Colegio de Postgraduados

The use of native yeasts to improve the organoleptic characteristics and yield of artisanal mezcal

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ABSTRACT

Objective: To assess the use of native yeasts on the yield and on the organoleptic characteristics of artisanal mezcal in Guerrero, Mexico.

Design/Methodology/Approach: Native yeasts from fermentation vats containing *Agave angustifolia* and *A. cupreata* were subjected to molecular isolation and identification in three regions of the state of Guerrero. A consortium of yeasts was designed and used to produce artisanal mezcal, using *A. cupreata* as a substrate. The experimental mezcal (with yeasts) and the control (without yeasts, only producer lore) were compared. Finally, the physicochemical and sensory characteristics of the final product were assessed at the Concours Mondial de Bruxelles 2020.

Results: *S. cerevisiae* A5GM, *T. delbrueckii* A6GM, *S. cerevisiae* A3GM, and *K. marxianus* A2GM were identified and used in a consortium that produced 46% more yield (8.3 kg L^{-1} compared to 10.5 kg L^{-1}). The mezcal produced with this consortium obtained the Grand Gold medal based on its sensory characteristics.

Study Limitations/Implications: To work with the producer in both systems with and without yeast.

Findings/Conclusions: The consortium improved the production yield by 26%, along with the sensory characteristics; therefore, the use of native yeasts is feasible and recommended for the improvement of processes in the production of artisanal mezcal from Guerrero.

Keywords: agave, artisanal mezcal, native yeasts, yields, sensory characteristics.

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INTRODUCTION

Artisanal mezcal in Mexico is generally produced on a small scale in different states that are protected by the Denomination of Origin Mezcal (DOM), through the spontaneous fermentation of agave must (Torres-Velázquez *et al.*, 2022; Domínguez-Coronado *et al.*, 2021). However, this type of fermentation results in productions with varying organoleptic characteristics, which generates different yields per production (Aldrete-Tapia *et al.*, 2020).

The spontaneous fermentation of mezcal is carried out by microorganisms from the agave or the environment of the production system (Pérez-Hernández *et al.*, 2022). These specialized native microorganisms participate throughout the process and their different fermentative capacities can be modified by pH, type of substrate, and temperature (Alcázar-Valle *et al.*, 2019, Núñez-Guerrero *et al.*, 2019). Therefore, *mezcaleros* (mezcal producers) consider that the yields and organoleptic characteristics of the production of artisanal mezcal must be standardized and increased, using native yeasts from the agave must through starter cultures and defined production methodologies (Aldrete-Tapia *et al.*, 2020). The most important conventional yeasts are *S. cerevisiae* —which has a high tolerance to ethanol production— and non-*Saccharomyces* yeasts —due to their influence on the organoleptic composition of the drink, given their perfect adaptation to the environment and because they frequently are more numerous than *S. cerevisiae* (Aldrete-Tapia *et al.*, 2018, Nuñez-Guerrero *et al.*, 2019). The presence of non-*Saccharomyces* native yeasts in the must leads to a competition for nutrients (Alvarez-Ainza *et al.*, 2021). The diversity of native microbiota during fermentation has been described in different regions that produce agave distillates. The prevailing species was *S. cerevisiae*, followed by *Kluyveromyces marxianus*, *Zygosaccharomyces rouxii*, *Torulaspora delbrueckii*, *Pichia membranifaciens*, and other species (Aldrete-Tapia *et al.*, 2018; Pérez-Hernández *et al.*, 2022; Núñez-Guerrero *et al.*, 2019; Kirchmayr *et al.*, 2017; Alvarez-Ainza *et al.*, 2021; Vera-Guzmán *et al.*, 2018). *T. delbrueckii* produces a lower concentration of acetic acid than *S. cerevisiae*, which benefits alcoholic beverages, since a >0.8 g/L concentration could generate a vinegar odor (Ogawa *et al.*, 2022). Likewise, *K. marxianus* and *S. cerevisiae* can be found at the end of the fermentation stage, with similar levels of resistance to ethanol in the fermentation medium (Martínez-Estrada *et al.*, 2019). In addition, the microbiota in the most of the agave can be affected or favored by various factors such as: species, maturity, geographical location of the fields, factories, climatic conditions, and the artisanal production experience of each mezcalero (Lappa *et al.*, 2020; Ruiz-Teran *et al.*, 2019). The objective of this study was to assess the potential use of native yeasts in fermentation processes to increase the yield (kg) of agave per liter and the sensory quality of artisanal mezcal.

