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Optimization of *Agave cupreata* juice fermentation process for mezcal production using statistical experimental design

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Abstract

Mezcal is a traditional alcoholic beverage in Mexico obtained from fermentation of juices, coming from mature agave pineapples, and their subsequent distillation, *Agave cupreata* juice as natural sugar source is the commonly used. This study aims to investigate mezcal production from *Agave cupreata* juice by optimizing fermentation at flask level temperature; initial content sugar; pH and initial cell concentration, using single strains, factorial fractional design was applied and for fermentation at bioreactor level 2.5 Liters a Simplex centroid design was developed to determine the most appropriate yeast consortium for producing mezcal. The fermentation process was carried out for 52 h with varied input variables, and all the models showed significant *p*-values for interaction of variance (< 0.05). It was produced at an optimized temperature of 32.5 °C, initial quantity sugar of 14 °Brix, pH of 5.5 and stirring agitation of 150 rpm was found that it is possible to reach ethanol concentration levels of about 5.9, 5.2, 7.3 and 4.9% v/v for *S. cerevisiae, C. lusitaniae*, *K. marxianus* and *Z. bailii*, respectively. Therefore *K. marxianus* and *Z. bailii* strains reached the highest production of ethanol. Fermentation broths from the mixture design were distilled and rectified determining alcohol concentration; mixtures of *K. marxianus* and *Z. bailii* reached ethanol content in their distillates, about 42.82% v/v. The developed models could predict the quality of mezcal developed from *Agave cupreata* using a yeast consortium.

Keywords: *Agave cupreata*; fermentation; mezcal production; optimization; yeast consortium.

Practical Application: This study can be implemented by alcoholic beverages producers in the processes currently being carried out to develop the mezcal drink with high quality. Based in the results of this work with the using a yeast consortium and *Agave cupreata* juice.

1 Introduction

The production process of mezcal includes the following stages: selection and cutting of the raw material, agave cooking, milling of the cooked agaves, juice fermentation, distillation, rectification and in some cases distillate maturation. Agaves juices contain high concentrations of fructans, which are hydrolysed during the cooking step to obtain simple sugars like fructose, glucose, and sucrose, the initial contain sugar concentration in fermentation is between 40 and 160 g/L (Segura-García et al., 2015). During mezcal production both, natural and spontaneous fermentation, are common in practice. Nevertheless, in some cases, low quality distillates could be obtained in natural fermentation, caused by the presence of several microorganisms in the juice, such as *Saccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Kluyveromyces* and *Hanseniaspora* and some bacteria genus, which compete for sugars consumption in medium and generally these last produce undesirable compounds in the fermentation and the sensory quality varies between product batches (López et al., 2012; Perini et al., 2013; Nolasco-Cancino et al., 2018; Ficagna et al., 2020). Spontaneous fermentations were carried out from the yeasts presents in agave plants and in the materials of the production areas, without any external inoculation. These fermentations are not products of the action of a single species or yeast strain, but a succession of different species and yeast strains during fermentation (Zambonelli, 1988). In the first days of fermentation, the majority genus that proliferate are non- *Saccharomyces* yeasts as *Hanseniaspora, Kloeckera*, *Candida* and in a lower amount *Hansenula*, *Pichia*, *Rhodotorula* y *Metschnikowia* (Querol et al., 1990; Lachance, 1995). Non-*Saccharomyces* yeast has been considered as contaminants of alcoholic fermentation and, for years, practitioners attempted to avoid their presence during the process. After firsts days, these genera are drastically reduced, their growth is rapidly inhibited due to their low ethanol tolerance (Segura-García et al., 2015; Kunkee & Amerine, 1970) and a nutritional limitation (Valle-Rodríguez et al., 2012), giving way to other species growth more tolerant to ethanol as *Saccharomyces*. In fact, it is considered the main responsible for the alcoholic fermentation (Ribéreau, 1985). However, some studies have shown that although non-*Saccharomyces* yeasts are active for short periods in the fermentation, they contribute significantly to the aromatic quality of the final beverage (Romano et al., 2003,).

Studies by Fleet et al. (1984) and Heard & Fleet (1986), have shown that some species of non-*Saccharomyces* also contribute during wine fermentation, these species survive more than initially thought, and can achieve significant growth that influence the organoleptic composition of alcoholic beverages. In the case of tequila production, they are particularly important because they synthesize a variety of volatile compounds that contribute

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greatly to the bouquet of the drinks (Díaz-Montaño et al., 2008; Lappe-Oliveras et al., 2008). These types of yeast have ethanol yields of 25 to 49 g/L but can produce ethanol quantities equal of the of the genus *Saccharomyces*, when they are cultivated in a suitable medium that meets the nutritional and physical characteristics for optimal development (Valle-Rodríguez et al., 2009), by the addition of nutrients, to improve the efficiency of fermentation, increasing consumption of reducing sugars and ethanol production (Valle-Rodríguez et al., 2012). Besides, modifications that produce them in the composition of the must influence the kinetic fermentation and biochemical behavior of *Saccharomyces*. Diversity, composition and evolution of yeast strains and other microorganisms presents in the fermented must depends on several factors such as geographic location, climatic conditions, and the variety and maturity of the agave.

