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THE USE OF RESPONSE SURFACE METHODOLOGY TO EVALUATE THE FERMENTATION CONDITIONS IN THE PRODUCTION OF TEPACHE

EL USO DE LA METODOLOGÍA DE SUPERFICIE DE RESPUESTA PARA EVALUAR LAS CONDICIONES DE FERMENTACIÓN EN LA PRODUCCIÓN DE TEPACHE

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Abstract

Due to the artisan nature of the production process of tepache, there is no uniformity in fermentation conditions and raw material used. The fermentation is crucial to the final characteristics of tepache and there is a certain degree of ignorance of related organisms and the level of acceptance among consumers. In this study, tepache fermentation conditions (concentration of sugars, initial pH, temperature and fermentation time) were evaluated to enable correlating the formation of products of fermentation (lactic, acetic and ethanol) with the degree of acceptance of the beverage among consumers. Sensory evaluation was measured on a 9-point hedonic scale. Results were analyzed using the response surface methodology (RSM) which showed that the fermentation conditions for higher acceptance were: 22°C, 10% (mass/volume) of sugars (brown sugar), 72 h of fermentation and an initial pH of 5. According to this study, to have a wider acceptance the beverage must contain about 7 g/L of ethanol, no more than 5 g/L of lactic and acetic acid, and 70 g of sucrose/L. Likewise, yeasts present in the fermentation were identified and it was found that *Saccharomyces cerevisiae* is the predominant species.

Keywords: tepache, fermentation, response surface methodology, hedonic scale, yeast identification.

Resumen

Debido a la naturaleza artesanal del proceso de producción del tepache, no hay uniformidad en condiciones de fermentación y materia prima utilizadas. La fermentación es crucial para las características finales del tepache y existe cierto grado de desconocimiento de los microorganismos relacionados y el nivel de aceptación entre los consumidores. En este estudio, las condiciones de fermentación (concentración de azúcar, pH inicial, temperatura y tiempo de fermentación) fueron evaluadas para correlacionar los productos de la fermentación (etanol, ácido láctico y acético) con el grado de aceptación de la bebida. La evaluación sensorial se realizó con una escala hedónica de 9 puntos. Los resultados fueron analizados utilizando una metodología de superficie de respuesta (MSR), la cual mostró que las condiciones de fermentación, para una mayor aceptación, fueron: 22°C, 10% (masa/volumen) de azúcar morena (piloncillo), 72 horas de fermentación y un pH inicial de 5. De acuerdo con este estudio, para tener una mayor aceptación la bebida debe contener alrededor de 7 g/L de etanol, no más de 5 g/L de ácido acético y láctico, y 70 g/L de sacarosa. Asimismo, se identificaron las especies de levaduras presentes en la fermentación y se encontró que *Saccharomyces cerevisiae* es la especie predominante.

Palabras clave: tepache, fermentación, metodología de superficie de respuesta, escala hedónica, identificación de levaduras.

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1 Introduction

Long time ago, since pre-Hispanic era, fermented beverages were widely consumed in Mexico. Among the most popular are tepache, pulque, tejuino, and pozol, all considered soft drinks with low alcohol content (Godoy *et al*., 2003). Tepache is the most popular traditional fermented drink, prepared using traditional methods from pineapple peels, sugar (brown sugar, a type of unrefined sugar cane), water and spices (cinnamon and pepper). Fermentation of tepache takes place in wooden barrels, at room temperature, from 1 to 4 days (Alvarado *et al*., 2006). In distilled spirits, such as tequila and wine, ethanol is desirable as the major product and total sugar consumption during fermentation. In contrast, tepache is a not distilled beverage, ethanol concentration should be low and there is partial consumption of sugars to provide a sweet taste, and a production of lactic, acetic and other volatile compounds that give it its organoleptic characteristics. Tepache fermentation is affected by environmental, chemical and biological factors, such as temperature, concentration of sugars, pH, and fermentation time, among others, which modify the final product characteristics (Aidoo *et al*., 2006). If the fermentation continues for more than four days, the concentration of acetic acid increases and the taste is unpleasant (Moreno-Terrazas, 2005; Swiers *et al*., 2005).

