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Optimisation of fermentation conditions in the production of ethanol from Palmer mango

Otimização das condições de fermentação na produção de etanol a partir da manga Palmer

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Abstract

The objective of this work is to analyse the production potential and optimize the aqueous pulp extract of Palmer mangoes for the production of ethanol. The concentration of total sugars obtained was measured by phenol sulphuric acid method in the pulp and by chromatography; the results showed 83.33 gL^{-1} and 80.51 gL^{-1} respectively. By means of preliminary tests at 30°C, it was established that the period of 10 hours presented the biggest yield of ethanol. This was followed by fermentation, with 13 tests and two repetitions in the central point, after which the samples were centrifuged to determine the alcohol content. The optimisation indicated a formula containing 3.0 gL^{-1} of yeast extract, 8.0 gL^{-1} of yeast, and 0.35 gL^{-1} of $NH_4H_2PO_4$, for an alcohol content of 34.5 gL^{-1} with a yield of 88.27%.Sugar cane juice, diluted under the same conditions, was also fermented and passed through the same procedures; however the resulting alcohol content of 35.68 gL^{-1} with a yield of 70.74% proved to be inferior value to that presented when the aqueous extract of Palmer mango was used.
Keywords: Alcoholic Fermentation, Ethyl Acohol, Response Surface Methodology.

Resumo

O objetivo deste trabalho foi analisar o potencial de produção e otimização do extrato aquoso da polpa da manga Palmer para a produção de etanol. Foi determinada a concentração de açúcares totais, na polpa pelo método do fenol sulfúrico e por cromatografia, sendo o valor obtido de açúcares totais de $83,33\ gL^{-1}$ e $80,51\ gL^{-1}$, respectivamente. Por meio de ensaios preliminares a 30°C, estabeleceu-se que o tempo de 10 horas apresentou o maior rendimento em etanol. Em seguida foi feita a fermentação, com 13 ensaios e duas repetições no ponto central, após, as amostras foram centrifugadas e o teor de álcool foi determinado. A otimização indicou uma formulação contendo $3,0\ gL^{-1}$ de extrato de levedura, $8,0\ gL^{-1}$ de levedura e $0,35\ gL^{-1}$ de $NH_4H_2PO_4$, para um teor de álcool de $34,5\ gL^{-1}$ e rendimento de 88,27%. O caldo da cana-de-açúcar, diluído nas mesmas condições, também foi fermentado e passou pelos mesmos procedimentos, mas o resultado do teor de álcool foi de $35,68\ gL^{-1}$ com rendimento de 70,74% valor inferior ao apresentado quando se utilizou o extrato aquoso da manga Palmer.
Palavras-chave: Fermentação Alcoólica. Álcool Etílico. Metodologia de Superfície de Resposta.