The results suggested that these strains were affected by nutritional limitation and/or to toxic compounds present in the juice of agave. *S. cerevisiae* strains produced predominantly amyl alcohol, isoamyl alcohol, n-propanol, 2-phenylethanol, succinic acid, glycerol, methanol, isoamyl acetate, ethyl hexanoate, acetaldehyde and isobutanol, Ruiz-Terán et al. (2019). While the strains of the genus *Koleckera* showed high production of acetic acid, 2-phenylethyl acetate and ethyl acetate. The methanol concentration was significantly different among the yeasts studied. The diversity between the three strains of *S. cerevisiae* was higher in the flavor profile compared with genetic and kinetic level. On the other hand, the diversity among strains of the genus *Kloeckera* was lower regarding of *S. cerevisiae*, despite belonging to two different species. López-Alvarez et al. (2012) studied the *K. marxianus* yeast and demonstrated that it was also able to ferment the juice of *A. tequilana*, and that the concentration of volatile compounds and the ethanol yield were higher than those obtained with *S. cerevisiae*. Segura-García et al. (2015) evaluated the fermentative capacity of *K. marxianus* (DUE3) and *Pichia kluyveri* (GRO3), isolated from traditional mezcal fermentation and assesses their production of volatile compounds, their values founded were compared with those of the same attributes of a *S. cerevisiae* (AR5) isolated from tequila fermentation. They demonstrated the potential of non-*Saccharomyces* yeasts, which fermented the agave juice in the same manner as *S. cerevisiae* but with higher ester production.

This study aims to investigate mezcal production from *Agave cupreata* juice by optimizing finding ideal conditions for the process to improve the fermentation efficiency. The main contribution is based on the study of pure culture fermentation, analyzing four experimental factors (temperature, the contain sugars, pH and the initial cell concentration) simultaneously in three levels using *Agave cupreata* juice and comparing the individual behavior *K. marxianus, C. lusitaniae* and *Z. bailii* and *S. cerevisiae* of the yeast at flask level with the behavior consortium with *K. marxianus* and *Z. bailii* in bioreactor level. Two experimental designs were developed, a Fractionated Factorial design for the study of individual strains and another Centroid Simplex for cultures mixtures. The use of these designs allowed to apply statistical tools to identify and quantify the causes of several effects within the fermentation stage, for which several variables with significant effects on the fermentation process were manipulated and its effects were measured in the interest variables.

2 Materials and methods

2.1 Microorganisms

Yeast strains

Three unconventional yeasts strains were used: *K. marxianus* (ITMLB29), *C. lusitaniae* (ITMLB26) and *Z. bailii* (ITMLB31) and another belonging to *S. cerevisiae* (ITMLB21) genus, all yeast isolated from mezcal producing region. The strains were molecularly characterized by Restriction Fragment Length Polymorphism (RFLP) reported by Damián (2012) and deposited in the laboratory of biochemistry at the National Technological Institute of México/Technological Institute of Morelia, Michoacán, México.

Strains propagation and maintenance

The strains were seeded in a supplemented with *A. cupreata* juice adjusted to 14 ºBrix (the volume necessary to add another 10 g/L) for the better yeast adaptation, 28 °C and pH of 5. Conservation was performed by keeping microorganisms in YPD liquid medium (BD Bioxon™) and glycerol (Sigma Aldrich®) (1:1) at -20 °C.

Inoculum media

Inoculums were incubated at 30 ºC and 150 rpm for 18 h, before fermentation. Bioreactor medium was inoculated with liquid inoculums grown in agave juice overnight (adjusted at 12º Brix and pH 5) to start at concentration of 1-3 million cells/mL.

2.2 Agave cupreata juice

Agave cupreata juice, previously hydrolyzed thermically and filtered was used as substrate for fermentations. The thermal hydrolysis of the agave was carried out in an autoclave (Felisa) at a temperature of 121 ºC and a pressure of 15 lb/in^2 for 8 h approximately This juice came from 8-year mature agave, cultivated in "mezcal denomination region" in México, in Etúcuaro, Michoacán State.

2.3 Fermentation conditions and monitoring

Were placed into 250 mL Erlenmeyer flask (Kimax™), 100 mL of agave juice previously filtered. The sugar quantity was adjusted to 10 °Brix, 12 °Brix and 14° Brix using a refractometer ATC®. The culture media was enriched with 1% of $(NH_4)_2 H_2PO_4$ (JT Baker®) and the pH were adjusted to values 4.5, 5 and 5.5, respectively, checking in a potentiometer (Hanna Instruments®).

Cell growth determination

The cell concentration in the liquid medium was determined by microscopic counting using a Neubauer chamber (Loptik, Labor), 1:10 dilutions; non-viable cells were stained using methylene blue (Sigma Aldrich®).

2.4 Experimental design

Experimental factors and flask fermentation conditions

For each individual microorganism, a factorial fractionated design 2^{4-1} with three replicates at the central point (Table 1) was applied at flask level; *Agave cupreata* juice was used as substrate. Four factors were established: A: Temperature (25, 30, 35) °C; B: Initial sugars (10, 12, 14) °Brix; C: pH (4.5, 5.0, 5.5) and D: Initial cells concentration (1, 2, 3) million cells/mL. Four response variables were followed during the fermentation process: 1. Cell growth, 2. Sugar consumption, 3. pH variation, and 4. Ethanol production. All fermentations were carried out in 250 mL Erlenmeyer flask with 150 mL of medium, at 150 rpm stirring for 52 h. These parameters were determined every 2 h at the beginning of fermentation and then every 4 h. With the data obtained were built a kinetic graphics over time. For statistical analysis, the maximum values of each response variable in the fermentation were considered.

