

Article

## Application of the Super-modified Simplex Optimization Method in the Fermentation of Butiá Palm (*Butia eriospatha* (Martius) Beccari) Aqueous Extract

Caroline de Souza Cardoso, Kelly Roberta Spacino, Elisângela Tavares da Silva, Karina Gomes Angilelli, Olívio Fernandes Galão, Ivanira Moreira, Dionisio Borsato\*

State University of Londrina, Chemistry Department, Fuels Analyses and Research Laboratory, P.O. BOX 10.011, 86.057-970, Londrina, PR, Brazil

\* Author to whom correspondence should be addressed to; E-Mail: [dborsato@uel.br](mailto:dborsato@uel.br), Tel.: +55 43 33714878

Article history: Received 2 May 2014, Received in revised form 22 May 2014, Accepted 27 May 2014, Published 4 June 2014.

**Abstract:** The aim of this study was to determine the best conditions for alcoholic fermentation of butiá pulp aqueous extract using experimental mixtures with restrictions and simplex optimization. Yeast concentration, yeast extract concentration, and  $\text{NH}_4\text{H}_2\text{PO}_4$  concentration were used as the independent variables, with the ethanol production yield as the response. The validity of the model for predictive purposes was confirmed by analysis of variance, it was significant ( $p = 0.001$ ), presenting an experimental coefficient of determination ( $R^2$ ) of 0.97, adjusted  $R^2$  of 0.89, and non-significant lack of fit ( $p = 0.1082$ ). The simplex optimization indicated a formulation containing  $4.0 \text{ g L}^{-1}$  yeast extract,  $5.65 \text{ g L}^{-1}$  yeast and  $0.35 \text{ g L}^{-1}$   $\text{NH}_4\text{H}_2\text{PO}_4$  for the optimum yield of ethanol production (66.75%). In order to validate the predictive equation, the assay was repeated in triplicate under the optimum conditions, resulting in an average yield of 68.29%. Applying the t-test, it was found that there was no significant difference ( $p = 0.4552$ ) between the mean value and that obtained by simplex optimization.

**Keywords:** alcoholic fermentation, experimental design, simplex optimization.

## 1. Introduction

As a pioneer in biofuel production, Brazil accounts for 40% of the world's ethanol fuel. There are currently over 400 distilleries with an annual production of about 22 million m<sup>3</sup> (Mapa, 2012) produced almost exclusively from the fermentation of worts constituted by sugar cane juice or molasses, or a mixture of both elements (Silva et al., 2008).

Different raw materials have been investigated as alternatives to ethanol production, aiming to reach a yield and viability similar to sugar cane. One of these alternatives is palm trees, since the fruits have an important quality for ethanol production, i.e. the presence carbohydrates or sugars that enable fermentation. Palm trees represent one of the largest existing plant families in abundance, occupying almost all habitats. In Brazil, they include around 119 species, and belonging to 39 different genera (Lorenzi and Souza, 1996).

*Butia eriospatha* (Martius) Beccari belongs to the family Arecaceae (= Palmae), native to South America (Henderson et al., 1995), and is popularly known as butiá or butiá-da-serra (Reitz, 1974). According Lorenzi et al. (2004), *Butia* is a small genus of about eight species in South America, of which seven occur endemically and naturally in Brazil, in the states of Paraná, Santa Catarina and Rio Grande do Sul (Reitz, 1974). This plant produces globose and succulent fruits, 1.7 to 1.9 cm in average diameter, which transform into a yellowish epicarp at maturity (Henderson et al., 1995). The butiá palm bears fruit once a year, flowering from October to December and fruiting from January to April. There are rare cases of flowering in June and fruiting in July (Büttow, 2009). The concentrated juice can be frozen and stored and can be industrialized throughout the year until the next harvest (Büttow, 2009).

In the ethanol production process, several factors affect the yield, including the presence of nutrients such as nitrogen, phosphorus and magnesium, the amount of yeast added, and the treatment conditions of fermentation (Cesar et al., 1987). In order for ethanol to be produced, it is necessary that the sugars found in the aqueous extract of butiá pulp undergo a chemical transformation called alcoholic fermentation. This process involves the action of micro-organisms that turn the main sugars available in the pulp extract like sucrose, fructose and glucose into ethanol. Favorable conditions are required for this to occur, in particular regarding the temperature and pH. Currently, the most commonly used fermenting microorganism is a yeast-like fungus, *Saccharomyces cerevisiae* (Stupiello, 1985).