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Introduction

Ethanol raises the attention of researchers, companies and governments. This is due to price pressures and the prospect of depleting non-renewable sources of fossil fuels, as well as concerns over the emission of substances that compromise the environment (BASTOS, 2007). Brazil has taken leadership in the use of ethanol as an automotive biofuel in the international scene of carbon sequestration and mitigation of the greenhouse effect. This fuel, however, can be produced not only with sugarcane or corn, but also with another biomass that has a significant amount of sugar, which is one of the main requirements in the production of alcohol (SPACINO et al., 2013). However, the production of ethanol can be achieved from the aqueous pulp extract of some high sugar fruits that can be grown for this purpose or those that are discarded during regular production. Moreover, fruits contain sugar molecules formed by one or two monosaccharides, which are fermented by yeasts (FORTES et al., 2012; MAIA et al, 2014). The higher the sugar concentration in the biomass, the greater the amount of ethanol that is obtained, as its production occurs through yeast fermentation of sugars (CARDOSO et al., 2014). The mango (Mangifera indica L.), is one of the most sought after fruits in the world (FOLEGATTI et al., 2002). The Palmer variety is characterized by yellowish pulp with firm flesh, good flavor (21.6^0 Brix) and little or no fiber. It is a fruit that has gained attention and has become preferred because of its sharp, sweet taste and its propensity for consumption. Another advantage of the variety is that it has a small core, which guarantees a consistent pulp and a better use of the fruit with a pulp/fruit ratio of 72%, thin skin and medium fiber content (COSTA, 2004). The substrates must be adequate for the development of the microorganism and the purpose of its activity, which is to produce a particular substance. For better performance the composition of the medium must be able to meetthe requirements of the microorganism, such as pH level, temperature, asepsis or sterility among others (LIMA, 2001). Alcoholic fermentation is a biological process, yeast being its main agent, in which sugar is transformed into alcohol and carbon dioxide. The most used species in the production of alcohol is Saccharomyces cerevisiae.This yeast, when in contact with a medium containing glucose and under anaerobic conditions, produces ethyl alcohol and carbon dioxide, along with several other byproducts. During the fermentation process several factors can arise which may alter the ethyl alcohol production by interfering with the cellular activity of the yeast. The pH level plays an important role in fermentation, and in order to favor the development of yeasts it must be in the range of 4.5 to 5.5 (JONES, PAMMENT; GREENFIELD, 1981). Through experimental planning and the analysis of response surface it is possible to investigate the influence of the variables in a process, as well as the interaction between them, and arrive at the variable value that optimizes the results (SCHIRMANN, et al., 2018). Therefore, as the world demand for ethanol increases and Brazil continues to stand out as a leader in both fruit and ethanol production, this work proposes to determine the sugar concentration and the best conditions for the alcoholic fermentation process of the aqueous extract of the Palmer mango pulp for the production of ethanol, using response surface methodology.

Materials and methods

Preparation of the sample

The Palmer mango was peeled, stripped and transformed into an aqueous extract with the addition of 50% distilled water in a low-speed blender and the fibers were separated with the aid of a sieve. The aqueous extract obtained after milling was packed in a plastic bottle and one portion was diluted with distilled water in the same proportion as the mangoes. Both were frozen in and kept in a freezer until the moment of the use in fermentation.

Yeast

Blocks of commercial yeast Saccharomyces cerevisiae (ITAIQUARA brand) were allowed to rest at room temperature ($25°C$) for one hour. The inner part of the yeast was cut into small pieces until it became a powder and was added to the wort at the set time for fermentation.

Nutritional Supplementation

Yeast extract (Acumedia) and monobasic ammonium phosphate P.A. (Vetec) were used as source of nitrogen and phosphorus. Magnesium sulphate (Nuclear) and zinc sulphate (Anidrol) were added to the entire culture medium (CRUZ; BORZANI, 1980).

Chromatography

Sigma-Aldrich standard solutions of nysthosis, sucrose, glucose, fructose and maltose were used, along with an $8\mu m$ particle RCM type REZEX column. Other accessories of the chromatograph were a Shimadzu LC-20AT pump, SIL-20AC HT automatic injector, RID-10A

detector and a CTO-20AS oven. Ultra-pure Mili-Q sonicated water was used in the mobile phase. The analyses were processed in Shimadzu's LC Solutions software

Determination of total sugars

For the determination of total sugars, the Dubois et al. (1956) method was used with concentrated sulfuric acid (Vetec), 5% phenol solution (Vetec), and 0.1% (w/v) standard solution of glucose.

