologia de superfície de resposta e otimização simplex. Foi empregado o delineamento fatorial incompleto 3^3 tendo o extrato de levedura, $NH_4H_2PO_4$ e levedura como variáveis independentes e o rendimento da produção de álcool como resposta. A análise da regressão mostrou que o modelo é preditivo e a otimização simplex indicou uma formulação contendo 0,35 g L^{-1} de extrato de levedura, 6,33 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 85,40%. Para a validação da equação preditiva o experimento foi conduzido em triplicata, nas condições ótimas estabelecidas, obtendo-se um valor médio de 87,15% de rendimento. Aplicando-se o teste t, verificou-se que não houve diferença significativa, em nível de 5%, entre o valor médio obtido e aquele indicado na otimização simplex.

Palavras-chaves: otimização simplex, delineamento experimental, levedura.

Introduction

Brazil is one of the most technologically advanced countries in terms of the production and use of ethanol as a fuel, positioning itself internationally as one of the largest producers and exporters of sugar cane and the largest producer and consumer of alcohol and as the only country to introduce the large-scale use of alcohol as an fuel alternative to petrol. Ethanol has attracted increasing attention from researchers, businesses and governments due to the pricing pressures, the prospects for depleting non-renewable fossil fuel resources and environmental concerns relating to the production of emissions (SILVA et al., 2006, 2008).

In Brazil, ethanol is produced almost exclusively from the fermentation of wine, consisting of sugarcane juice, molasses, or a mixture of the two. As an alternative to ethanol production, researchers are seeking new raw materials with similar yields and viability to sugar cane. Among these alternatives, natural palm trees and their fruits could provide another resource for ethanol production because they contain the carbohydrates or sugars to enable alcoholic fermentation. Palm trees represent one of the largest plant families, not only in diversity but also in abundance, and are found in nearly every habitat. In Brazil, approximately 119 species can be found belonging to 39 different genera (SILVA et al., 2008).

The palm *Syagrus romanzoffiana*, belonging to the *Palmae* family, grows throughout South America (in Paraguay, Argentina and Uruguay) and in the brazilian states: Bahia, Espírito Santo, Minas Gerais, Goiás and Paraná to the Rio Grande do Sul and Mato Grosso do Sul. This palm thrives in various habitats such as forests and subtropical pines, Atlantic, savannas, steppes and coastal marshes, in dirty fields, young secondary forests and in late secondary and mature forests (LORENZI et al., 2010).

Commonly known as jerivá, coqueiro-gerivá, coqueiro, coco-de-cachorro, baba-de-boi or coco-de-babão, the palm grows to a height of 8-15 meter and reproduces is sexually, presenting inflorescence that is branched and can reach 150 cm in length with hundreds of rachis. Its yellow or orange fruit is a globose drupe and is approximately three centimeters in diameter with a thin exocarp and fibrous mesocarp, like a coconut with juicy, sweet flesh that surrounds a single seed (LORENZI et al., 2010). The jerivá fruit contains a great quantity of oils and carbohydrates and according to Jorge and Coimbra (2011), the fruit pulp is composed of approximately 49% carbohydrates.

In ethanol production many factors affect the expected yield including the presence of supplemental nutrients such as nitrogen, phosphorus, zinc and magnesium, the quantity of yeast added, and the fermentation conditions used to make the alcohol (BARBOSA et al., 2012; GARDE-CERDÁN, et al., 2011; SILVA et al., 2008).

To produce ethanol, the sugars found in the aqueous extract of jerivá pulp must undergo a chemical transformation called fermentation. This process involves the action of micro-organisms called yeasts, which transform the sugars in the pulp extract such as sucrose, fructose and glucose into ethanol; however, conditions, such as temperature and pH, must be favourable for this to occur. Current, the micro-organism favoured as a fermentation agent is a yeast-like fungi called *Saccharomyces cerevisiae* (MENDES-FERREIRA et al., 2010).