MATERIALS AND METHODS

Selection of native yeasts from agave must

Must was obtained from various fermentation stages in five mezcal factories of the following two municipalities: Eduardo Neri and Mochitlán. Tlanipatla (TI) in Eduardo Neri is located at −99.451667 longitude (dd) and +17.808056 latitude (dd). Mochitlán (Mo) in Mochitlán is located at −99.369167 longitude (dd) and +17.471389 latitude (dd).

To isolate and identify the yeasts most frequently found in the must, it was subjected to serial dilutions until a 1:1,000 ratio was reached; they were inoculated on yeast extract peptone dextrose agar (YPD) (Becton Dickinson, USA) and incubated at 30 °C for 48 h. Colonies were expressed in CFU/mL (Mambuscay *et al.*, 2013).

Amplification of the ITSI1-5.8S-ITSI2 region of the 5.8S rRNA gene

The total DNA of the yeast was extracted according to Querol *et al.* (1992). Subsequently, two variable regions that flank the 5.8S ribosomal RNA (rRNA) gene were amplified, using:

- 1 μL of DNA plus 24 µL of PCR master mix for a final volume of 25 μL: 50 mM ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) (White *et al.*, 1990; Suárez *et al.*, 2007),
- 2 μL of dNTP, 1X Taq Buffer with $(NH_4)_2SO_4$ (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20),
- 3 μL of $MgCl_2$, and
- 0.5 μL of Taq DNA Polymerase (Thermo Scientific, USA).

The amplification was carried out in a GeneAmp® PCR 2400 thermal cycler (Applied Biosystems) following the initial denaturation: 94 °C for 5 min, 94 °C for 1 min (30 cycles), 55 °C for 1 min, and 72 °C for 2 min, with a final extension of 72 °C for 10 min. PCR outputs were analyzed by electrophoresis in 1% agarose gel, stained with ethidium bromide, and visualized and recorded with a GVM30UV transilluminator (Syngene, UK). Finally, the size of the bands was analyzed using a Generuler 1Kb molecular weight marker (Fermentas, USA). To obtain the sequences, the PCR outputs were sent to the Institute of Biotechnology of UNAM. Once the sequences were obtained, they were analyzed with the Chromas 2.6 software (Technelysium Pty. Ltd.). The results were input into the database of Blast program, in order to search for homology with reported yeast sequences. Finally, the identified species were subjected to a phylogenetic analysis in the Mega 7.1 software (Mega Ltd), using the neighbor-joining method based on the distance matrix specified by Jukes and Cantor (1969).

Fermentation stage

Obtaining raw materials

Four tons of cooked *Agave cupreata*, with 8 to 10 years of maturation, were used. They were harvested at the El Calvario community, municipality of Chilpancingo, Guerrero. Agave was cooked in a ground oven for 6 days. Once the agave hearts were cooked, they were fragmented into ≈15 cm pieces and the bagasse were obtained using a 13 Hp hammer mill (CIATEJ, 2014).

Pre-inoculum preparation

A single consortium of yeasts (A5GM, A6GM, A3GM, and A2GM) was formed and cultured for 48 h at 30 °C in a Sabouraud Dextrose Agar (SDA) growth medium (MCD-

Lab). Two pre-inocula were prepared using 20 L of *A. cupreata* juice with bagasse, in clean and new 20 L containers (one container per pre-inocula). They were adjusted to 6 °Bx with an AT-HHR2N optical refractometer (Twilight) (CIATEJ, 2014).

Inoculum preparation

The final volume of the inoculum for each experimental vat was 50 L of agave juice with consortium adjusted to 6 °Bx. The control vats were not inoculated. The containers with the inoculum were incubated for 12 h prior to the preparation of the vats (CIATEJ, 2014).

Fermentation vats

The control and the experimental samples were established in 1,000 L wooden fermentation vats with the following preparation quantities: 650 kg of cooked agave and 350 L of water in triplicate, adjusted to 12 °Brix following the conditions of the CIATEJ (2014). During the fermentation process, °Brix were measured every 12 h, with an AT-HHR2N optical refractometer (Twilight). The entire process was based on the *mezcalero* lore (characteristic smell of the must) and °Brix measurements (CIATEJ, 2014).