Galvan et al. (2013) showed that system analysis can be performed theoretically by formulating mathematical models based on physical fundamentals and biological phenomena that occur empirically, by observing responses to various external stimuli. Although these theoretical models

present advantages, the relative lack of knowledge of biological system behavior makes development difficult. In these cases, empirical models should be the preferred alternative (Barbosa et al., 2010).

Experimental design and sequential analysis are fundamental requirements to obtain satisfactory data with fewer experimental trials. To increase the efficiency of finding the optimal solution to a problem, various statistical techniques have been proposed, especially multifactorial design (Berté et al, 2013). Among these, mixture designs have been used by a significant number of researchers (Borsato et al., 2010, Maia et al., 2011, Cini et al., 2013).

The mixture design methodology allows for developing mathematical models based on practical experiences that have already been carried out, which provides cost minimization and improved process quality (Hill and Hunter, 1966). In these designs, two or more components are mixed in various proportions and the characteristics of the resulting products are recorded. Responses are independent of the physical state, depending only on the ingredient proportions present in the mixtures (Cornell and Deng, 1982, Breitzkreitz et al., 2009, Galvan et al., 2013).

This study aimed to assess the influence of some variables, including the yeast extract, yeast content, and  $\text{NH}_4\text{H}_2\text{PO}_4$  supplementation, in the optimization of ethanol production by discontinuous fermentation of a butiá pulp aqueous extract by combining experimental mixtures with the supermodified-simplex optimization method.

## **2. Materials and Methods**

### *2.1. Fruits*

The fruits were harvested from native plants of *Butia eriospatha* (Martius) Beccari, in February in the city of Laguna, situated in the south of Santa Catarina State (Brazil) with diverse vegetation typical of the Atlantic Forest. The average elevation is 4 meters and the average annual temperature is 19.5°C. According to Rosa et al. (1998), maturation usually occurs from November to May, peaking in the summer, in the month of February.

The harvested fruits were frozen and transported to the chemistry laboratory at the State University of Londrina.

### *2.2. Butiá aqueous Extract Preparation*

Once harvested, the fruits were pulped in a low rotation liquefier in a proportion of 200 g butiá to 50 mL of distilled water. After the fruits were depulped, the pulp was sieved to separate it from the endocarp.

### 2.3. Substrate

The pulp of butiá aqueous extract, pH 3.5, was filtered through a cotton cloth filter and the pH was adjusted with 1 M sodium hydroxide to 4.5.

### 2.4. Chromatography

Chromatographic analysis were performed by high performance liquid chromatography (HPLC) on a Shimadzu apparatus with a LC-10AD pump, a CTO-10A oven, an RID-10A refractive index detector, and a C-R6A integrator at a flow rate of 0.6 mL.min<sup>-1</sup>. The oven temperature was 80°C and the pressure was 48 atm. An AMINEX HPX 87C carbohydrate column (300 mm x 7.8 mm) was used with ultra-pure MILLI-Q water as the mobile phase. Standard solutions of glucose, sucrose, fructose and fructooligosaccharides (FOS) were employed.

### 2.5. Determination of Sugar

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

### 2.6. Yeast

Commercial yeast blocks of *Saccharomyces cerevisiae* (brand Itaiquara) were left in equilibrium at ambient temperature for 1 h. The external layer of the block was discarded to avoid possible contamination (Jones, 1981). The stabilized yeast was added to the aqueous extract in quantities corresponding to the tests set by the mixture design with restrictions.

### 2.7. Nutrient Supplementation

Was used yeast extract and NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> as nitrogen and phosphorus source and MgSO<sub>4</sub>.7H<sub>2</sub>O (0.25 g L<sup>-1</sup>) and ZnSO<sub>4</sub> (0.2 g L<sup>-1</sup>) were added to the medium as magnesium and zinc source, respectively (Cruz and Borzani, 1980, Cordenunsi et al., 1985, Silva et al., 2006).

### 2.8. Fermentation

Pre-sterilized 125 mL Erlenmeyer flasks were filled with the aqueous extract supplemented and yeast at concentrations defined by the mixture design. The flasks were sealed with hydrophobic cotton and incubated for eight hours at 30°C in a stabilized oven. After centrifugation and the interruption of fermentation, the alcohol content was measured.