Optimising the fermentation process by selecting a set of optimal factors at various stages of the process is a difficult task that requires many experiments. To increase the efficiency of finding the optimal factors, various statistical techniques have been proposed, particularly factorial designs. Among these techniques, response surface methodology (RSM) has been used by a significant number of researchers (AGUIRRE-GANZÁLES et al., 2011; BARBOSA et al., 2010; SILVA et al., 2008).

The RSM allows optimised mathematical models to be developed that can improve the quality of process; however, to improve production and optimise a fermentation process, to either increase yield or productivity, experimental studies must be conducted to observe the influence of the variables on the process (SILVA et al., 2008).

The objective of this research was to study the influence of the following variables: yield, supplementation ($NH_4H_2PO_4$) and yeast extract in the optimisation of ethanol production through the discontinuous alcoholic fermentation of aqueous jerivá pulp extract, combining the response surface methodology with a super-modified simplex method.

Material and methods

Fruits

The jerivá fruits were collected in Ponta Grossa city (located at 950 m altitude), in Paraná State, on the campus of the State University of Ponta Grossa (UEPG).

Jerivá aqueous extract preparation

Once harvested, the fruits were pulped in a ratio of 10 kg fruit mixed with 10 kg water per batch. The pulping device consisted of a 0.55-kW propeller-driven motor simulating a low-speed blender. After the fruits were pulped, the pulp was separated from the endocarp using a sieve.

Substrate

The aqueous jerivá pulp extract, pH 4.8, was filtered through cotton cloth.

Yeast

Commercial yeast blocks containing *S. cerevisiae* (ITAIQUARA brand) were kept at equilibrium at room temperature for one hour. The external layer of the blocks was discarded to avoid contamination. The stabilised yeast (2, 6, and 10 g L^{-1}) was added to the medium.

Supplemental nutrients

A yeast extract was used in concentrations of 1.5, 2.5 and 3.5 g L^{-1}, and $NH_4H_2PO_4$ (0.3, 0.35, and 0.4 g L^{-1}) was added as a phosphorous and nitrogen source. $MgSO_4{\cdot}7H_2O$ (0.25 g L^{-1}) and $ZnSO_4$ (0.2 g L^{-1}) were added to the media as a magnesium and zinc source, respectively (SILVA et al., 2006).

Experimental design

To optimise the alcoholic fermentation conditions for aqueous jerivá pulp extract, the Box-Behnken incomplete factorial design (3^3) was applied. Each independent variable (X_1, X_2, and X_3) was transformed into coded variables (x_1, x_2, and x_3), respectively. Thirteen experiments were performed, with the central point being repeated twice, resulting in 15 experiments. The independent variables-yeast extract (X_1), $NH_4H_2PO_4$ (X_2) and yeast (X_3)-were analysed in three equidistant variation levels and coded to -1, 0 and +1, with the reaction yield chosen as the response variable.

Fermentation

Pre-sterilised test tubes (20 × 150 mm) containing the supplemented medium were used. The fermentation occurred the tubes with 2, 6, and 10 g L^{-1} inoculate yeast. The tubes were sealed with hydrophobic cotton and incubated for eight hours at 30°C, stabilised on a stove (SILVA et al., 2008). After centrifugation and interruption of the fermentation, the alcohol content was determined.

Chromatography

The obtained samples were analysed via high-pressure liquid chromatography (HPLC) in a SHIMATZU chromatograph with an LC-10AD pump, CTO-10A oven, RID-10A refraction index detector and an C-R6A integrator in a 0.6-mL·min^{-1} flux at an oven temperature of 80°C and 48 atm pressure. The column used for the carbohydrates was an AMINEX HPX 87C (300 mm × 7.8 mm), and the mobile phase was ultra purified MILLI-Q water. Glucose, sucrose, fructose and fructo-oligosaccharide (FOS) standards were used.