Simplex-Centroid mix design bioreactor conditions

For these fermentations were considered as components, the three most ethanol productive yeasts, selected by the statistical analysis of factorial designs. Fermentations were carried out in a stirred tank bioreactor (Applikon system®), working volume of 2.5 L, with two Rushton agitators on the same support and three baffles; this bioreactor is equipped with bio-controller, pH, temperature, and oxygen electrodes and three peristaltic pumps. The operating conditions were held constant at 32.5 °C, 14° Brix, pH of 5.5, 150 rpm and 1 vvm; these values were drawn from analysis of results obtained in flask fermentation. The three yeasts that reached the highest ethanol production, in flask fermentation, were selected as entries of a Simplex-Centroid mix design. Two output variables were followed: 1. Cellular growth and, after distillation of the musts, 2. Alcohol content (%v Alc.).

2.5 Distillation and determination of percent volume of ethanol

The fermented musts from each test were subjected to distillation in a Heidolph Rotavapor© maintaining constant volume (500 mL), temperature (80 °C), agitation (90 rpm) and distillation time (30 min). All distillates were rectified to concentrate volatile compounds. Determination of alcohol content was determined by a volumetric method using a breathalyzer lab (NMX-V-013-NORMEX-2005 – Norma Mexicana, 2005). Distillates were poured into a test tube where the breathalyzer was carefully introduced along with a thermometer tending to float freely at ideal temperature of 293 K (20 °C); both measures were taken. The alcohol content is the amount of ethanol in 100 volumes of distilled product.

2.6 Analytical procedures

Determination of sugar consumption

The quantification of reducing sugars concentration (fructose and glucose) was determined using a colorimetric reaction with 3, 5-dinitrosalicylic acid (DNS) Sigma Aldrich®, using a UV-Vis spectrophotometer (UNICO® model 1000) at 540 nm of wavelength (Miller, 1959).

Determination of the pH variation

pH values were determined every 4 h, using a potentiometer (Hanna Instruments).

Ethanol determination in fermented musts

Ethanol concentration was determined by a gas chromatograph (Varian 3800®), with a flame ionization detector (FID). The samples injected into the chromatograph were separated on a polyethylene capillary column (HP-FFAP 50 × 0.32 m; 60°-240°/250 °C; Agilent Technologie®) with high polarity under the following conditions: 40 °C for 5 min, heated at 5 °C/min up 75 °C, heated at 10 °C/min to 200 °C, and maintained at 200 °C for 10 min. Injector and detector temperatures were set at 200 °C and 250 °C respectively. The carrier gas was high purity nitrogen at flow rate of 1.7 mL/min and constant pressure of 0.67 atm. Ethanol was identified according to their retention time obtained from a calibration curve using a pure standard (JT Baker®).

2.7 Statistical data analysis

The experimental designs and the statistical analysis were carried out using the STATGRAPHICS Plus® and Minitab® software, to interpret the results of each experiment in a statistical way.

Table 1. Randomized 2^{4-1} factorial experimental design.

Test	Temperature (°C)	Sugars (°Brix)	pH	Inoculum (cells/mL)/10^6
1	25	10	4.5	1
2	35	14	4.5	1
3	25	14	4.5	3
4	35	14	5.5	3
5	25	14	5.5	1
6	30	12	5.0	2
7	35	10	4.5	3
8	25	10	5.5	3
9	35	10	5.5	1
10	30	12	5.0	2
11	30	12	5.0	2

3 Results and discussion

Results are divided into two parts. Firstly, the analysis of the kinetics of cell growth and ethanol production by pure cultures of *S. cerevisiae, C. lusitaniae*, *K. marxianus* and *Z. bailii*, respectively at flask level: a) Output variables, b) Kinetic parameters and c) Statistical analysis. Secondly, the analysis of mixed cultures of *K. marxianus*, *S. cerevisiae* and *Z. bailii* at bioreactor level is described, and a chemical characterization of distillates obtained using mixed cultures is discussed: a) Response variables and b) Statistical analysis.

3.1 Analysis statistic at flask level

A factorial fractionated design 2^{4-1} at flask level (Table 1) was applied to each pure yeast strain to know its individual behavior, using *Agave cupreata* juice as substrate; cell growth and ethanol production are the response variables (Table 2). It is noteworthy that these strains were isolated from spontaneous ferments of mezcal producing region meaning that are adapted to this type of substrate.

3.2 Monitoring of kinetics

Kinetics of biomass growth and reducing sugars consumption were monitored every four hours, along 50 h of fermentation. All the strains used agave juice as substrate and cultivation conditions were those belonging to the central point: 30 °C, 12 °Brix, pH = 5 and inoculum of 2 million cells/mL; these are almost ideal conditions for fermentation as reported some authors, who found through response surface methodology very similar values (León-Rodríguez et al., 2008; Pérez et al., 2013).

