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Yeasts population in fermented sotol must, fermentative capacity and the higher alcohols production potential of isolated yeasts

Población de levaduras en mosto de sotol fermentado, capacidad fermentativa y potencial de producción de alcoholes superiores de levaduras aisladas

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Technological innovation: Isolation of native sotol yeasts with biotechnological potential.

Industrial Application Area: Alcoholic beverage industry: sotol and mezcal.

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Resumen

El sotol es una bebida alcohólica mexicana obtenida de la destilación de jugos de plantas fermentadas de sotol (*Dasylirion* spp.). Es fermentado naturalmente por levaduras autóctonas, quienes juegan un papel importante en la formación del *flavor* de las bebidas alcohólicas. Este estudio tuvo como objetivo identificar la población de levaduras de mostos de sotol fermentados, evaluar la capacidad fermentativa y el potencial de producción de alcoholes superior de levaduras aisladas para contribuir a la comprensión del papel que juegan las levaduras en la fermentación natural para producir sotol (bebida alcohólica destilada). Las levaduras aisladas se identificaron mediante análisis 5.8S-ITS-RFLP. Se detectaron *Saccharomyces cerevisiae* y cuatro especies de levaduras no *Saccharomyces*, incluidas *Pichia kudriavzevii*, *Pichia kodamaea*,

Schizosaccharomyces pombe y *Candida glabrata*. Se seleccionaron dos especies de levaduras autóctonas: *S. cerevisiae* (LTS12) y *P. kudriavzevii* (LTS8) para evaluar su perfil de desempeño en términos de crecimiento, consumo de azúcar, etanol y producción de alcoholes superiores en cultivo mixto inoculado en medio sintético, jugos de sotol *Dasylirion* spp. y maguey (*Agave angustifolia*). Los resultados indicaron que se favoreció el crecimiento de cultivos mixtos en jugos de maguey y sotol. En estos mostos naturales, las fermentaciones terminaron a las 120 h (5 días), las levaduras consumieron la mayor parte de los azúcares (≥96%), mientras que en medio sintético solo se consumió menos del 50% del azúcar (57,58 ± 0,25 g/L de azúcar residual) después de 384 h (16 días), por lo que se considera una fermentación estancada. El cultivo mixto tuvo un gran potencial de fermentación en jugo de maguey con un rendimiento de etanol de 0.33 g de etanol/g de sustrato y una mayor eficiencia (65%) que el jugo de sotol (51.54%) y medio sintético (41.89%). Los jugos fermentados de sotol y maguey se caracterizaron por una mayor cantidad de 2-feniletanol (42% y 70% del área de pico), un alcohol superior que contribuye a los atributos florales de las bebidas alcohólicas. Este alcohol superior fue producido principalmente por *P. kudriavzevii* como se evidenció en el experimento de cultivos puros en medio sintético. Los hallazgos sugieren que el rendimiento alcohólico, la eficiencia de la fermentación y la mayor producción de alcohol dependen de la naturaleza y composición del medio de cultivo. Estos resultados sugieren que el co-cultivo de *S. cerevisiae* LTS12 con *P. kudriavzevii* LTS8 podría ser una alternativa para fermentar jugos de sotol y maguey.

Palabras clave: Medios de cultivo, alcoholes superiores, levaduras autóctonas, *Pichia kudriavzevii*, sotol.

Abstract

Sotol is a Mexican alcoholic beverage obtained from the distillation of fermented sotol plants (*Dasylirion* spp.) juices. It is naturally fermented by autochthonous yeasts, which play an important role in the flavor formation of alcoholic beverages. This study aimed to identified yeasts population of fermented sotol, evaluation of the fermentative capacity, and higher alcohols production potential of isolated yeasts to contribute to the understanding of the play role the yeasts in the natural fermentation to produce this spirit. Isolated yeasts were identified by 5.8S-ITS-RFLP analysis. *Saccharomyces cerevisiae* and four non-*Saccharomyces* yeast species, including *Pichia kudriavzevii*, *Pichia kodamaea*, *Schizosaccharomyces pombe* and *Candida glabrata* were detected. Two autochthonous yeast species: *S. cerevisiae* (LTS12) and *P. kudriavzevii* (LTS8) were selected to evaluate their performance profile in terms of growth, sugar consumption, ethanol and higher alcohols production in mixed culture inoculated in synthetic medium, sotol (*Dasylirion* spp.) and maguey (*Agave angustifolia*) juices. The results indicated that the growth of mixed culture was favored in maguey and sotol juices. In these natural musts, fermentations finished after 120 h (5 days), the yeasts consumed most of the sugars (≥96%), while in synthetic medium only less than 50% of the sugar was consumed (57.58±0.25 g/L of residual sugar) after 384 h (16 days), so it is considered as a stuck fermentation. Mixed culture had a great fermenting potential in maguey juice with an ethanol yield of 0.33 g ethanol/g substrate and a higher efficiency (65%) than that of sotol juice (51.54%) and synthetic medium (41.89%). Fermented sotol and maguey juices were characterized by higher amount of 2-phenylethanol (42 and 70% of peak area), a higher alcohol that contribute to the floral attributes of the alcoholic beverages. This higher alcohol was produced mainly by *P. kudriavzevii* as evidenced in the experiment of pure cultures in synthetic medium (supplementary material). Findings suggest that the alcoholic yield, fermentation efficiency and higher alcohol production depend on the nature and composition of the culture medium. These

results suggest that the co-culture of *S. cerevisiae* LTS12 with *P. kudriavzevii* LTS8 could be an alternative to ferment sotol and maguey juices.