Alcoholic fermentation

To perform the fermentation, pre-sterilized 125 mL erlenmeyer flasks were used containing supplemented culture medium and yeast at concentrations defined by the experimental design (Table 1). The erlenmeyer flasks were closed with hydrophobic cotton and incubated for ten hours at a stabilized at $30°C$ temperature in an oven. After removal from the oven the samples were centrifuged for 10 minutes at maximum rotation (4000 rpm), distilled, and the alcohol content was measured

Determination of alcohol content

The following solutions were used: 0.15 N sulphochromic solution containing potassium dichromate ($K_2Cr_2O_7$), sulfuric acid (H_2SO_4) and distilled water; ferrous ammonium sulfate solution $[Fe(NH_4)_2(SO_4)_2.6H_2O]$. As an indicator, the solution used was orthophenanthroline, iron sulphate heptahydrate and distilled water. To determine the alcohol content, the Zimmerman method (1970) was used. A 1 mL aliquot of the fermentation was distilled in a Tecnal TE-012 microdistiller, to which water was added, yielding a 20 mL sample that was placed in a 125 mL Erlenmeyer flask, followed by an addition of 5 mL of distilled water, 20 mL (3.7 g of potassium dichromate, 280 mL of sulfuric acid and sufficient distilled water to make 500 mL). The solution was allowed to stand for 25 minutes in a water bath at $60°C$. Then 0.5 ml of orthophenanthroline indicator (1.45 g of orthophenanthroline, 0.69 g of ferrous sulfate heptahydrate and sufficient distilled water to make up to 100mL) was added to the solution. Shortly after it was titrated with ammoniacal ferrous sulfate solution (67.55 g), sulfuric acid (10 mL) and distilled water sufficient to reach 500 mL, the alcohol content being expressed as gL^{-1} (ZIMMERMAN, 1970).

Alcohol Content

The alcohol content (E) was determined by equation (1) and the yield by equation (2)

$$E\left(gL^{-1}\right) = k.V.N.\left(1 - \frac{V_a}{V_b}\right), \qquad (1)$$

Where, $E\ \left(gL^{-1}\right)$ the alcohol content, k is the value of 11.5 a theoretical constant; V and N refer to the volume and molarity of the potassium dichromate reagent respectively: V_a is the volume of ammoniacal ferrous sulfate used in titration of the sample and V_b is the volume of ammoniacal ferrous sulfate spent in the titration of the white

$$Yield\,(\%) = \frac{Content\ of\ alcohol\ obtained}{Maximum\ alcohol\ content}.100. \qquad (2)$$

Experimental design

The incomplete factorial design (3^3) of Box-Behnken was used to optimize the alcoholic fermentation conditions of the aqueous extract of the samples. The independent variables; X_1, X_2 and X_3, were respectively transformed into and coded into three levels of variation, with the restrictions presented in table 1.

Table 1: Independent variables and levels of variation in gL^{-1}.

Independent variables	Coded variables		
	-1	0	1
X_1 = Yeast extract	1.50	2.50	3.50
X_2 = $NH_4H_2PO_4$	0.30	0.35	0.40
X_3 = Yeast	2.00	6.00	10.00

Source: The Author.

Mathematical Model

The function used was thus

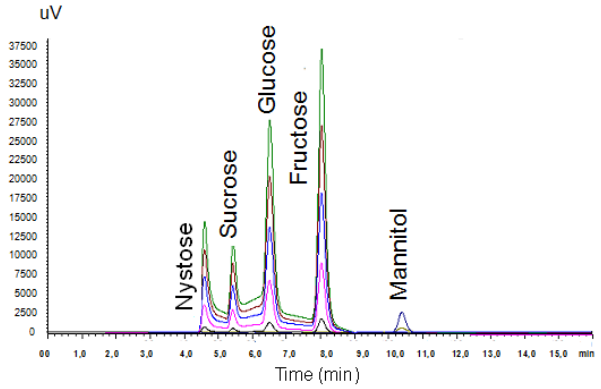
$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \beta_{ij} x_i x_j + \varepsilon \qquad (3)$$

In this case, Y, represents the response function of the experimental data, x_1, x_2 and x_3 are coded independent variables, corresponding to the concentrations of yeast extract, yeast and $NH_4H_2PO_4$ respectively, β the estimated parameters and ε the error observed (STATISTICA, 2009).