By its artisan nature, raw materials used, temperature and fermentation time, the tepache process has a high degree of variation that affects its fermentation; accordingly, the final characteristics of the drink are different among preparations of different origin (Moreno-Terrazas, 2005). Tepache has potential for industrial scale production but it is necessary to know the fermentation conditions to generate a product of wider acceptance among consumers. Furthermore, the shell of the pineapple represents about 40% of the fruit and, since it is a waste of industrialization, its decomposition pollute the environment (Moreno-Terrazas, 2005). It is likely that in the process of industrialization of tepache, pineapple by-products will be totally used, giving added value to this waste.

There are few studies about the characteristics of tepache and the effect of fermentation conditions on the features of the beverage and its acceptance. Sensory evaluation is often used to determine the degree of acceptance of a product; similarly, the Response Surface Methodology (RSM) is used to optimize production with respect to sensory evaluations (Deshpande *et al*., 2008). In this study, using a central composite design (CCD), we evaluated the main process variables for tepache fermentation (brown sugar concentration, fermentation temperature, initial pH and fermentation time) to correlate the formation of fermentation products (lactic acid, acetic acid, and ethanol) with the acceptance of the beverage among consumers. The results were analyzed using the RSM and the yeast in the fermentation was also identified. With this study we claim to increase the knowledge of the fermentation process of this type of beverages and also contribute to the discernment of the control of fermentation conditions for industrialization.

2 Materials and methods

2.1 Preparation of tepache

The fermentation was performed in closed stainless-steel containers with 10-liter capacities. The top part of the containers had a 7-mm hole, which was sealed with a cotton plug to simulate semi-anaerobic conditions. The *panela* (brown sugar from Pihuamo, Jalisco, Mexico) was dissolved in water by shaking, and subsequently 9% (w/v) of pineapple rind (Pineapples *Ananas comosus* from Diva, Veracruz, Mexico), which had previously been cut into pieces, was added. The fermentation was carried out without agitation. The initial pH of the fermentations was adjusted with NaOH or citric acid solutions.

2.2 Determination of sugar and fermentation products

Sucrose, glucose, fructose, ethanol, lactic and acetic acids, were determined by HPLC. Sugars were analyzed with a Bio-Rad Aminex HPX-87C (300 x 7.8 mm) with water, as mobile phase, at a flow rate of 0.5 mL/min and 60°C. Acids and ethanol were quantified using an Alltech OA-100 column of 300 mm x 6.5 mm at 60°C and at a flow of 0.5 mL/min, with a mobile phase of 0.01N H_2SO_4, filtered and degasified. The device used was integrated to a Waters 600 controller, a Waters 717 plus auto injector, and a Waters 2410 RI detector.

2.3 Experimental design

To evaluate the fermentation conditions of the tepache (temperature, concentration of *panela*, initial pH, and

fermentation time), a non-replicate central composite design was used. The distance of the axial points to the center of the design was $\alpha = \pm 1.61$ to allow the design to rotate. Four central points were established to provide a reasonably stable variance of the predicted response (Montgomery, 2010). The range and levels of the researched variables are presented in Table 1.

The behavior of the response surface was studied with respect to the response function (Y) using a polynomial regression equation. The generalized response surface model is given by Eq. (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (1)$$

Where Y is the response variable, X_1, X_2, X_3, and X_4 are the independent variables with respect to the fermentation temperature, the amount of *panela*, the initial pH, and the fermentation time, respectively, β_0 is the intercept term, β_1, β_2, β_3, and β_4 are the linear effects, β_{11}, β_{22}, β_{33}, and β_{44} are the quadratic effects, and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are the interaction terms (Nwabueze *et al*., 2010).