Total sugar determination

The phenol-sulfuric method was used to determine the total sugar content (HALL, 2013).

Alcohol content determination

The alcohol content, in g L^{-1}, was determined using the Zimmerman (1963) method and the yield was determined according to maximum alcohol content, in g L^{-1}, obtained from the initial total sugar content.

Yield

Maximum alcohol content was estimated on total sugar and Gay-Lussac equation for alcoholic fermentation, in which 1 mol of glucose (180 g) produced 2 moL of ethanol (92 g), 2 moL of carbon dioxide (CO_2) (88 g) and 238.26 kJ of energy (SILVA et al., 2006). Yield was determined according to the expression: Yield (%) = (obtained alcohol content (g L^{-1}) x 100) / maximum alcohol content (g L^{-1}).

Simplex optimisation

Optimisation was performed by combining the regression equation from response surface methodology with the super-modified simplex method (BONA et al., 2000).

Results and discussion

According to Köppen climatic classification, the region climate (where the fruits were collected) is pointed as temperate (Cfb), with average temperature lower than 18°C in the colder month (mesotermic), with fresh summer; average temperature in the hotter month lower than 22°C, and no defined dry season.

The obtained aqueous extract exhibited a pH of 4.8 with optimal yeast growth occurring at pH values between 4.5 and 5.5 and a total sugar concentration of 60 g L^{-1}. The chromatographic analysis indicated that the extract contained 30.6 g L^{-1} glucose, 22.0 g L^{-1} fructose and 7.3 g L^{-1} fructo-oligosaccharides (FOS) as the carbohydrate sources, with a notable absence of sucrose.

Preliminary tests with jerivá pulp extract containing 2.5 g L^{-1} yeast extract, 6.0 g L^{-1} yeast, 0.35 g L^{-1} $NH_4H_2PO_4$, with a set design central point, $MgSO_4 \cdot 7\ H_2O$ (0.25 g L^{-1}) and $ZnSO_4$ (0.2 g L^{-1}) were performed to determine the fermentation time at 30°C. Figure 1 shows a progressive increase in the ethanol production yield and a stabilisation of the response values after 6 hours of fermentation. No significant variation in pH was observed throughout the fermentation process.

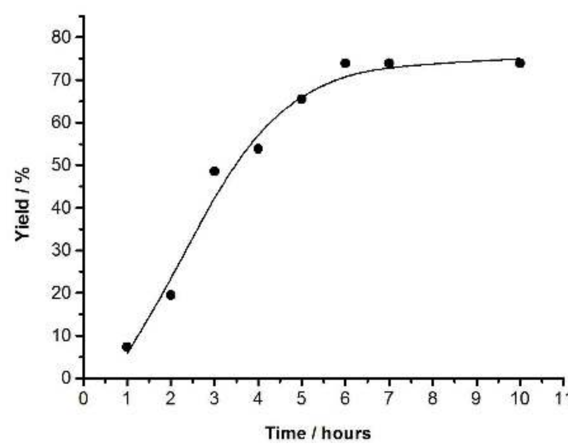

Figure 1. Ethanol production yield, expressed as a percentage, as a function of fermentation time. The line was adjusted and the points represent the experimental data.

To optimise the conditions for the fermentation of jerivá pulp extract, the response surface methodology (RSM) was applied using the incomplete factorial design 3^3. The experiment consisted of 15 trials, with two replications at the central point to estimate the error variance (CALADO; MONTGOMERY, 2003). The experimental region, with the minimum and maximum limits for each independent variable, was chosen through preliminary trials and the use of literature data (SILVA et al., 2008).

In the environment, yeasts thrive over a wide temperature range, but their range of optimum growth is between 26 and 35°C with an average of 30°C (GALANAKIS et al., 2012). Based on this information, the temperature used in the experimental trials was 30°C, controlled in an oven, and the fermentation time was 8 hours based on the data obtained in Figure 1.