Results and discussions

To identify and quantify the different types of sugars present in Palmer mango, a chromatography of the aqueous extract of its pulp was performed using the standards of nystose, sucrose, fructose, glucose and mannitol (Figure 1).

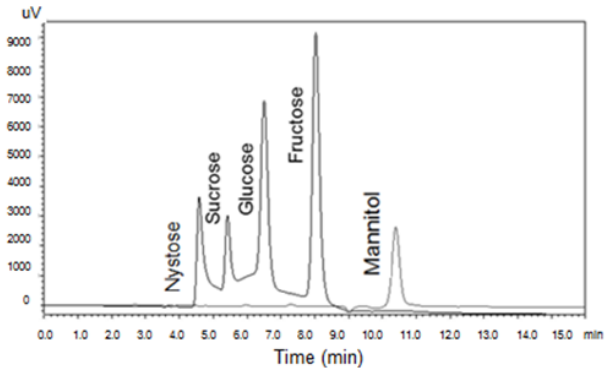
Figure 1: Chromatography of the standards of nystose, sucrose, fructose, glucose and mannitol.



Source: The Author.

In the chromatographic analysis, the aqueous extract of the Palmer mango pulp (Figure 2) presented the following contents for the different types of sugars: nystose (3.0 gL^{-1}), sucrose (40.71 gL^{-1}), glucose (7.36 gL^{-1}), fructose (16.93 gL^{-1}) and polyalcohol mannitol (5.26 gL^{-1}). Some compounds that were not identified reached the value of 7.24 gL^{-1} and the sum of all compounds was 80.51 gL^{-1}.

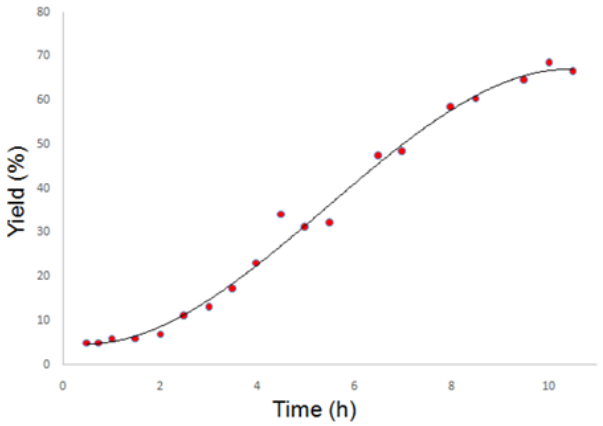
Figure 2: Chromatogram of the aqueous extract of the Palmer mango with identification of the substances nystose, sucrose, glucose, fructose and mannitol.



Source: The Author.

After the determination of the total sugars by the phenol sulphuric method (83.33 gL^{-1}) and by chromatography (80.51 gL^{-1}), preliminary tests were carried out on the fermentation of the aqueous extract of the Palmer mango pulp to determine the fermentation time using the central point of delineation (Table 1). Figure 3 shows the progressive increase in alcohol production yield, where the points represent the experimental data and the continuous line the mathematically adjusted data. The polynomial model used showed the stabilization of the response values after 10 hours of fermentation, thus being determined as the best time period for the fermentation of the aqueous extract of the Palmer mango pulp.

Figure 3: Graph of the time (h) of fermentation as a function of alcohol yield.



Source: The Author.

Identified the best time period, the incomplete factorial design of Box-Behnken 3^3 was used in order to optimize the alcoholic fermentation of the aqueous extract of the Palmer mango pulp, with 13 experiments and two repetitions at the central point (CALADO, MONTGOMERY, 2003) Table 2 shows the coded independent variables, together with the levels of variation in original values, the production responses in gL^{-1}, and the yield in %. Average values are presented of two repetitions of the fermentation tests, as well as the central point repetitions that were used to estimate error variance. The average central point in relation to the alcohol content of the aqueous extract of the Palmer mango was 33.98 gL^{-1} and the yield was 88.27%.