2.4 Sensory evaluation

150 untrained panelists evaluated batches of tepache, prepared according to the CCD combinations, with regard to their overall acceptance. The panelists were selected to participate based on their preference for fermented drinks, interest, and availability. Samples of approximately 50 mL were served at a temperature of 4-9°C; an evaluation sheet accompanied the samples. The sessions were performed at room temperature (24-28°C). The tepache was evaluated according to a 9-point hedonic scale (1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = neither like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much, 9 = like extremely). Finally, the mean value of the evaluations was used for statistical analysis (Deshpande *et al*., 2008; Duarte *et al*., 2011; Valim *et al*., 2003).

2.5 Statistical analysis

A statistical analysis was performed using the Statgraphics Centurion XV program (StatPoint, Inc., 2005). The adjusted response surface model was statistically evaluated using the ANOVA F statistic, and the significant effects of the dependent variables were determined using a P-value with a probability value of less than 0.05.

2.6 Identification of yeasts

The yeasts were isolated from tepache after 72 h of fermentation. They were grown using GPYA medium at 30°C for 48 h, to subsequently select the different colonies and identify them according to their morphology and frequency in the culture medium.

The selected colonies were inoculated into tubes with 800 μl of GPY broth to extract the DNA after 12 h of incubation at 30°C and agitation at 100 rpm. Yeast DNA was extracted according to Querol *et al*. (1992). Colonies isolated were identified by PCR amplification of the region spanning internal transcribed spacers 1 and 2 (ITS-1, and ITS-2) and the 5.8S rRNA gene (5.8S-ITS region) and subsequent restriction analysis according to and compared with Esteve-Zarzoso *et al*. (1999).

3 Results and discussion

3.1 Conditions of fermentation and sensory evaluation

In the traditional process of preparing tepache, the temperature is not controlled during fermentation and it may vary from 10°C to 35°C throughout the year, which could significantly affect the final products of the drink and its acceptance. Likewise, the concentration of sugars may vary because fermentation time. The fermentation time also depends on temperature, and at different times the final products will have different concentrations. The pH depends on the acidity of the pineapple so it could also affect the formation of products in the beverage. A preliminary study determined the choice of all these variables. The complete experimental design is shown in Table 1.

The analysis of variance of CCD showed that the overall acceptance was significantly affected by all of the studied factors (P-value < 0.05). The most significant statistical factors were the fermentation time and the amount of *panela* used, both for the linear coefficients and for the quadratic coefficients. The analysis also shows that there is an optimal point of acceptance for tepache located among the levels of the factors or variables considered for fermentation; this is suggested by the fact that three quadratic terms in the model were statistically significant.

Table 1. Matrix and summary of the results of the central composite design

Run #	Temp. (°C) X_1	Conc. (%) X_2	Initial pH X_3	Time (h) X_4	Acceptance[a]	Ethanol (g/L)	Lactic acid (g/L)	Acetic acid (g/L)	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)
1	25	5	4	48	6.57	2.06	1.12	ND	32.53	ND	2.70
2	35	5	4	48	3.93	3.57	14.12	4.94	10.71	10.75	10.53
3	25	15	4	48	7.47	7.23	2.98	ND	92.12	16.57	15.61
4	35	15	4	48	6.87	6.05	18.45	6.18	87.29	21.78	21.36
5	25	5	6	48	5.93	ND	2.53	ND	35.46	1.28	4.42
6	35	5	6	48	5.17	1.72	9.97	3.22	39.58	ND	ND
7	25	15	6	48	6.13	4.42	6.61	ND	101.87	18.09	20.28
8	35	15	6	48	6.37	6.64	20.88	5.28	88.87	11.03	12.80
9	25	5	4	96	4.18	16.06	17.39	6.01	ND	0.26	3.70
10	35	5	4	96	3.18	22.17	25.05	4.49	1.85	0.81	1.38
11	25	15	4	96	7.18	27.31	33.92	6.50	5.24	30.46	45.52
12	35	15	4	96	3.93	52.72	21.64	1.12	3.96	9.51	23.50
13	25	5	6	96	3.73	12.27	20.10	6.31	ND	1.36	6.76
14	35	5	6	96	3.27	18.26	16.34	3.03	2.07	0.89	1.02
15	25	15	6	96	4.12	22.09	15.06	15.23	6.99	29.87	44.05
16	35	15	6	96	5.40	30.25	30.24	6.36	0.73	4.13	26.52
17	22.0	10	5	72	7.73	7.30	5.25	4.16	67.63	4.48	1.09
18	38.0	10	5	72	6.87	5.71	8.45	5.59	57.20	12.87	8.86
19	30	2.0	5	72	2.80	9.85	6.04	4.37	0.49	ND	1.50
20	30	18.0	5	72	6.47	11.05	24.35	7.28	61.34	12.19	30.41
21	30	10	3.4	72	6.00	21.59	9.44	ND	2.72	15.73	27.26
22	30	10	6.6	72	4.50	16.36	7.79	6.35	7.28	16.15	20.83
23	30	10	5	33.4	6.93	1.18	6.49	2.77	73.86	7.44	3.76
24	30	10	5	110.6	2.67	27.56	25.23	10.30	ND	0.80	5.52
25	30	10	5	72	7.40	7.00	17.73	3.58	18.69	20.06	26.33
26	30	10	5	72	7.23	3.80	15.99	5.53	29.93	19.73	18.08
27	30	10	5	72	5.80	11.95	14.39	ND	10.91	18.29	26.76
28	30	10	5	72	6.63	9.56	10.65	ND	8.90	24.97	31.38