Table 1 shows the coded independent variables, the levels of variation in the original values, the amount of alcohol produced and yield responses, expressed as average values of two replications of each fermentation test.

Table 1. Variation level, coded independent variables (x), originals (X) and alcohol yields (Y) obtained from the aqueous jerivá pulp extract.

Experiments	Coded variables		Alcohol Production ($g\ L^{-1}$)	Yield (%)
	x_1 x_2	x_3		Y
1	-1 -1	0	22.17	72.3
2	1 -1	0	16.31	53.2
3	-1 1	0	17.23	56.2
4	1 1	0	19.97	65.1
5	-1 0	-1	19.02	62
6	1 0	-1	26.71	87.1
7	-1 0	1	23.52	76.7
8	1 0	1	20.27	66.1
9	0 -1	-1	19.17	62.5
10	0 1	-1	15.89	51.8
11	0 -1	1	15.21	49.6
12	0 1	1	14.91	48.6
13	0 0	0	22.39	73
14	0 0	0	22.97	74.9
15	0 0	0	22.30	72.7

Independent Variables	Coded Levels		
	-1	0	1
X_1 = Yeast extract ($g\ L^{-1}$)	1.5	2.5	3.5
X_2 = Yeast ($g\ L^{-1}$)	2	6	10
X_3 = $NH_4H_2PO_4$ ($g\ L^{-1}$)	0.3	0.35	0.4

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

$$Y = 73.53 - 1.99x_2 - 2.8x_3 + 4.0x_1^2 - 15.84x_2^2 - -4.57x_3^2 + 7.00x_1x_2 - 8.92x_1x_3 + 2.43x_2x_3 \quad (1)$$

In the above equation, Y represents the estimated reaction yield and, in the coded form, x_1 represents the yeast extract concentration, x_2, the yeast concentration and x_3, the concentration of $NH_4H_2PO_4$. In the complete model, only the linear term of the yeast extract (p = 33.06%) was not significant at the 5% level and, therefore, removed from the equation.

The analysis of variance (Table 2), without the linear term of the yeast extract concentration, indicates that the proposed model was significant at the 5% level, and no significant lack of fit was found in the same level of variation.

Table 2. Analysis of variance for alcohol production obtained from aqueous jerivá pulp using the incomplete factorial design 3^3.

Variation source	G.L.	Square Sum	Square Medium	F_{calc}	F_{tab}
Regression	8	1695.314	211.9143	148.87*	19.37
Linear	2	94.321	47.1605	33.13*	19.00
Quadratic	3	1062.94	354.3133	248.90*	19.16
Interaction	3	538.146	179.382	126.01*	19.16
Lack of fit	4	97.609	24.4022	17.14(NS)	19.25
Error	2	2.847	1.4235		
Total	14	1795.912			

*Significant the 5% level; (NS)Not significant at the 5% level.

The overall observed coefficient of determination (R^2) and the percentage of variance provided by the model were 94.59 and 85.0%, respectively, which are appropriate because, in a model well fitted to the experimental data, the value of R^2 should be greater than 80% (BISHT et al., 2013). Therefore, the no significant lack of fit and the high value of R^2 indicate that the obtained equation, without the linear term of the concentration of yeast extract, can be used to predictive purposes and suitable for optimisation procedures.

The binary combination region between the original variables $NH_4H_2PO_4$ ($g\ L^{-1}$) and yeast ($g\ L^{-1}$) content can be observed through the level curves depicted in Figure 2. It was obtained using MATLAB® software (MATSUMOTO, 2006) and shows the contour regions of the response surface for the dependent variable, alcohol production yield, obtained from the mathematical model with the statistically less important variable X_1 fixed at 0.35 $g\ L^{-1}$. Figure 2 indicates that the optimum region for ethanol production (at an estimated ethanol yield near 85%) is situated near the minimum point for the $NH_4H_2PO_4$ variable and at the central point for the yeast concentration.