Experimental results showed that the strain belonging to the genus *C. lusitaniae* exhibited the highest cell growth, being 5.625 x 10^8 cells/mL. *S. cerevisiae, K. marxianus,* and *Z. bailii* reached 3.485 x 10^8 cells/mL, 4.490 x 10^8 cells/mL and 4.280 x 10^8 cells/mL populations respectively. These results point out that non-*Saccharomyces* yeast growth is similar to or greater than that of *S. cerevisiae*, results also confirmed by Segura-García et al. (2015) studies, who found final populations in agave juice to range from 6.90 x 10^7 cells/mL to 3.13 x 10^8 cells/mL, where the strain with the highest population was *P. kluyveri* compared to *K. marxianus* and *S. cerevisiae*.

Comparing growth rates, *S. cerevisiae* did not present lag phase or adjustment to culture medium phase (Figure 1a); this because the inoculums medium was similar to the treatment one, and because the sample is taken in inoculum logarithmic phase. In contrast, *C. lusitaniae* (Figure 1b), *K. marxianus* (Figure 1c), and *Z. bailii* (Figure 1d) presented adaptation phase within 2-4 h. The exponential growth phase ended about 13 h for *S. cerevisiae* (Figure 1a), and 24 h for *C. lusitaniae* (Figure 1b), *K. marxianus* (Figure 1c), and *Z. bailii* (Figure 1d), which indicates that non-conventional strains are adaptive and growth slowly in comparison to *S. cerevisiae*. According to the literature (Querol et al., 1990), *C. lusitaniae*, *K. marxianus* and *Z. bailii* proliferate in the early days of fermentation favored by the low alcohol content in the musts. Regarding to the medium acidification, *S. cerevisiae* and *C. lusitaniae* concluded in a pH value of 4.2, while *Z. bailii* and *K. marxianus*, in 3.79 and 3.73, respectively; these pH value correlates with the content of acetic acid produced in the musts, as it was observed by Ramírez & Molina (2005) who reported final pH values of 3.5 and 4 for *S. cerevisiae*.

Reducing sugars consumption by *S. cerevisiae* was faster than the non-conventional yeast, consuming almost all the sugars at 16 h of fermentation (Figure 1a). *C. lusitaniae* consumed most of the reducing sugars in about 28 h (Figure 1b), and *K. marxianus* (Figure 1c) and *Z. bailii* (Figure 1c) required about 50 h consuming most of the sugars. These results are consistent with those reported by Segura-García et al. (2015), who found that after 36 h of fermentation, *S. cerevisiae* and *K. marxianus* had consumed nearly all the sugar present in the agave juice medium, while *P. kluyveri* took a further 48 h to achieve this consumption.

3.3 Comparative analysis of the response variables

The four strains exhibit different behavior during alcoholic fermentation. Non-*Saccharomyces* yeasts proved to be great ethanol producers. To find the best strains for ethanol production, a comparative study of the four yeast strains, analyzing cell

Table 2. Response variables for each strain*.

Essay	*S. cerevisiae*		*C. lusitanea*		*K. marxianus*		*Z. bailii*	
	Cells/ mL	Ethanol (% v/v)	Cells/ mL	Ethanol (% v/v)	Cells/ mL	Ethanol (% v/v)	Cells/ mL	Ethanol (% v/v)
1	**3.485 x 10^8**	2.67	5.075 x 10^8	4.42	**4.490 x 10^8**	2.67	3.835 x 10^8	3.81
2	1.645 x 10^8	5.77	3.600 x 10^8	4.24	2.475 x 10^8	**7.30**	2.790 x 10^8	4.42
3	2.625 x 10^8	2.38	5.075 x 10^8	**5.13**	3.255 x 10^8	2.42	3.255 x 10^8	3.27
4	1.520 x 10^8	4.08	3.600 x 10^8	4.67	3.005 x 10^8	7.73	2.395 x 10^8	3.72
5	1.635 x 10^8	5.90	**5.625 x 10^8**	3.00	3.545 x 10^8	2.65	**4.280 x 10^8**	3.91
6	1.320 x 10^8	3.31	4.205 x 10^8	3.43	2.665 x 10^8	4.99	2.435 x 10^8	**5.91**
7	1.395 x 10^8	3.04	3.725 x 10^8	2.14	3.180 x 10^8	4.26	1.820 x 10^8	2.40
8	1.880 x 10^8	5.24	3.900 x 10^8	2.56	3.975 x 10^8	4.66	3.095 x 10^8	2.51
9	2.060 x 10^8	**5.96**	3.875 x 10^8	3.09	3.745 x 10^8	1.47	3.725 x 10^8	3.97
10	1.960 x 10^8	4.90	4.450 x 10^8	3.98	3.745 x 10^8	5.37	2.750 x 10^8	4.58
11	2.170 x 10^8	3.69	4.225 x 10^8	4.53	3.035 x 10^8	4.19	2.910 x 10^8	5.24

*Conditions given in Table 1. The values presented in this table are the maximum observed during the fermentation phase.