According to Table 3, the variance analysis indicated that the proposed model is significant at a 5 % level. However the regression deviance is not significant at the same level of variation.

The quadratic model, containing the coded independent variables and adjusted for the yield of the alcoholic fermentation, is represented by equation 4, where the regression coefficients (β) were obtained by $\beta = (A'A)^{-1}A'B$, where A is the delineation matrix containing the linear, quadratic and interaction terms and B is the response vector.

Table 2: Level of variation, original independent variables in gL^{-1}, alcohol content in gL^{-1} and yield of the fermentation process in %.

Tests	Independent variables			Alcohol Content (gL^{-1})	
	X_1	X_2	X_3		
	Yeast extrac	$NH_4H_2PO_4$	Yeast		
1	-1(1.50)	-1(0.30)	0(0.60)	28.62	74.36
2	1(3.50)	-1(0.30)	0(0.60)	31.36	81.49
3	-1(1.50)	1(0.40)	0(0.60)	29.02	75.38
4	1(3.50)	1(0.40)	0(0.60)	30.58	79.45
5	-1(1.50)	0(0.35)	-1(2.00)	25.61	66.55
6	1(3.50)	0(0.35)	-1(2.00)	28.23	73.34
7	-1(1.50)	0(0.35)	1(10.00)	30.58	79.45
8	1(3.50)	0(0.35)	1(10.00)	30.84	80.12
9	0(2.50)	-1(0.30)	-1(2.00)	27.71	71.98
10	0(2.50)	1(0.40)	-1(2.00)	25.61	66.54
11	0(2.50)	-1(0.30)	1(10.00)	29.79	77.41
12	0(2.50)	1(0.40)	1(10.00)	31.36	81.48
13	0(2.50)	0(0.35)	0(0.60)	32.93	85.55
14	0(2.50)	0(0.35)	0(0.60)	34.50	89.63
15	0(2.50)	0(0.35)	0(0.60)	34.50	89.63

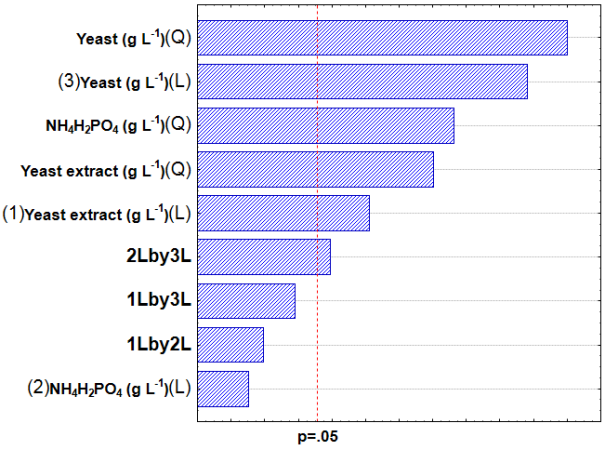
Source: The Author.

$$Y = 88.274^{*} + 2.333^{*}x_1 - 0.298x_2 + 5.006^{*}x_3 - 0.766x_1x_2 - 1.530x_1x_3 + 2.375^{*}x_2x_3 - 5,047x_1^2 - 5.459^{*}x_2^2 - 8.362^{*}x_3^2. \quad (4)$$

In the equation, asterisks represent the significant terms, at 5% level, and Y represents the estimated yield of the fermentation reaction. In coded form, x_1 represents the concentration of yeast extract, x_2 the concentration of $NH_4H_2PO_4$ and x_3 the yeast concentration. In addition, the coefficient of determination (R^2) was equal to 0.98 and the adjusted coefficient equal to 0.95, which can be considered adequate, as according to Joglekar and May (1987), to obtain a proper adjustment from the model to the experimental data the R^2 value must be greater than 80%. The non-significant regression deviance ($p = 0.95$) and the high R^2 revealed that the obtained equation, without the non-significant terms, can be used for predictive purposes, showed itself to be useful for optimization procedures.