[a]Mean values of 30 evaluations; ND: Not detected

Using the results of the experiments and the statistical analysis, a second-order polynomial equation was obtained (Equation 2) for the estimated value of acceptance (Y) as a function of the temperature (X_1, °C), the concentration of *panela* (X_2, %), the initial pH (X_3), and the fermentation time (X_4, h).

$$Y = 4.97493 - 0.567992X_1 + 0.728927X_2 + 1.73046X_3 + 0.11907X_4 + 0.097375X_1X_3 - 0.0282162X_2^2 - 0.465171X_3^2 - 0.00110431X_4^2 \quad (2)$$

The value of the correlation coefficient (R^2) was 0.826; this value was considered sufficiently high. Some authors indicate that an R^2 value of at least 0.80 is sufficient to explain the variability of a model, and to explain the variance of sensory data (Prasad and Nath, 2002). Therefore, the model developed to predict the sensory scores of the tepache produced under different fermentation conditions was acceptable, considering that the response variable is a hedonic measurement, which may often display considerable variations (Valim *et al*., 2003).

The quadratic model (Equation 2) for overall acceptance was used to generate the response surface graphs. Fig. 1 illustrates the influence of the fermentation time and the amount of *panela* on the overall acceptance of the tepache. It can be observed by the curving of the graph (Fig. 1a) that both, the linear and the quadratic coefficients of these variables, affect the overall acceptance. In addition, the optimal levels of each variable can be determined: between 40 h and 60 h for the fermentation time, and between 10% and 15% for the amount of *panela* (Fig. 1a). To help visualize the shape of the response surface (Fig. 1b),

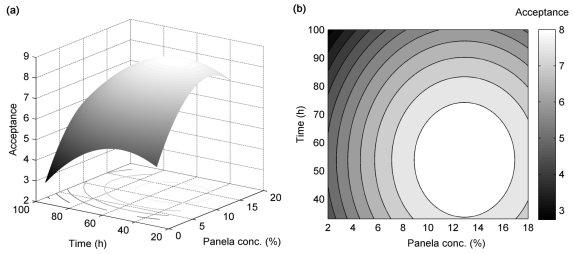

Fig. 1. Effect of time and amount of panela on the overall acceptance of tepache, (a) Response surface, (b) surface contours.