### 2.9. Determination of Alcohol

The alcohol content in  $\text{g L}^{-1}$  was determined by the Zimmerman method (1970) and the yield was calculated based on the maximum alcohol content in  $\text{g L}^{-1}$  which would be obtained from the initial total sugar content.

### 2.10. Experimental Design

To optimize the conditions for butiá aqueous extract fermentation, a simplex mixture design was used with restrictions. Nine assays were performed in triplicate resulting in a total of 27 experiments. Table 1 shows the restrictions used to establish the mixture design.

**Table 1.** Lower and upper limit to original variables used to establish the experimental mixture design.

	Lower limit ( $\text{g L}^{-1}$ )	Upper limit ( $\text{g L}^{-1}$ )
Yeast extract	2	4
Yeast	2	10
$\text{NH}_4\text{H}_2\text{PO}_4$	0.25	0.35

### 2.11. Mathematical Model

The function used was:

$$Y_n(x) = \sum_{1 \leq i \leq q} \beta_i x_i + \sum_{1 \leq i < j \leq q} \beta_{ij} x_i x_j + \beta_{123} x_1 x_2 x_3 \quad (1)$$

where  $Y_n$  represents the response function of the experimental data,  $x_1$ ,  $x_2$  and  $x_3$  are the coded independent variables corresponding to the concentrations of yeast extract, yeast and  $\text{NH}_4\text{H}_2\text{PO}_4$ , respectively, and  $\beta$  represents the estimated parameters (Breitkreitz et al., 2009, Statistica, 2009).

### 2.12. Statistical Analysis

The regression coefficients and analysis of variance were obtained using the software Statistica V.9.0 (Statistica, 2009).

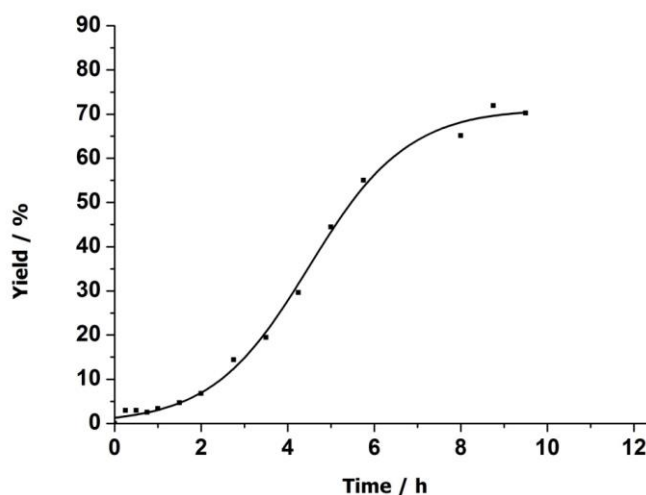
### 2.13. Simplex Optimization

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

### 3. Results and Discussion

The aqueous extract had a pH of 3.5 that was adjusted to 4.5 by adding sodium hydroxide (40 g L<sup>-1</sup>), according to Maia et al. (2013), for optimal yeast growth in relation to the variation of pH, the limits are between 4.5 and 5.5. Additionally, the total sugar concentration determined by the phenol-sulfuric acid method was 91.20 g L<sup>-1</sup>. Chromatographic analysis showed that the initial extract presented 29.97 g L<sup>-1</sup> (32.86%) glucose, 39.84 g L<sup>-1</sup> (43.69%) fructose, 3.43 g L<sup>-1</sup> (3.76%) sucrose and 17.96 g L<sup>-1</sup> (19.69%) fructooligosaccharides (FOS) as carbohydrate sources.

Preliminary tests with the butiá pulp extract contained 3.0 g L<sup>-1</sup> of yeast extract, 6.0 g L<sup>-1</sup> of yeast, and 0.30 g L<sup>-1</sup> NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, i.e. intermediate values to those shown in Table 1, with MgSO<sub>4</sub>·7H<sub>2</sub>O (0.25 g L<sup>-1</sup>) and ZnSO<sub>4</sub> (0.2 g L<sup>-1</sup>). Tests were performed to determine the fermentation time. Figure 1 shows a progressive increase in ethanol production yield and response value stabilization after 8 hours of fermentation.



**Figure 1.** Ethanol yield in percentage, as a function of fermentation time. The points and curve represent the experimental data and the fitted data, respectively.

The experimental region (Table 1), i.e. the upper and lower limits of each independent variable, were chosen by preliminary tests and based on literature data (Cordenunsi et al., 1985, Silva et al., 2008).