Distillation and product refinement processes

The distillation in each fermentation vat (with and without yeasts) was carried out when the °Brix reached 2.0. First, a 600 L stainless steel still was used and the distillate (ordinary) was received after it began to boil. Subsequently, at the end of the first distillation, the equipment was washed before a second distillation (refining) started. This stage was done over a low heat to avoid smoking or burning the product. The heads, hearts, and tails were separated. Finally, the mezcal was adjusted to 48° ABV (CIATEJ, 2014). During distillation, alcohol content was measured using a PCE-ALK handheld refractometer (PCE Instruments) (CIATEJ, 2014).

Mezcal quality analysis

One L of the control and one of experimental mezcal were sent to the Centro de Innovación y Desarrollo Agroalimentario de Michoacán (CIDAM) (accredited by the EMA, the Mexican accreditation agency), where the following analyses were according to the NOM-070-SCFI-2016 (Bebidas alcohólicas-Mezcal-Especificaciones) Official Mexican Standard: alcohol volume at 20 °C, dry extract, higher alcohols, methanol, furfural, aldehydes, and esters.

Sensory analysis

The sensory analysis was based on the participation of the control and experimental mezcals in the Spirits Selection by Concours Mondial de Bruxelles. The drinks were assessed by a panel of internationally renowned experts, with a total of 103 international judges of 28 nationalities. The number of samples tasted per session is deliberately restricted to 35; the samples were assessed anonymously, hiding the shape of the bottle and the name of the drink from the testers. The appearance, aroma, and flavor of the drink were assessed.

Three types of medals are awarded in this competition: Grand Gold Medal, Gold, and Silver Medals.

RESULTS AND DISCUSSION

Five manufacturing factories were visited in Tlanipatla (Eduardo Neri) and Mochitlán, Guerrero. A letter code (A to E) was assigned to each factory in the order of the sampling. Additionally, a number was assigned according to the vat from which the sample was taken; the said number included day of fermentation, place, and raw material data. Once the samples were sent to the research laboratory, the colony forming units were determined for each sample on YPD agar after 48 hours of incubation. The number of microorganisms decreased as the days of fermentation passed —*i.e.*, the samples corresponding from days 1 to 3 recorded more microorganisms (36×10^4 CFU/mL of yeasts per factory) than the samples from days 4 to 12 ($2\text{-}3\times10^3$ CFU/mL of yeasts per factory).

Based on the macro and microscopic morphology, close to 20 different yeast phenotypes were identified, with approximately 80 strains in the different factories. The 1% carbohydrate fermentation (glucose, fructose, maltose, and others) was carried out and eight representative profiles were obtained and selected for molecular identification (Table 1).

Some researchers report that, in the first days of fermentation, the most proliferate genera are non-conventional yeasts. After the first days, a supposed intolerance to ethanol and/or a nutritional limitation cause a drastic reduction in these genera (Kunkee, 1984); this phenomenon allows the growth of other species which have greater tolerance to ethanol —such as *Saccharomyces*, which is considered the main species responsible for alcoholic fermentations (Ribéreau 1985, Aldrete-Tapia *et al.*, 2018).

Only eight strains were selected from the two mezcal producing regions. They were subjected to a molecular identification and the analysis of the resulting sequences identified only four genera (Table 2): *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, and *Pichia kluyveri*.

Table 1. Colony forming units during the fermentation process.

Agave angustifolia				***Agave cupreata***			
Sample		**CFU/mL**	**Time (h)**	**Sample**		**CFU/mL**	**Time (h)**
AX	A1	65.5×10^4	24	TL	B1	29×10^4	24
	A2	14.5×10^4	72		B2	33×10^4	48
	A3	11.5×10^4	144		B3	30.5×10^4	72
	D1	4.5×10^4	24		C1	10.5×10^4	168
	D2	12×10^4	96		C2	7×10^4	192
	E1	5.5×10^4	72	MO	C3	3.5×10^4	216
	E2	13.5×10^4	96		C4	3×10^4	240
					C5	8×10^4	264
					C6	6×10^4	96

CFU/mL results on average, (h): fermentation time at sample collection. Sampling location: AX: Axaxacualco, TL: Tlanipatla, MO: Mochitlán.