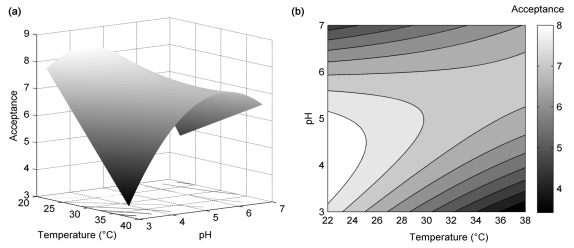

Fig. 2. Effect of pH and fermentation temperature on the overall acceptance of tepache.(a) Response surface, (b) surface contours.

the contours of the response surface are graphed. In the contour graph, the constant response lines are graphed in the x_1, x_2 plane. Each contour corresponds to a particular height of the response surface (Montgomery, 2010). In Fig. 1b, it can be observed that the maximum overall acceptance is found at the center of the circle, at 54 h of fermentation and with 13% *panela*.

Figure 2a illustrates the response surface between the fermentation temperature and the pH. It can be observed that the overall acceptance decreased with low temperature and high pH of the drink; moreover, for high temperature and low pH, the overall acceptance decreased even more. This behavior can be explained in terms of the linear effect of the fermentation temperature and the quadratic effect of the pH on the overall acceptance of the tepache. The contour graph (Fig. 2b) shows that the maximum acceptance is found between 22°C and 25°C, with an initial pH of 3.5 to 5.0.

RSM is a procedure that allows a general idea of the optimal conditions to be rapidly and efficiently obtained (Duarte *et al*., 2011). The levels of each factor estimated by the statistical model (Equation 2) for the optimization of the overall acceptance of the tepache were a temperature of 22°C, 13% *panela*, an initial pH of 4, and 54 h of fermentation.

Microbiologically, low values of pH in the fermentation of beverages are considered to be a selective and favorable factor. Most bacteria, with the exception of acetic and lactic bacteria, prefer a neutral to slightly alkaline pH and do not develop at the low pH of must. However, for yeast, a pH range of between 3 and 6 is more favorable for their growth and fermentation activity. The fermentation temperature of wine varies widely for temperatures that may range from 10°C to 30°C. The advantages of low-temperature fermentation are the development of a more fruity and fresh character of the drink, the formation and lower losses of ethanol, in addition to a reduction in the risk of contamination by bacteria and, consequently, a lower risk of producing volatile acids. Finally, wines must contain between 120 to 250 g/L of sugar because at concentrations greater than 300 g/L, the osmotic pressure may have a negative effect on the yeast, which may decrease yeast growth and fermentation activity (Benda, 1984).

Our results coincide with those found by other authors for other similar fermented beverages; for instance, the optimal values for the fermentation of mango wine were 22.53°C and a pH of 3.8 (Suder *et al*., 2009), and the optimal fermentation temperature of Jabuticaba (*Myrciaria cauliflora*) was 20°C (Duarte *et al*., 2011). Moreover, the conditions of pH, temperature, and even the concentration of sugar that was predicted to be optimal by the model, were similar to those found in the preparation of wine. The pH of grapes must vary between 3.0 and 3.9.

Results obtained in this study are innovative since sugar contents, to be contained in the drink for its acceptance, was unknown (both ends, few, or too many sugars are unpleasant). Similarly, as to the fermentation time concerns, tepache producers quite often use as far as 96 h; however, we found that large fermentation times are not acceptable to consumers.

3.2 Products formation of fermentation and sensory evaluation

In Table 1 is shown the results of the sensory analysis, in addition to the final concentration of sugars, ethanol and acids. More than half of the combinations got mean values greater than 5 (neither like nor dislike); among those, five obtained values greater than 7 (like moderately). The best score was 7.73 a value close to "like very much" on the hedonic scale. The fermentation conditions for this tepache were 10% *panela*, an initial pH of 5.0, a fermentation

temperature of 22°C, and a fermentation time of 72 h. It can be observed (Table 1) that, in general, the concentrations of lactic acid and ethanol were greater with prolonged fermentation times (96 h). In contrast, over short fermentation periods (48 h), the concentrations of these products, mainly of ethanol, were lower. In most treatments, the acetic acid content did not exceed 5 g/L, regardless of the fermentation time. The amount of residual sugars in the tepache was also different for each treatment. However, there was a tendency for the nearly total consumption, of sucrose at 96 h of fermentation, regardless of the initial amount of *panela*. In the majority of treatments, the presence of fructose and/or glucose was found, even with prolonged fermentation times. The concentrations measured for the treatment with the greatest acceptance (T = 22°C, *panela* = 10%, pH = 5, and time = 72 h) were (g/L): 7.30 of ethanol, 5.25 of lactic acid, 4.16 of acetic acid, 67.63 of sucrose, 4.48 of glucose, and 1.09 fructose.