In the environment, yeasts thrive in a wide temperature range, but optimum growth is between 26 and 35°C, with an average of 30°C (Lima et al., 1975). Based on this information, the temperature used in the experimental trials was 30°C in a controlled oven, and the fermentation time was 8 hours based on the data obtained in Figure 1.

The experimental mixture design was used to evaluate and optimize the effect of the yeast extract concentration ( $x_1$ ) in  $\text{g L}^{-1}$ , the yeast concentration ( $x_2$ ) in  $\text{g L}^{-1}$ , and the  $\text{NH}_4\text{H}_2\text{PO}_4$  concentration ( $x_3$ ) in  $\text{g L}^{-1}$ , against ethanol yield ( $Y_1$ ) in the fermentation of an aqueous extract of butiá pulp.

Table 2 shows the combinations of the original variable mixtures (X) and codes (x) obtained by the restrictions presented in Table 1, two replicates provided averages (%). The variables were encoded with respect to the proportions such that the sum of the components in each test was equal to 1, or 100% (Calado and Montgomery, 2003).

**Table 2.** Experimental design and responses obtained.

Test	Coded variables			Original variables ( $\text{g L}^{-1}$ )			Yield (%)
	$x_1$	$x_2$	$x_3$	$X_1$	$X_2$	$X_3$	Y
1	0.200	0.775	0.025	2.000	7.750	0.250	64.86
2	0.400	0.575	0.025	4.000	5.750	0.250	65.62
3	0.200	0.765	0.035	2.000	7.650	0.350	61.64
4	0.400	0.565	0.035	4.000	5.650	0.350	68.30
5	0.200	0.770	0.030	2.000	7.700	0.300	63.35
6	0.400	0.570	0.030	4.000	5.700	0.300	65.57
7	0.300	0.675	0.025	3.000	6.750	0.250	61.37
8	0.300	0.665	0.035	3.000	6.650	0.350	68.96
9	0.300	0.670	0.030	3.000	6.700	0.300	65.46

The predictive equation (2) was fitted to the experimental data, containing only the significant terms at 5%. This represents the model where  $Y_1$  is the yield in alcohol percentage, yeast extract concentration is  $x_1$ , the yeast concentration is  $x_2$  and  $x_3$  is the  $\text{NH}_4\text{H}_2\text{PO}_4$  concentration, where the binary mixture ( $x_1.x_2$ ) influences the response negatively.

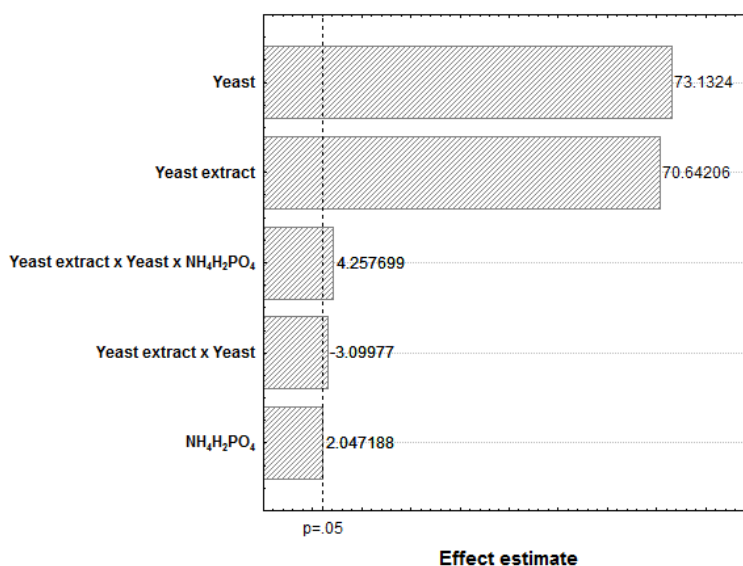
$$Y_1 = 67.3135x_1 + 63.6878x_2 + 48.5931x_3 - 16.6980x_1.x_2 + 747.0958x_1.x_2.x_3 \quad (2)$$

The model validity for predictive purposes was confirmed by analysis of variance (Table 3), which was significant ( $p = 0.001$ ). It also showed that the determination coefficient ( $R^2$ ) was 0.97, the adjusted experimental coefficient was 0.88, and the regression deviation ( $p = 0.1082$ ) was not significant. According to Joglekar and May (1987), in order to consider a model as having a good fit to the experimental data, the value of  $R^2$  must be greater than 0.80.