Table 2. Molecular identificación of yeasts.

Isolation	5.8S rDNA	Maximum identified
A1GM	*Torulaspora delbrueckii*	99%
A2GM	*Kluyveromyces marxianus*	98%
A3GM	*Saccharomyces cerevisiae*	99%
A4GM	*Pichia kluyveri*	98%
A5GM	*Saccharomyces cerevisiae*	99%
A6GM	*Torulaspora delbrueckii*	98%
A7GM	*Torulaspora delbrueckii*	99%
A9GM	*Torulaspora delbrueckii*	99%

The following yeasts were used to form the consortium for the inoculation of fermentation vats: *S. cerevisiae* A5GM, *T. delbrueckii* A6GM, *S. cerevisiae* A3GM, and *K. marxianus* A2GM. During the fermentation process, the sugar consumption was observed and measured in °Brix. The initial (control and experimental) fermentation vats were adjusted to 12 °Brix. The consortium was formed according to the characteristics attributed to each species. *K. marxianus* has a higher growth rate and lower production of ethyl acetate and acetaldehyde in *A. cupreata*; it can also produce the same or more ethanol than *S. cerevisiae* (Alcázar-Valle *et al.*, 2019; Martínez-Estrada *et al.*, 2019). *T. delbrueckii* has been more studied in wines and it is known to increase the concentration of the thiol 4MMP (4-mercapto-4-methylpentan-2-one), which is related to floral and herbaceous aromas (notes of boxwood, cassis, or broom flower) (AGROVIN, 2020; Ogawa *et al.*, 2022). *S. cerevisiae* is the largest producer of esters and it is the yeast with the greatest resistance to alcohol levels; likewise, it is considered the largest producer of ethanol (Alcázar-Valle *et al.*, 2019; Pretorius, 2000; Gabriel *et al.*, 2012). Between 6 and 7 days, the fermentation process reached 2 °Brix in both conditions, with a temperature between 28 to 33 °C. Pérez-Hernández *et al.*, (2022) reported similar results, finding that the optimal fermentation condition is an optimized temperature of 32.5 °C, which favors the production of biomass.

Once the refinement was completed, the alcohol levels were adjusted to obtain the mezcal at 48% ABV. Finally, 123.5 L±0.7 and 156 L±1 of mezcal were obtained for the control and the experiment, respectively.

Once the total L of mezcal produced in both cases was determined, the calculations were made considering the amount of raw material and distillation —*i.e.*, the amount of previously cooked and weighed agave, related to the liters of total ethanol obtained—, in order to observe the yield (L) of mezcal kg^{-1} of agave used. On the one hand, for every 10.5 kg L^{-1} of agave in the control vats, one liter of mezcal was obtained; on the other hand, for every 8 kg L^{-1} of agave in the experimental vats, one liter of mezcal was obtained. In conclusion, the use of native yeasts results in a 26% increase in yield. The quality analysis was based on the NOM-070-SCFI-2016 (Bebidas alcohólicas-Mezcal-Especificaciones) Mexican official standard. Table 2 shows the parameters of both mezcals within the range established by the said standard. The physicochemical analysis and its results allowed the research team to proceed with the sensory and consumer acceptance

analyses. For this purpose, the mezcal was entered into the Spirits Selection by Concours Mondial de Bruxelles, a competition where tasting judges assess drinks from different parts of the world. The 103 judges awarded this experimental mezcal the Grand Gold medal (Table 3).

According to the quality analysis (Table 4), the experimental mezcal showed values within the NOM-070-SCFI-2016 Mexican official standard; the concentrations of higher alcohols were better in the experimental unit (191.63 mg/100 mL) which is related to the sensory descriptors. The production of higher alcohols depends on the species of agave and yeast used (Vera *et al.*, 2009). For example, fermentation the juice with bagasse of *A. cupreata* generates a high production. Meanwhile, the three species used in the consortium can produce high concentrations of 1-propanols.

Meanwhile, only *T. delbrueckii* did not record a good isobutanol and amyl alcohol production, unlike *S. cerevisiae* and *K. marxianus* —the former of which provided the highest concentration. Regarding the aldehydes, low values were obtained in both mezcals (Alcázar-Valle, 2019). The medium was suitable for the growth and development of the yeasts, given the imbalance in the medium (*e.g.*, a lack of nutrients) resulting from a high concentration of aldehydes. This situation affects the activity of the yeasts.