Figure 3 shows graphs of response surfaces for production of ethanol, lactic acid and acetic acid in tepache. Alcoholic fermentation is the result of many interactions; not only depends on the strain but also on physicochemical environmental factors, sugars, acidity, temperature and others. The ethanol content can impact on the perception of the "body", the viscosity and, to a lesser extent, on the sweetness, acidity, aroma, flavor intensity and textural properties (Suder *et al*., 2009). Figure 3a shows the quadratic effect of pH and the linear effect of brown sugar on the amount of ethanol production; at pH values near 5, ethanol levels are lower than at higher pH values below 5. Ratman *et al*. (2003, 2005) using a CCD found that the most important physical factors, which affect fermentative production of ethanol, are temperature, initial pH and fermentation time. Although statistical analysis of this study showed no significant effect of temperature on ethanol production in the tepache, we found that time, the initial pH, and additional brown sugar concentration, did affect the response of this variable. Problems caused by the retention or loss of alcohol and aromatic substances at low and high temperatures, respectively, can also occur, affecting the optimal temperature for the growth and physiological activity of the yeast (Benda, 1984); furthermore, the lactic acid production was affected by the fermentation temperature, the initial amount of brown sugar and the fermentation time ($P < 0.05$) but not by the initial pH ($P > 0.05$). Figure 3b shows the response surface for lactic acid; note that only the linear coefficients of percentage of brown sugar and the fermentation temperature affect the response, and that the concentration of lactic acid is directly proportional to the increase the percentage of brown sugar and temperature. Figure 3c shows the linear effect of time and fermentation temperature (the only significant factors) on the production of acetic acid. At shorter fermentation times and lower temperatures, the amount of this acid is reduced; likewise, as reported by Suder *et al*. (2009), acetic acid concentration shows variation with temperature.

With the data obtained from the sensory evaluations and the final concentration of ethanol, acids, and sugars, multivariate analysis was performed to correlate the concentrations of these components in the tepache with the degree of the drink's acceptance. Table 3 shows the Pearson product-moment correlation coefficients (r) for each compound, with overall acceptance of the tepache.

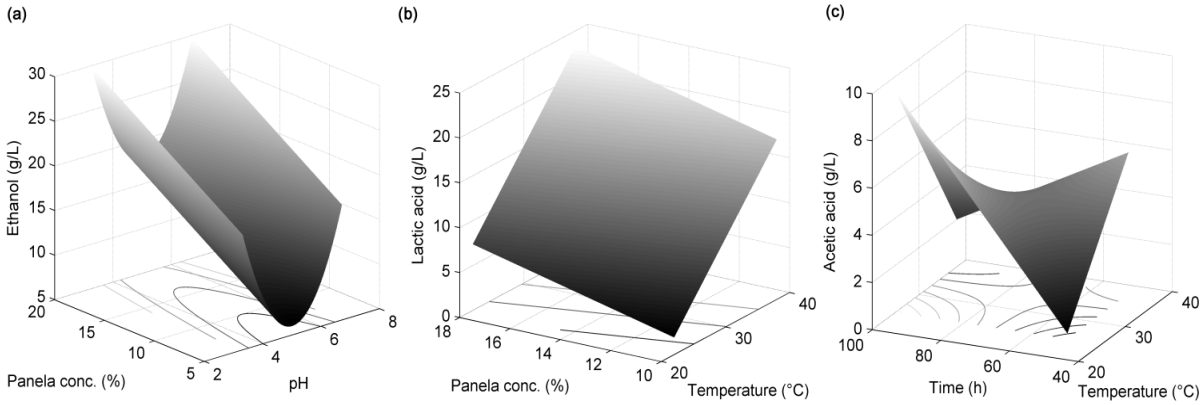

Fig. 3. Response surface for production of (a) Ethanol, (b) Lactic acid, and (c) Acetic acid.