**Table 3.** Analysis of variance for alcohol yield response (%)

	G.L.	S.Q.	Q.M.	F	p-value
Model	4	137.4459	34.3615	9.4421	0.0001
Total Error	22	80.0616	3.6392	-	-
Lack of fit	4	26.4104	6.6026	2.2152	0.1082
Pure Error	18	56.6512	2.9806		
Total	26	217.5075	8.3657		

The Pareto chart containing only significant terms (Figure 2) showed that the statistically most important variable in fermentation process was the yeast concentration, followed by the yeast extract concentration and the ternary interaction. The numbers represent the values of the t-test.

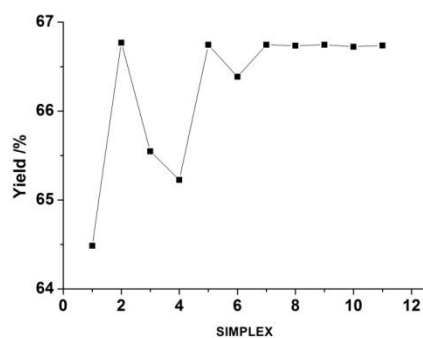


**Figure 2.** Pareto chart showing the most significant terms of the predictive model.

A predictive equation was optimized using the simplex sequential method forming a regular figure. In the case of three variables, a tetrahedron is formed, which moves on a surface to prevent unsatisfactory response regions. It is a recurrent procedure, which tends to achieve the optimal value through reflection movements in the direction opposite to the worst vertex. Once in the neighborhood of the optimum, the simplex may suffer contraction in order to determine a more precise position. To avoid a rapid expansion or contraction of the simplex, which would isolate it from the optimal point, a better movement vector was generated (Walters et al., 1999, Bona et al., 2000). This procedure was repeated until a satisfactory point was found. The upper and lower limits of the simplex optimization

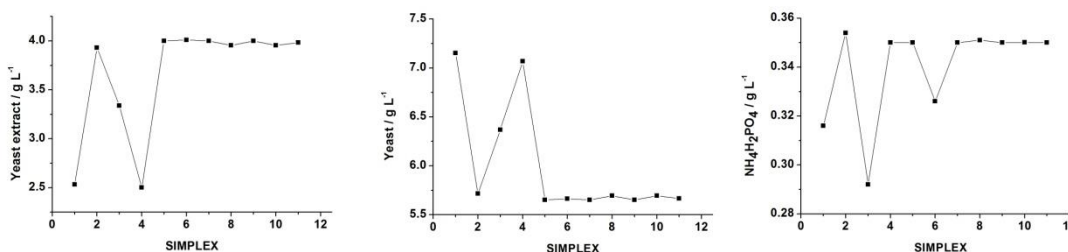
were applied to the lowest and highest values found in Table 2, for the independent variables, always respecting that the sum of the three components has to be equal to 1 or 100% while maintaining the ratio.

A progressive increase in the alcohol yield towards the optimal, convergence, and stability of the response values can be seen in Figure 3. The maximum yield of 66.75% was found in simplex 7. The figure shows that the yield tended rapidly to the optimum value and convergence was achieved due to the response function which began to grow at a rate lower than  $10^{-3}$ . This was used by the simplex method as the convergence criterion for the studied parameters.



**Figure 3.** Ethanol yields as a function of simplex stabilization.

In Figure 4, the stabilization of the independent variables can be observed. The maximum ethanol yield (66.75%) was obtained in simplex 7 at the upper limit of yeast extract ( $4.0 \text{ g L}^{-1}$ ), a value close to the lowest concentration to yeast ( $5.65 \text{ g L}^{-1}$ ) and at the upper limit of  $\text{NH}_4\text{H}_2\text{PO}_4$  ( $0.35 \text{ g L}^{-1}$ ).



**Figure 4.** Stabilization of independent variables values in the simplex function to optimize the performance of alcoholic fermentation process.

The predictive equation was validated by fermenting the aqueous butiá extract under the optimal conditions. The average yield, in triplicate, was 68.29% with a standard deviation of  $\pm 2.89\%$ . Applying the t-test, no significant difference was found ( $p = 0.4552$ ) between the mean value obtained and the simplex optimization response (66.75%). The Tukey test also showed no significant difference

( $p = 0.6894$ ) and the Levenes test ( $p = 0.3764$ ) indicated that the variance was homogeneous (Calado and Montgomery, 2003).