Table 3. Mezcal quality analysis according to the NOM-070-SCFI-2016 (Alcoholic beverages-mezcal-specifications) .

Essay	Mezcal			Applied standard
	Control	Experimental		
Alcohol volumen at 20 °C	46.70	40.55	% alcohol/Vol. A 20 °C	NMX-V-013-NORMEX-2013
Dry extract	0.06	0.07	G L^{-1}	NMX-V-017-NORMEX-2014
Higher alcohols	19.61	191.63	mg 100^{-1} mL AA	NMX-V-005-NORMEX-2013
Methanol	218.53	164.05	mg 100^{-1} mL AA	NMX-V-005-NORMEX-2013
Furfural	3.70	2.94	mg 100^{-1} mL AA	NMX-V-004-NORMEX-2013
Aldehydes	<4.61	5.55	mg 100^{-1} mL AA	NMX-V-005-NORMEX-2013
Esters	15.97	45.58	mg 100^{-1} mL AA	NMX-V-005-NORMEX-2013

note: results obtained in the distillation process, reported as means and with standard deviation (S) in each of the tests.

Table 4. Tasting notes obtained in the competition Spirits Selection by Concours Mondial de Bruxelles.

Mezcal	Qualification	Tasting Note
Control	66/100	**Appearance**: Transparent **Nose**: Dried and exotic fruit with a citrus and smoky touch on the back. **Palate**: Spicy tone continues with interesting sweetness on the palate. Tasty but luxurious alcohol tone that adds an intense spicy character. **Overall**: lack of elegance and grace, robust style with a warm but moderate length. Sound product with a discreet finish.
Experimental	Gran Gold Medal	**Appearance**: Clear and bright, without faults. **Nose**: A nice palette of fruity agave, harmonious florality, spicy and nutty clove and well-integrated smoky impressions caress the nose. **Paladar**: On the palate, a round, full, and soft body with characterful, earthy aromas is accompanied by a powerful, complex spiciness that makes you want to drink more. **Overall**: A lovely mezcal that feels like a relaxed stroll in a Winter Christmas market! ¡Bravo!

Meanwhile, the production of esters takes place in the final phase of fermentation. They provide fruity notes to alcoholic beverages and are related to the species of yeast and agave used. Ogawa (2022) has recently shown that *T. delbrueckii* may be related to ester production. The methanol values can indicate a correct cooking process, since they are produced by the degradation of the pectins found in the agave heart. Species of yeast with the pectin-methyl-esterase enzyme have been found in tequila, which allows them to hydrolyze pectins in fermentation and generate methanol; therefore, the presence of yeasts with this enzymatic capacity can be taken into consideration (Gallardo Valdez *et al.*, 2020). Furfural involves a procedure similar to that of methanol, except that its production is based on the degradation of carbohydrates; in the case of mezcal, they are generated during cooking and therefore also function as an indicator that verifies the use of agave as a raw material (Gallardo Valdez *et al.*, 2020). This phenomenon could be related to the results obtained, since relatively higher values for both compounds are observed in the control mezcal than in the experimental one (CIATEJ, 2014). Compared with the results obtained in the distillation, the tails obtained were low —if the standard deviation is taken into account. In terms of yield, a 46% increase was recorded in the experimental mezcal —higher than the result obtained by CIATEJ (2014), which studied the application of yeast in three different factories. In the said study, the B factory has a higher yield (11 kg L^{-1}) and the yeasts found (*S. cerevisiae*, *T. delbrueckii*, *Kazachstania exigua*, and *K. marxianus*) were similar to the consortium used in this study. In the case of the tasting descriptors, the aroma characteristics were better in the experimental mezcal (a more diverse palette of flavors) than in the control. In conclusion, these sensory descriptors seem to be provided by the consortium of yeasts used.

CONCLUSIONS

The consortium of native yeasts used achieved a 46% increase in yield (liters of mezcal) and improved the organoleptic and physicochemical characteristics of the product, leading to the award of the Grand Gold Medal in Brussels 2020. Therefore, the use of native yeasts is a good biotechnological proposal for the agave-mezcal production chain.

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