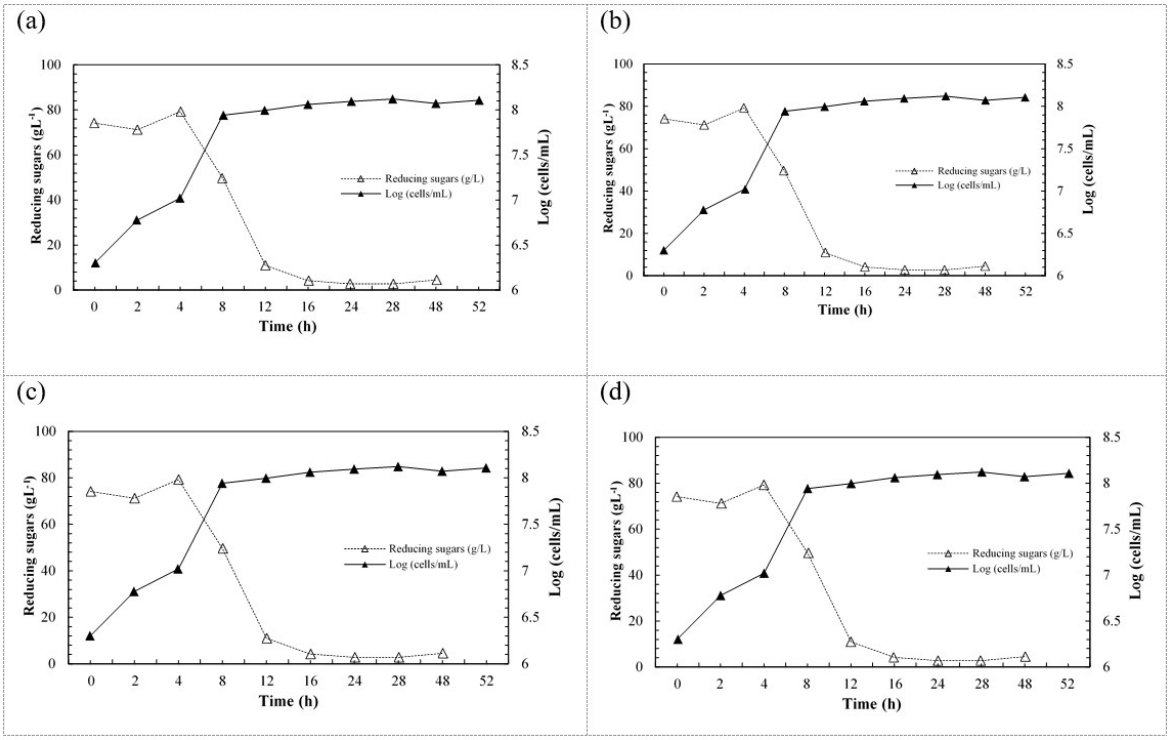

Figure 1. Growth and sugar consumption kinetics for (a) *S. cerevisiae*; (b) *C. lusitaneae*; (c) *K. marxianus*; and (d) *Z. bailii*.

growth, reducing sugars consumption and ethanol production, was performed (Table 2).

The strain with the highest cell growth was *C. lusitaniae*, however it obtained the lowest ethanol concentration. The strains with the highest consumption of sugars were *C. lusitaniae* and *Z. bailii*, which does not correlate with ethanol production.

K. marxianus strain reached higher ethanol concentrations than *S. cerevisiae*, these results agreed with those by Segura-García et al. (2015), who used *A. tequilana* Weber var. azul as substrate. López et al. (2012) also studied a yeast isolated from native agave must and was identified as *K. marxianus* UMPe-1 by 26S rRNA sequencing. They compared this strain with the baker's yeast *S. cerevisiae* Pan 1 and their findings demonstrated that the UMPe-1 yeast was able to support the sugar content of agave must and glucose up to 22% (w/v) and tolerated 10% (v/v) ethanol concentration in the medium with 50% cells survival. Carried out a pilot and industrial fermentation of agave must and found that *K. marxianus* UMPe-1 produced ethanol with yields of 94-96% compared with *S. cerevisiae* with 70-76% yields.

3.4 Determination of kinetic parameters

For determination of kinetic parameters, the cell growth data for each strain were adjusted to a logistic model (Equation 1) described by Zwietering et al. (1990); kinetic parameters obtained are shown in Table 3.

$$y = \frac{A}{\left\{1 + exp\left[\frac{4\mu_m}{A}(\lambda - t) + 2\right]\right\}} \quad (1)$$

It is interesting to note that *S. cerevisiae* exhibited the lowest cell growth, although seemed to be the best adapted strain. In contrast, *C. lusitaniae* exhibited the largest growth, which correlates with the high sugar's consumption and the low ethanol productivity. *K. marxianus* and Z. *bailii* exhibited values of growth in the middle of the other two strains. Maximum growth rate of *K. marxianus* was the highest one, followed closely by the rate of Z. *bailii*; *S. cerevisiae* and *C. lusitaniae* exhibited maximum growth rates that are less than one t//hird of the other two rates. Strains with largest adaptation phase are *K. marxianus* and Z. *bailii* with 2.7687 h and 3.5627 h, respectively.

León-Rodríguez et al. (2008) applied the Response Surface methodology to optimize the fermentative phase for the mezcal production from *Agave salmiana*, during the kinetic fermentation study, they determined the maximum specific growth rate being of 0.6 h^{-1}, this result is similar to that found for *S. cerevisiae*, 0.582 h^{-1}. Other values obtained were 0.5528, 1.9650 and 1.3246 h^{-1} for *C. lusitaniae*, *K. marxianus* and Z. *bailii* respectively.