The predictive equation was optimised using the sequential simplex method (BONA et al., 2000). The upper and lower limits applied to the simplex optimisation were same as those used for the experimental design (Table 1). The progressive increase in the ethanol production yield toward the optimal response, the convergence and the stabilisation of the response values can be seen in Figure 3. A maximum yield of 85.40% was found at the vertex 13. The figure indicates that the yield of the reaction quickly reaches its optimum value after the first six simplex calculations. The convergence (the stopping criterion) was achieved because of the response function to the reaction yield and because the independent variables failed to grow at a rate less than 10^{-3}, which were used by the simplex method as the convergence criteria.

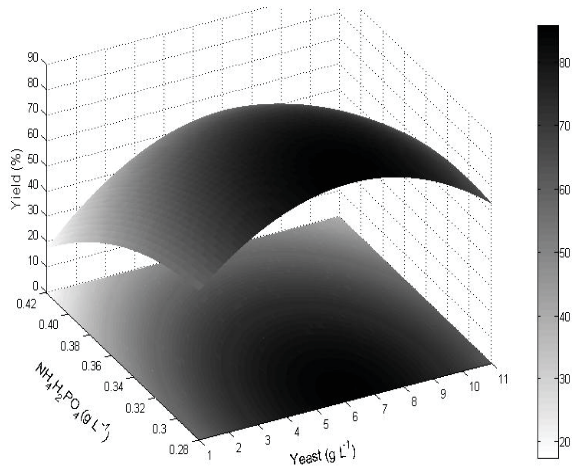

Figure 2. Response surface of the MATLAB software for alcoholic fermentation yield; the yeast extract concentration was fixed at 0.35 g L^{-1}.

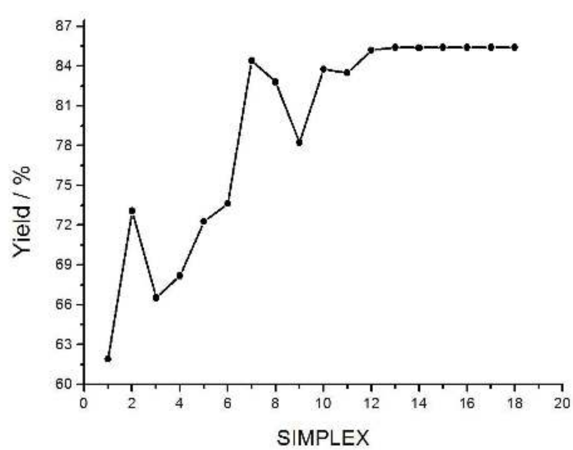

Figure 3. Ethanol yield as a function of the simplex stabilisation.

In Figures 4, 5, and 6 the stabilisation of the independent variables can be observed. The maximum ethanol (85.40%) yield was obtained from simplex 14, as the upper limit of the yeast extract (0.35 g L^{-1}), a value near the middle of the yeast (6.33 g L^{-1}) and the lower limit of the $NH_4H_2PO_4$ (0.30 g L^{-1}).

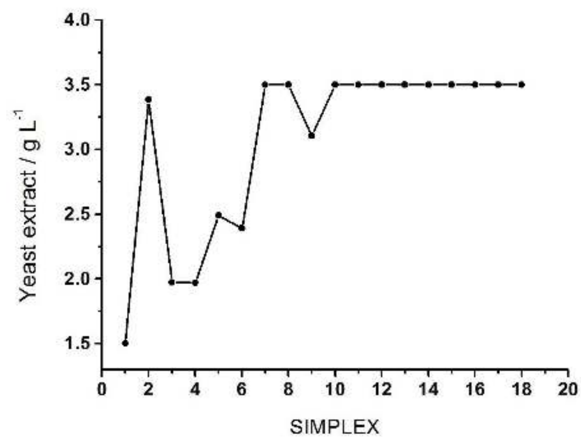

Figure 4. The independent variable $NH_4H_2PO_4$ as a function of the simplex stabilisation in the optimisation of the yield in the alcoholic fermentation process.