Chromatographic analysis of the fermented product, using the optimal conditions established by the simplex method, showed that FOS present in the extract did not undergo fermentation,  $0.75 \text{ g L}^{-1}$  of fructose, traces of glucose and the absence of sucrose were also found. The presence of FOS ( $17.96 \text{ g L}^{-1}$ ) in the aqueous extract indicates that the butiá had not yet reached the required maturity level, which may have contributed to a lower ethanol yield since this type of carbohydrate is not fermented by *Saccharomyces cerevisiae*.

The experimentally obtained and optimized yields were lower than that typically found in alcoholic discontinuous or semi-continuous fermentation when using sugar cane juice or molasses, or even with a mixture of these two components (Prescott and Dunn, 1949, Silva et al., 2006). Silva et al. (2008) optimized grape cane fermentation, using response surface methodology, and obtained a yield of 88% in batch fermentation. However, these comparisons should be treated with caution, since the media used are not the same.

#### 4. Conclusions

Modeling using mixture design combined with the simplex method with restrictions was an efficient and relatively simple optimization strategy and can be considered useful in the research and development of fermentation processes.

The butiá pulp aqueous extract was an adequate and potential substrate for ethanol production due to the amount of carbohydrates and the good yield, making it an alternative to sugar cane.

#### Acknowledgments

The authors wish to express their gratitude to Fundação Araucária, by financial support and CAPES for the Master's scholarship.

#### References

- Barbosa AM., Giese EC, Dekker RFH, Borsato D, Briones A, Úbeda J. (2010). Extracellular  $\beta$ -glucosidase production by the yeast *Debaryomyces pseudopolymorphus* UCLM-NS7A: Optimization using response surface methodology. *New Biotechnol.*, **27**:374-381.
- Berté SD, Borsato D, Silva PB, Vignoli JA, Celligoi MAP. (2013). Statistical optimization of levansucrase production from *Bacillus subtilis* ATCC 6633 using response surface methodology. *Afr. J. Microbiol. Res.*, **7**:898-904.

- Bona E, Borsato D, Silva, RSSF, Herrerra R. (2000). Software for optimization using a sequential simplex method. *Acta. Sci.*, **22**:1201-1206.
- Borsato D, Dall'Antonia LH, Guedes CLB, Maia ECR, Freitas HR, Moreira I, Spacino KR. (2010). The simplex-centroide design applied to study of the kinetics of the oxidation of B100 biodiesel in blend with syntetic antioxidants. *Quim. Nova*, **33**:1726-1731.
- Breitkreitz MC, Jarsim ICSF, Bruns RE. (2009). Combined column-mobile phase mixture statistical design optmization of high- performance liquid chromatographic analysis of multicomponent systems. *J. Chromatog. A*, **1216**:1439-1449.
- Büttow MV, Barbieri RL, Heiden G. (2009). Traditional knowledge associated with the use of butia palm (*Butia* spp., *Arecaceae*) in the southern of Brazil. *Rev. Bras. Frutic.*, **31**:1069-1075.
- Calado V, Montgomery DC. (2003). *Design of Experiments using the Statistica*. E-papers Serviços Editoriais Ltda: Rio de Janeiro, Brazil, pp. 159-191.
- Cesar MAA, Delgado AA, Camargo AP, Bissoli BMA, Silva FC. (1987). Capacity of natural and artificial phosphorus content in phosphates lift in juice of cane sugar (plant cane), targeting the industrial process. *STAB*, **6**:32-38.
- Cini JRM, Borsato D, Guedes CLB, Silva HC, Coppo RL. (2013). Comparison of methods for determination of oxidative stability of B100 biodiesel mixed with synthetic antioxidants. Application of simplex-centroid design with process variable. *Quim. Nova*, **36**:79-84.
- Cordenunsi BR, Silva RSSF, Srivastava KC, Fabre-Sanches S, Perre MA. (1985). Mathematical model for the alcoholic fermentation in batch culture: Comparison between complete and incomplete factorial ( $3^3$ ) designs. *J. Biotechnol.* **2**:1-12.
- Cornell JA, Deng JC. (1982). Combining process variables and ingredient components in mixing experiments. *J. Food Sci.*, **47**:836-843.
- Cruz MRM, Borzani W. (1980). Alcoholic fermentation of molasses and sugar cane juice sugar by *Zymomonas*: Preliminary assays. *Rev. Bras. Technol.*, **11**:51-57.
- Dubois N, Gilles KA, Hemilton JK, Rebers BA, Smith F. (1956). Colorimetric method determination of sugars and substances. *Anal. Chem.*, **23**:350-356.
- Galvan D, Orives JR, Coppo RL, Silva ET, Angilelli KG, Borsato D. (2013). Determination of the kinetics and thermodynamics parameters of biodiesel oxidation reaction obtained from an mixture of vegetable oil and animal fat. *Energy Fuel*, **27**:6866-6871.
- Henderson A, Galeano G, Bernal R. (1995). *Field guide to the palms of the Americas*; Princeton University Press: New Jersey, United States, pp.1- 351.
- Hill WJ, Hunter WG. (1966). A review of response surface methodology: a literature survey. *Technometrics*, **8**:571-590.