The cell division is an important part in the cell life cycle, this division occurs during growth. Doubling time is similar for the four strains (Table 4), and they can be transformed into the division rate. In the case of *S. cerevisiae* in batch fermentation, Muñoz & Catrilaf (2013) estimated Dt = 3.68 h, which means larger doubling time than in this study. The same happens with another important kinetic parameter, specific growth rate, which was estimated by the same authors as $\mu = 0.188\ h^{-1}$, and in this work the value is lower (Table 4). For the other strains there are not data about kinetic parameters.

3.5 Analysis statistic for ethanol production

By drawing the main effects of the four factors studied on each strain, it is possible to find the combination of levels that result in the highest production of ethanol (Table 5).

Response surfaces (Figure 2) were used to know the experimental region, and to detect the best direction to move temperature and the initial sugars concentration. Using these conditions for each individual strain, it is possible to maximize ethanol concentrations at levels of about 5.99% v/v, 5.25% v/v, 7.33% v/v and 4.92% v/v for *S. cerevisiae, C. lusitaniae*, *K. marxianus* and *Z. bailii*, respectively.

Finally, statistic models were estimated for ethanol production, on the base of the significant factors determined for each strain. In these models Y_S means ethanol concentration (%v/v) reached when using strain 'S', X_1 = temperature (°C), X_2 = initial sugars concentration (°Brix), X_3 = pH, X_4 = initial cell concentration (cells/mL/10^6). Models for *S. cerevisiae* (Equation 2), *C. lusitanie* (Equation 3), *K. marxianus* (Equation 4), and *Z. bailii* (Equation 5), are shown below.

$$Y_{S.cerevisiae} = 4.267 + 0.332X_1 + 0.915X_2 - 0.695X_4 - 0.607X_1X_3 - 0.457X_1X_4 + 0.060X_3X_4 \quad (2)$$

$$Y_{C.lusitaniae} = 3.744 - 0.121X_1 + 0.604X_2 - 0.326X_3 + 0.316X_1X_2 + 0.671X_3X_4 - 0.099X_2X_3 \quad (3)$$

Table 3. Kinetic parameters obtained from the logistic mathematical model: μ_{max} = maximum growth rate; λ = lag phase time.

Yeast	Maximum growth (cells/mL)/10^6	μ_{max} (h^{-1})	λ (h)
S. cerevisiae	170 ± 41.4	0.5823 ± 0.03	1.3803 ± 0.30
C. lusitaniae	329 ± 19.0	0.5528 ± 0.10	2.2207 ± 0.30
K. marxianus	226 ± 2.56	1.9650 ± 0.96	2.7687 ± 0.54
Z. bailii	258 ± 24.5	1.3246 ± 0.79	3.5627 ± 0.99

Table 4. Kinetic parameters obtained to determine the kinetic behavior over fermentation time. Dt: Doubling time; δ: Division rate; μ: Specific growth rate.

Yeast	Dt (h)	δ (h^{-1})	μ (h^{-1})
S. cerevisiae	3.1271 ± 0.0298	0.3198 ± 0.0030	0.0877 ± 0.0026
C. lusitaniae	2.9371 ± 0.0205	0.3405 ± 0.0024	0.1061 ± 0.0022
K. marxianus	2.9958 ± 0.0479	0.3339 ± 0.0053	0.1001 ± 0.0048
Z. bailii	3.0258 ± 0.0149	0.3305 ± 0.0016	0.0970 ± 0.0014

$$Y_{K.marxianus} = 4.199 + 0.855X_1 + 0.69X_2 + 0.432X_4 + 1.255X_1X_2 - 0.0075X_1X_4 - 0.7625X_2X_4 \quad (4)$$

$$Y_{Z.bailii} = 3.976 + 0.126X_1 + 0.328X_2 - 0.526X_4 + 0.114X_1X_2 - 0.041X_1X_4 + 0.191X_2X_4 \quad (5)$$

3.6 Analysis of mixed cultures at bioreactor level

Once the individual strains were characterized, it was possible to select ideal blends of strains to perform an alcoholic fermentation to produce mezcal; and to establish the culture conditions to achieve ethanol yield and to reduce processing time.

To analyze the fermentation process but with different strains simultaneously an experimental mix design was formulated. The function of the response of interest depends on the relative proportions of each component (yeast), not the absolute amount. A Simplex-Centroid design was constructed for the study of yeast consortia. According to results of the analysis of pure cultures, three strains were selected: *K. marxianus*, *S. cerevisiae* and *Z. bailii*, based on their high ethanol production. The design was constructed with three components (3 yeasts) and operating conditions were held constant at 32.5 °C, 14 °Brix, pH = 5.5, 150 rpm, and 1 vvm; these values were determined using the results of analyzes of fractional factorial designs. The response variables were cellular growth and once distilled the musts, the alcoholic strength (% v/v ethanol) (Table 6). The regulatory standard establishes a range of 35-55% v/v of ethanol, for a proper distilled to marketing. A linear plus interactions model (Equation 6) was selected, assuming a confidence level of 95.0%.

$$Y = 9.820X_1 + 42.820X_2 + 31.8203X_3 + 61.595X_1X_2 - 32.405X_1X_3 - 38.405X_2X_3 \quad (6)$$

Here Y is ethanol concentration (%v/v), X_1 = *S. cerevisiae*, X_2 = *K. marxianus*, X_3 = *Z. bailii*.