Table 2. Correlation for acceptance

	Acetic acid	Lactic acid	Ethanol	Fructose	Glucose	Sucrose
r	-0.2487	-0.0004	-0.3643	0.4105	0.5240	0.5624
P-value	0.0672	0.9975	0.0063	0.0019	0.0000	0.0000

The range of these correlation coefficients, ranging from -1 to $+1$, measures the strength of the linear relationship between variables. A correlation of $+1$ means that there is a perfect direct linear relationship (positive) between the two variables, while a correlation of -1 means that there is a perfect inverse linear relationship (negative) between the two variables. Finally, a correlation of 0 is interpreted as the absence of a linear relationship between two variables (Lincoln, 1985).

It can be observed that the sugars exerted a great deal of influence in their linear relationship with acceptance. The table also shows the P-value that establishes the statistical significance of the estimated correlations. P-values below 0.05 indicate correlations that are significantly different from zero, with a confidence level of 95.0%. The ethanol, sucrose, glucose, and fructose have shown some correlation with overall acceptance of the tepache. These results show the type of relationship between the final concentration of sugar and ethanol on the acceptance of the tepache. In some drinks, the sensation of acidity is perceived less frequently when the products are sweeter. For this reason, some producers of tepache often compensate for the high degree of fermentation with the addition of sugar, with the goal of decreasing the sensation of acidity, given their extensive experience in balancing the flavor of the drink (Moreno-Terrazas, 2005). However, the ethanol content may impact the perception of the "body", the viscosity, and to a lesser degree the sweetness, acidity, aroma, flavor intensity, and textural properties (Suder *et al*., 2009).

These results can be explain the linear and quadratic effect of time on the overall acceptance of the tepache, given that in the initial stages of fermentation ($<$ 48 h), the sugar remains practically unconsumed and the compounds (lactic acid and ethanol) that provide the tepache with better sensory characteristics are not yet formed. However, with prolonged fermentation times, the sugars are almost entirely consumed, and the concentration of the products of fermentation is very high, leading to a decrease in the acceptance of the tepache after long fermentation times ($>$ 72 h).

The chemical composition of the tepache was affected by the fermentation conditions, given that many biosynthetic routes adopted by yeasts and bacteria are related to the formation of the aroma and flavor and are affected by various factors, such as the composition, the pH of the medium, concentration of sugar, dissolved oxygen and the prevailing temperature of the fermentation (Swiers *et al*., 2005; Estela-Escalante *et al*., 2012; Téllez-Mora *et al*., 2012). Other authors have already reported (Duarte *et al*., 2011; Swiers *et al*., 2005) that the fermentation temperature, the initial sugar concentration, and the pH significantly affect the chemical composition of different alcoholic drinks.

The temperature affects the yeast and as a result considerably affects the course of the fermentation. The death of yeast at high temperatures causes the fermentation to stop, which is accompanied by the danger of contamination by thermophilic microorganisms. However, low temperatures may produce problems in delaying the beginning of fermentation. In addition, to the optimal temperature for the growth and physiological activity of the yeast, problems caused by the retention or loss of alcohol and aromatic substances at low and high temperatures, respectively, can also occur (Benda, 1984).

The pH influences the growth of microorganisms and their metabolism, which is frequently modified as a result of a change in pH of up to 1-1.5 (Scragg, 2009). Some studies mention that the growth of acetic bacteria during fermentation is correlated with the initial pH of the medium. When the pH of the must is relatively high, the amount of acetic bacteria is greater at the end of fermentation, which is an indication that these bacteria may grow during alcoholic fermentation (Escalante *et al*., 2004). The presence of acetic bacteria leads to souring, brown discoloration, a bittersweet taste, and turbidity of fermented drinks due to the production of acetic acid

(Manca *et al*., 2006). Concentrations of 0.3-1.1 g/L may become undesirable, depending on the type of drink (Swiers *et al*., 2005; Flores-Ramirez *et al*., 2005).