The Pareto chart (Figure 4) containing all terms shows the most significant variables and their order of importance

Figure 4: Pareto chart showing the most significant variables in the fermentation.



Source: The Author.

in the obtained predictive model. The values next to the rectangle represent the values of the t test statistics. The letter "L" indicates the linear terms, the letter "Q" the quadratic ones that do not present physical significance but give an indication of the graph's curvature. Through

Table 3: Analysis of variance of the alcoholic fermentation reaction yield of the aqueous extract of the Palmer mango pulp, using the incomplete factorial design 3^3.

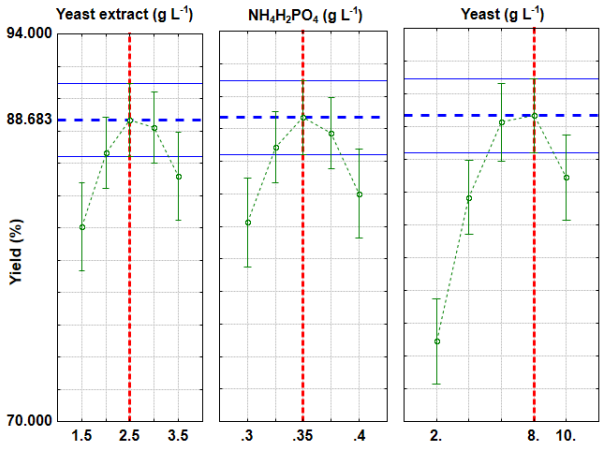
Source of variation	df	**Sum of squares**	Mean square	$\mathbf{F}_{cal}$	$\mathbf{F}_{tab}$
	X_1	X_2	X_3		
	Yeast extrac	$NH_4H_2PO_4$	Yeast		
Regression	9	688.252	76.472	29.640*	4.77
$X_1(L)$	1	43.545	43.545	16.878*	6.61
$X_2(L)$	1	0.708	0.708	$0.274^{(NS)}$	6.61
$X_3(L)$	1	200.486	200.486	77.707*	6.61
$X_1(Q)$	1	94.046	94.046	36.452*	6.61
$X_2(Q)$	1	114.081	114.081	44.217*	6.61
$X_3(Q)$	1	258.194	258.194	100.075*	6.61
X_1X_2	1	2.344	2.344	$0.909^{(NS)}$	6.61
X_1X_3	1	9.361	9.361	$3.628^{(NS)}$	6.61
X_2X_3	1	22.564	22.564	8.746*	6.61
Error	5	12.898	2.580		
Total	14	701.150			

Source: The Author.

the graph it is possible to determine the most significant variables.

Figure 5 shows the optimization of the variables, showing a yield of 88.68% that can be obtained when using 8.0 gL^{-1} of yeast, 2.5 gL^{-1} of yeast extract and 0.35 gL^{-1} of $NH_4H_2PO_4$.

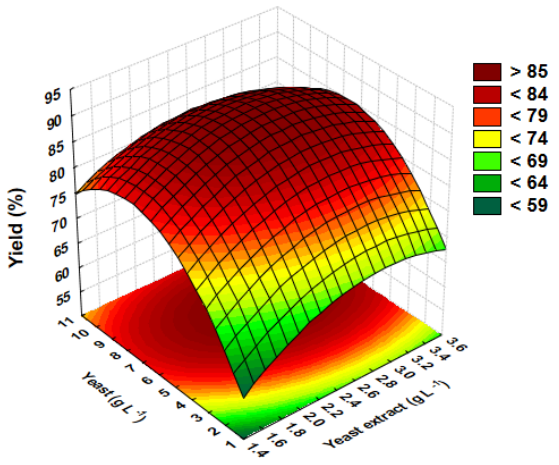
Figure 5: Optimization of the dependent and independent variables of the aqueous extract of the Palmer mango pulp.