The response surface analysis and the surface contours (Figure 3a) allowed to determine the best yeast blend for higher alcohol yield. According to these graphs, to obtain higher concentrations of alcohol is necessary to involve blends between *S. cerevisiae* and *K. marxianus*, with distillates of 41% of alcohol volume approximately, which was corroborated with a contour plot (Figure 3b). The higher cell growth found was 3.76 x 10^8 cells/mL (test 4), with cultures of *S. cerevisiae* and *Z. bailii*.

The values of cell concentration and ethanol concentration in the fermented musts were reduced in the mix design (ethanol = 2-4%v/v), this because the yeasts were subjected to hydrodynamic stress, due to the agitation and aeration

Table 5. Best values for maximum ethanol concentration.

Factor	High	Low	*Ideal*			
			S. cerevisiae	*C. lusitaniae*	*K. marxianus*	*Z. bailii*
Temperature (°C)	25	35	35	25	35	35
Initial sugars concentration (°Brix)	10	14	10	14	14	10
pH	4.5	5.5	5.5	4.5	4.5	4.5
Initial cell concentration (cells/mL)/10^6	1.0	3.0	1.0	3.0	1.0	3.0

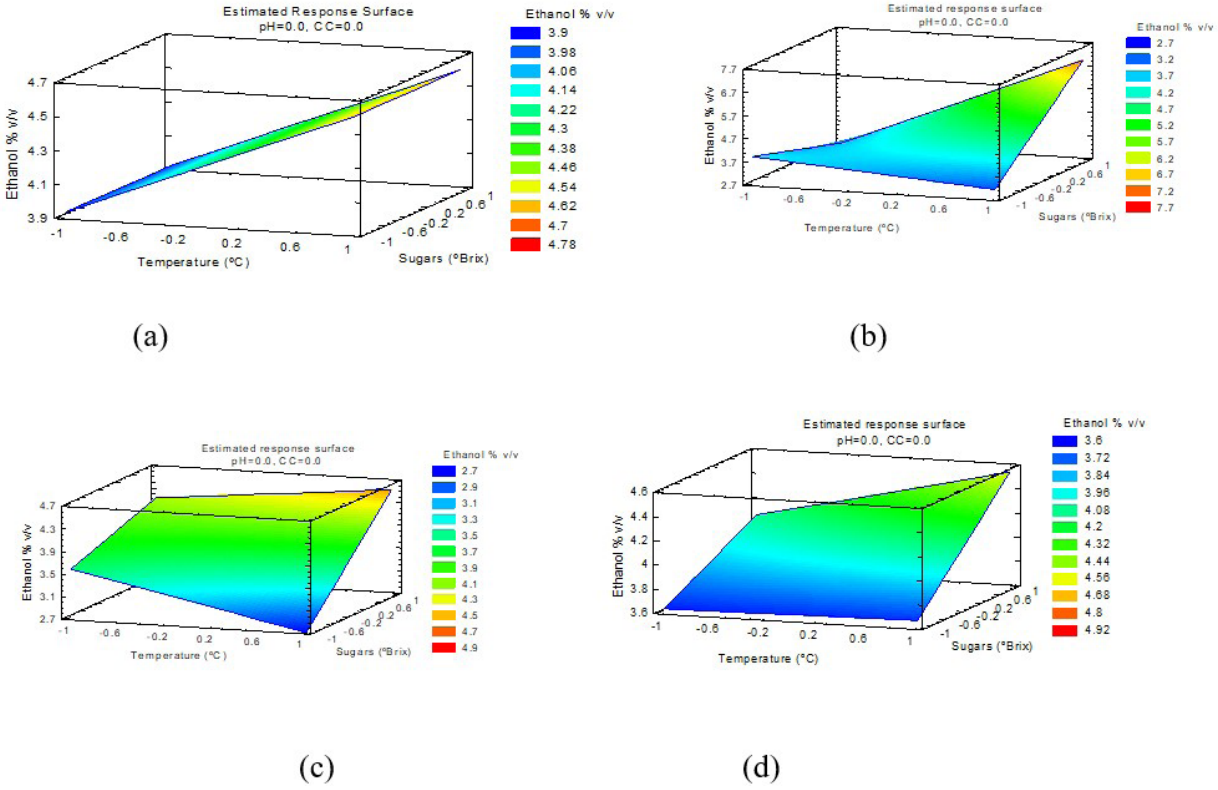

Figure 2. Response surfaces for ethanol production: (a) *S. cerevisiae*; (b) *C. lusitaniae*; (c) *K. marxianus;* and (d) *Z. bailii*. CC = Cell concentration. pH and CC are in their center points.