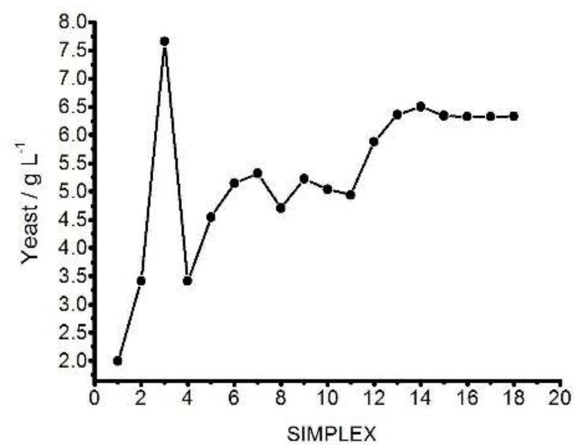

Figure 5. The yeast extract independent variable as a function of the simplex stabilisation in the optimisation of the yield in the alcoholic fermentation process.

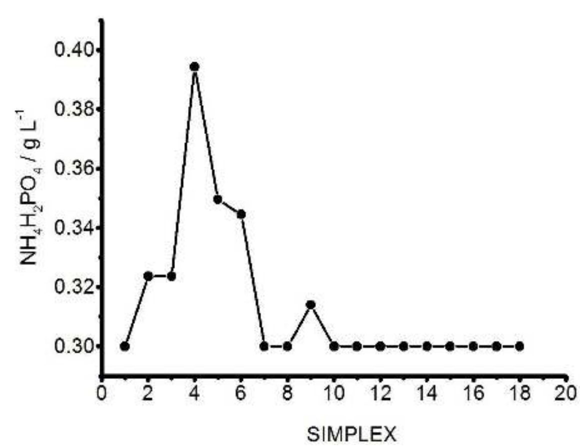

Figure 6. The yeast independent variable as a function of the simplex stabilisation in the optimisation of the yield in the alcoholic fermentation process.

The predictive equation was validated by fermenting the aqueous jerivá extract at 30°C, for 8 hours, under the optimal conditions. The average yield of the experiment, performed in triplicate, was

87.15%. Applying the t-test, no significant difference was found at the 5% level between the average value of 87.15% and that obtained using the simplex optimisation, which was 85.4%.

The presence of 7.3 g L^{-1} FOS in the aqueous extract may have contributed to a lower yield because this type of carbohydrate is not completely fermented by *S. cerevisiae*. A fermentation test with only 10 g L^{-1} FOS, yeast and the supplements yielded a concentration of 4.87 g L^{-1} ethanol under the presented optimised conditions after 8 hours of incubation.

The yield of 87.15% is lower than that typically found in the discontinuous or semi-continuous fermentation of alcohol when using a solution consisting of sugar cane juice, molasses, or a mixture of the two (SILVA et al., 2006). Silva et al. (2008) optimised the fermentation of grape cane using the response surface methodology as a statistical tool and obtained, through discontinuous fermentation, a yield of 88%, which is close to the value obtained in experiment 6 (Table 1) using 3.5 g L^{-1} yeast extract, 6 g L^{-1} yeast and 0.3 g L^{-1} $NH_4H_2PO_4$. However, these comparisons must be treated with caution as the employed media are not the same. In addition, a distillation assay of the material-fermented under the optimum conditions established by the simplex method-using a 60-cm-long Vigreux column yielded an aqueous solution containing 56% ethyl alcohol.

Conclusion

The model using the response surface combined with the simplex method is an efficient and relatively simple optimisation strategy and can be considered useful in the research and development of alcoholic fermentation processes.

The aqueous jerivá extract proved to have adequate potential for ethanol production due to its ability to produce large quantities of carbohydrates. Thus the aqueous jerivá extract can be used as an alternative to cane sugar.

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