Source: The Author.

Figure 6 shows the level curves of the response surface, the binary combination between the original variables, yeast concentration (gL^{-1}) and yeast extract (gL^{-1}). On the diagram, one can easily see the contour regions of the response surface for the dependent variable, yield of alcohol production obtained by the mathematical model, and that the least significant linear variable x_2 ($NH_4H_2PO_4$) in 0.35 gL^{-1} was set according to the Pareto diagram (Figure 4). It was observed that the optimum region for the yield of ethanol production is close to the central point of the experimental design.

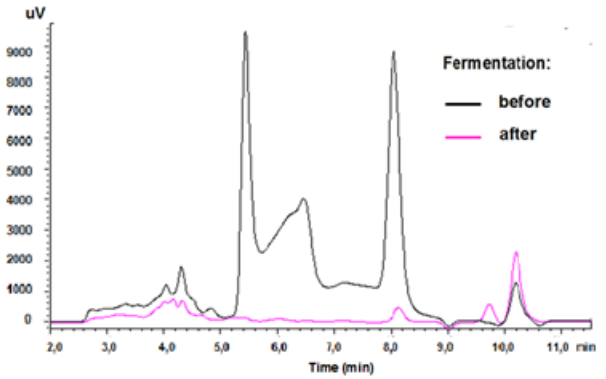
Figure 6: Level curves, Response surface, binary combination between original variables, yeast concentration (gL^{-1}) and yeast extract (gL^{-1}) and % alcohol yield.



Source: Statistica (2009).

The validation of the predictive equation was achieved by fermentation of the aqueous extract of the Palmer mango pulp under optimum conditions. The average value of the triplicate yield was 88.27%. Through the t-test, for simple sample, it was found that there was no significant difference at a level of 5% between this average value and that obtained in the optimization, which was 88.68%. The result of the chromatographic analysis of the product before and after the fermentation (Figure 7) under the optimum conditions established, shows that although the fermentation took place satisfactorily, not all the sugars were consumed in their entirety.

Figure 7: Chromatograms overlap of the aqueous extract of the Palmer mango pulp, before and after the fermentation under optimum conditions, in 10 hours.



Source: The Author.

In order to obtain a comparison of the alcohol content in gL^{-1} and the yield in % of the Palmer mango, fermentation with sugarcane was also carried out, which in Brazil is a parameter in the production of alcohol. Through the refractometer, it was evaluated that the cane juice presented a reading of 19.8°brix. Considering this value as the sugar concentration, its theoretical alcohol content after fermentation would be 101.26 gL^{-1}. Using the Zimmerman method (1970), the fermented broth was analyzed for ten hours in triplicate and its average was 28.62 gL^{-1}, thus the yield was 28.26%. Dilution was also carried out in the proportion of 1 part juice and 1 part water, the soluble solids content being 9.9°brix, yielding the theoretical alcohol content value of 50.63 gL^{-1}. In the experiment with the diluted juice, the value obtained after fermentation of the alcohol content was on average 35.68 gL^{-1}, with a yield of around 70.74%.

Conclusion

Palmer mango has shown itself to be an alternative in the production of alcohol, as it has favorable characteristics such as high sugar content, adequate pH and little or no fiber.

The fermentation of the aqueous extract of the Palmer mango pulp indicates that it can be used as an alternative source in alcohol production, if the values of 2.5 gL^{-1} of yeast extract, 0.35 gL^{-1} of $NH_4H_2PO_4$ and 8.0gL^{-1} of yeast are used at a constant temperature of 30°C for 10 hours. The diluted juice of sugarcane, which is the country's parameter in the production of alcohol, was fermented under the same conditions as the aqueous extract of the Palmer mango, and produced a lower alcohol content and yield, thus demonstrating that under the same conditions Palmer mangoes achieved more significant results than sugarcane in alcohol production.

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