- Joglekar AM, May AT. (1987). Product excellence through design of experiments. *Cereals Foods World*, **32**:854-868.
- Jones RP, Pamment N, Greenfield PF. (1981). Alcohol fermentation by yeasts – the effect of environmental and other variables. *Proc. Biochem*, **16**:42-49.
- Lima UA, Aquarone E, Borzani W. (1975). *Fermentation Technology*; Edgard Blucher: São Paulo, Brazil, pp.1-285.
- Lorenzi H, Souza HM. (1996). *Palms in Brazil: native and exotic*; Plantarum: Nova Odessa, Brazil. P.1-303.
- Lorenzi H. (2004). *Brazilian and exotic cultivated palms*; Plantarum: Nova Odessa, Brazil. p.1-416.
- Maia ECR, Borsato D, Moreira I, Spacino KR, Rodrigues PRP, Gallina AL. (2011). Study of the biodiesel B100 oxidative stability in mixture with antioxidants. *Fuel Process. Technol.*, **92**:1750-1755.
- Maia GAR, Borsato D, Rodrigues PRP, Payret Arrúia ME, Weirich Neto PH, Kurchaidt SM, Antunes AC, Pereira JAA, Antunes SRM. (2013). *Production of ethanol from, jerivá, Syagrus romanzoffiana*. In: *Materials and process for energy: communicating current research and technological developments*; Méndez-Villas A. Ed.; Formatex Research Center: Badajoz, Spain, pp. 335-339.
- MAPA, *Ministério da Agricultura, Pecuária e Abastecimento. Acompanhamento da Produção Sucroalcooleira, Safra 2011/2012, 2012.*
- Nelson NJ. (1944). A photometric adaptation of the Somogy method for determination of glucose. *J. Biol. Chem.*, **153**:375-380.
- Prescott SC, Dunn CG. (1949). *Industrial Microbiology*, 2nd ed.; Mac Graw-Hill: Nova Iorque, United States, pp.1-113.
- Reitz, R. (1974). *Palms*. In: REITZ, R. *Illustrated Flora of Santa Catarina*; Herbário Barbosa Rodrigues Itajaí, Brazil, pp. 1-189.
- Rosa L, Castellani TT, Reis A. (1998). Reproductive biology of *Butia capitata*(Martius) Beccari var. *odorata* in a coastal sandy shrub vegetation.; *Rev. Bras. Bot*, **21**: 281-287.
- Silva JA, Silva FLH, Alves RRN, Santana DP. (2006). Influence of nitrogen, phosphorus and °brix on the production of total secondary contaminant Metabolites (msct) of alcoholic fermentation. *Quím. Nova*, **29**:695-698.
- Silva JA, Damasceno BPGL, Silva FLH, Madruga MS, Santana, DP. (2008). Application of the factorial design and response surface methodology for optimization of alcoholic fermentation. *Quim.Nova*, **31**:1073-1077.
- Statistica, Statistica for Windows Software, v.9.0. Tulsa, United States, 2009.

Stupiello JP, Horii J. (1985). Conduct the alcoholic fermentation, *Saccharum*, *STAB*, **4**:43-46.

Walters FH, Morgan SL, Parker LR, Deming SN. (1999). *Sequential optimization*; Licensed electronic reprint by Multisimplex AB, Karskrona, Sweden, pp. 1-403.

Zimmerman HW. (1970). *Studies on the dichromate method of alcohol determination*. In: *Methods in Food Analysis*, 2nd ed.; Academic Press, New York, United States, pp. 457-458.