Information obtained in this study is important because it was previously expected that a higher alcohol content and lactic acid would have a greater acceptance among consumers and did not. On the other hand, it was found that for a fermentation time longer than 72 h, the acceptance of the beverage is reduced since the lower concentration of sugars and higher contents of alcohol and acetic acid.

3.3 Identification of yeast

A total of 33 colonies were obtained, from which DNA extraction was performed. *Saccharomyces cerevisiae* was the predominant species (70% of the total of the identified strains) and was found practically in all of the analyzed samples; for some samples, it was the only species that was isolated. The other species were as follows: *Candida apicola* (9%), *Cryptococcus skinneri* (9%), *Hanseniaspora* (6%), and *Saccharomyces* sp. (6%). The species of the genera *Hanseniaspora* were not differentiated because with these enzymes, it is not possible to do so and would require further digestion with an additional enzyme (*Dde* I). In studies performed on different fermented fruit products, it has been reported that the species *S. cerevisiae* is predominant during the final stages of fermentation. It has also been mentioned that in not inoculated fermentations, the final product is the result of the combined activity of various strains of yeast that grow more or less successively over the course of the fermentation (González-Hernández *et al*., 2012). The first stages of fermentation are dominated by non-*Saccharomyces* yeast, whose growth rapidly declines due to its sensitivity to ethanol; subsequently, the *S. cerevisiae* strains, which are more tolerant to ethanol, begin to dominate and are the yeast strains that complete the wine production process (Garde and Ancín, 2006). In the production of wine, the non-*Saccharomyces* yeast present in grape juice, such as *Hanseniaspora* (*Kloeckera*) and *Candida*, begin to experience a decrease in their population by half during fermentation when the ethanol produced by *S. cerevisiae* exceeds 5-7%. The production of ethanol by S. cerevisiae is the factor that most greatly affects the growth of non-*Saccharomyces* yeasts. However, when the fermentation is performed at temperatures below 15-20°C, the species *Hanseniaspora* and *Candida* experience a decrease in their sensitivity to ethanol, providing a significant contribution to the taste of the wine (Romano *et al*., 2006).

Yeasts of the genera *Candida* and *Hanseniaspora* have also been found in other natural fermentations. It was reported the presence of these genera in fermentations of pineapple juice; even the species *C. apicola* was identified, although only occasionally and at a very low concentrations during fermentation (Chanprasartsuk *et al*., 2010). However, the genus *Hanseniaspora* was one of the most frequently found genres during the entire fermentation process of pineapple juice. Moreover, although yeasts of the genus *Cryptococcus* were not found during fermentation, they were isolated and identified from the pineapple rind (Chanprasartsuk *et al*., 2010); the presence of this yeast may be associated with the pineapple rind used.

However, *S. cerevisiae* was the only species isolated in different samples it was found at least one different species. The variation in the species found could be attributed to the fermentation conditions, the fermentation stage in which the sample was taken, and/or the natural flora of the skins of pineapple and brown sugar concentration.

Conclusion

With the response surface methodology (RSM) it was possible to correlate the fermentation products with the degree of acceptance of the beverage, and to determine the optimal conditions for fermentation of tepache for a wider acceptance among consumers. With the graphs of contours, it is possible to predict the fermentation conditions to achieve that desired levels of ethanol, acetic acid, lactic acid and residual sugars. The optimum conditions for fermentation of tepache, predicted by quadratic model, were: temperature 22°C, brown sugar 13%, an initial pH of 4, and 54 h of fermentation. The yeast species identified in the different treatments were: *Candida apicola* (9%), *Cryptococcus skinneri* (9%), *Hanseniaspora* (6%), *Saccharomyces* sp. (6%) and *Saccharomyces cerevisiae*. The latter, about 70%, was the predominant species at the end of fermentation.

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