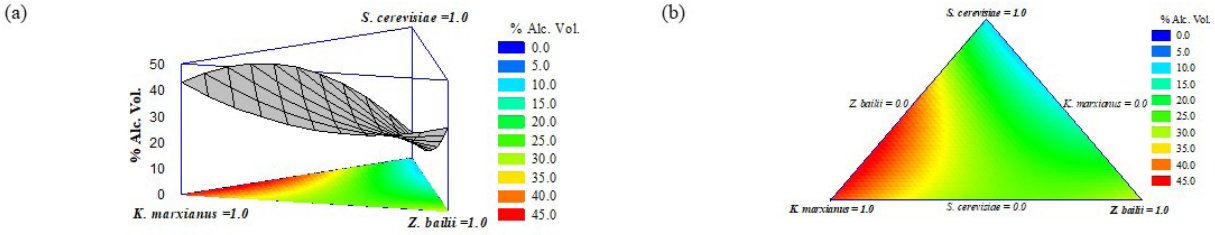

Figure 3. Diagrams estimated for %Alc. Vol. (a) Response surface; (b) Contours surface response.

Table 6. Simplex-Centroid experimental design and response variables.

Test	*S. cerevisiae*	*K. marxianus*	*Z. bailii*	Ethanol %v	Initial cell concentration (cells/mL)/10^6
1	0.0	1.0	0.0	43	185
2	0.5	0.5	0.0	41	238
3	0.0	0.0	1.0	32	323
4	0.5	0.0	0.5	12	376
5	1.0	0.0	0.0	10	331
6	0.3	0.3	0.3	24	355
7	0.0	0.5	0.5	27	198
8	0.3	0.3	0.3	29	261
9	0.3	0.3	0.3	30	204

Table 7. Concentrations of volatile compounds of mixed cultures distillates (mg/100 mL of anhydrous alcohol).

Test	Acetaldehyde	Methanol	S-Butanol	1-Propanol	Iso-Butanol	Iso-Amyl	Ethyl acetate
1	ND	216.24 ± 2.3	ND	103.95 ± 1.53	95.30 ± 1.36	270.67 ± 8.54	2.89 ± 2.29
2	ND	102.99 ± 42.9	ND	56.01 ± 22.01	93.77 ± 29.73	441.51 ± 13.60	ND
3	ND	484.047 ± 28.8	ND	89.31 ± 4.67	183.15 ± 4.27	489.63 ± 41.05	16.36 ± 0.54
4	ND	520.75 ± 62.7	ND	66.33 ± 11.51	53.96 ± 4.06	121.69 ± 35.86	ND
5	ND	410.36 ± 25.6	ND	118.00 ± 61.79	61.39 ± 13.92	49.73 ± 4.39	2.98 ± 27.04
6	ND	125.32 ± 18.1	ND	39.39 ± 28.52	61.64 ± 4.55	260.95 ± 24.04	ND
7	ND	491.38 ± 27.4	ND	66.78 ± 4.60	38.62 ± 0.38	98.71 ± 9.72	ND
8	ND	273.09 ± 8.8	ND	107.74 ± 9.81	86.32 ± 15.30	320.61 ± 1.11	ND
9	ND	235.08 ± 13.9	ND	139.48 ± 63.44	186.53 ± 73.80	314.75 ± 29.05	4.61 ± 8.57

Aldehydes (such as acetaldehyde): 0-40; Methanol: 3-300; Higher alcohols (such as S-Butanol, 1-propanol, and iso-Amyl): 20-500; Esters (ethyl acetate): 2-200. Values expressed in mg/100 mL of anhydrous alcohol. ND: Compound no measurable by equipment due to their low presence (NOM-006-SCFI-2005, NOM-070-SCFI-1994 – Norma Oficial Mexicana, 1994, 2005).

in the bioreactor, another factor that has effect in these two measurements is the interaction between yeast in the mixed cultures by an intolerance to ethanol produced or other volatile compounds generated, in addition to the rapid depletion of the substrate. The analysis of volatile compounds in distillates shown in Table 7. Internal standardization method was used. All values are within the ranges established by NOM-006-SCFI-2005 and NOM-070-SCFI-1994 (Norma Oficial Mexicana, 1994, 2005) standards, except for methanol which exceeds the permitted content, this because is not waste sufficient volume of the first distillate (first fraction) which contain most of the methanol produced.

In all test's methanol, propanol, Iso-butanol and Iso-amyl, are the most abundant compounds, contrary to ethyl acetate which is found in very small concentration. As the acetaldehyde and S-butanol were identified in the chromatograms but it was not quantification possible due to its low chromatographic area. In this case, a higher concentration to quantify them so it would be interesting to carry out an organoleptic study.

4 Conclusion

The developed models could predict the quality of mezcal developed from *Agave cupreata* using a yeast consortium. Experimental results showed that the strain belonging to the genus *C. lusitaniae* exhibited the highest cell growth, being 5.625 x 10^8 cells/mL. However, it does not produce enough ethanol, majority compound in an alcoholic beverage. *K. marxianus* reached higher ethanol concentrations than *S. cerevisiae,* 7.30% v/v and 5.96% v/v respectively, it was subsequently demonstrated with mixtures design, according to these results, to get higher alcohol content is advisable to use mixtures involving *K. marxianus* and *S. cerevisiae*, obtaining distillates about 41% of alcohol volume at optimal conditions of fermentation were temperature of 32.5 °C, initial sugar contain 14 °Brix, pH of 5.5, agitation of 150 rpm, and 1 vvm by 52 h. The composition of mezcal and its variability is complex, and each component requires additional studies to elucidate its origin and its effects on compliance with the standards and the sensory characteristics in the final product. To achieve it is essential to standardize the production processes to achieve the products of uniform and constant quality.

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Conflict of interest

The authors have no conflict of interest to declare.

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