Keywords: Culture media, higher alcohols, indigenous yeasts, *Pichia kudriavzevii*, sotol.

I. Introduction

Sotol is a regional spirit from the North of Mexico, it is produced from the fermentation and distillation of the stems of *Dasylirion* genus plants, particularly *duranguensis*, *cedrosanum* and *wheeleri* species. It is produced in the territory protected by the Appellation of Origin sotol (NOM-159-SCFI-2004, Bebidas Alcohólicas-Sotol-Especificaciones y Métodos de Prueba) which comprises the Mexican states of Chihuahua, Coahuila, and Durango. Sotol production must meet the following stages in the elaboration process: sotol plants with diameter greater than 30 cm are selected or when it reaches around six years of age (Flores-Gallegos et al., 2019; Zavala-Díaz de la Serna et al., 2020), then, leaves of the plant are removed (jimado) to obtain the stem called pineapple (Flores-Gallegos et al., 2019). Later, the pineapples are cooked in an underground stone oven which are previously heated using local woods. The cooking process is well described in Flores-Gallegos et al. (2019). The cooked pineapples are ground in a mill or cut into small pieces, which are placed into wood vats, underground concrete, or stainless-steel tanks where *Dasylirion* fibbers are left for few hours in the recipients and then, water at ca 50ºC is added until fibbers are covered, must is left to ferment by natural microbiota. Reducing sugar in the cooked sotol pineapple is around 109 g/L (Casas-Acevedo et al., 2021), in some places, the reducing sugars in the must are adjusted at 10.5% or 10ºBrix (Zavala-Díaz de la Serna et al., 2020). Spontaneous fermentation is developed by Non-*Saccharomyces* and *Saccharomyces* yeasts and it stands for 4 to 10 days (Buenrostro-Figueroa, et al., 2012). In addition to yeasts population, lactic acid bacteria and filamentous fungi have been detected (Zavala-Díaz de la Serna et al., 2020).

Saccharomyces cerevisiae, *Pichia kudriavzevii*, *P. fermentans*, *P. guilliermondii*, *Candida cellae*, *C. parapsilopsis*, *C. tropicalis*, *Clavispora lusitaniae*, *Dipodascus australiensis*, *Galactomyces geotrichum* and *Kodamaea ohmeri* have been detected in spontaneous fermentation of sotol (Zavala-Díaz de la Serna et al., 2020). Knowledge of autochthonous yeasts population is an important step in the exploration of yeasts to be used as inoculum in alcoholic beverages production (Nolasco-Cancino et al., 2018). Tequila and mezcal are Mexican alcoholic beverages like sotol, in these maguey spirits several studies have been performed to design pure and mixed starter culture of *Saccharomyces* and non-*Saccharomyces* yeasts (Díaz-Montaño et al., 2008; Núñez-Guerrero et al., 2016; Nolasco-Cancino et al., 2018). It has been demonstrated that non-*Saccharomyces* yeasts provide a positive impact on sensorial characteristics of mezcal (Segura-García et al., 2015; Núñez-Guerrero et al., 2016). In other fermented beverages such as wine, it is showed that non-*Saccharomyces* yeast strains contribute to the fruity aroma quality culture, thus, mixed culture of *Saccharomyces* yeasts with non-*Saccharomyces* yeasts is a good tool to improve the aromatic complexity of the wine (Pei-Tong et al., 2016; Yu et al., 2018).

The aim of this work was to identify the yeasts population presents in fermented sotol must, and evaluate the ethanol production, alcoholic yield, and efficiency of fermentation, as well as the potential to produce higher alcohols of the isolated non-*Saccharomyces* yeast, *Pichia kudriavzevii*, in mixed culture with the autochthonous *Saccharomyces cerevisiae* on three culture media: synthetic media, sotol juice and maguey juice.

II. Material and methods

Fermented sotol juice sampling and yeast isolation

Samples for the isolation and identification of yeasts were taken at the end of the natural fermentation of sotol produced in the municipality of Aldama, Chihuahua. Samples of fermented sotol were placed aseptically in sterile Falcon tubes and transferred to the laboratory in ice boxes. Samples were diluted by serial decimal dilutions, and 100 µL of each dilution was plating by surface inoculation on WL nutrient agar with 100 mg/L of chloramphenicol. Cultures were incubated at 30 °C for 3 to 5 days. According to the macroscopic characteristic, colonies of different types were subculturing by striking on YPD agar (1.0% yeast extract, 2.0% glucose, 2.0% peptone, and 2.0% agar).

Yeast identification

Yeast DNA was extracted as described by Santiago-Urbina et al. (2015). The 5.8S-ITS (internal transcribed spacer region) rDNA of the isolated yeast was amplified by PCR assay, using primers ITS1F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4R (5'-TCCTCCGCTTATTGATATGC-3') and digesting the PCR amplicon with *Hae* III, *Hinf* I and *Cfo* I restriction enzymes (Invitrogen, CA, USA) as described by Santiago-Urbina et al. (2015). Electrophoretic profiles were compared to a yeast database (www.yeast-id.org).

Yeast strains and culture media

The yeast strains *Saccharomyces cerevisiae* (LTS12) and *Pichia kudriavzevii* (LTS8) were used to determine their fermentative capacity and higher alcohols production on three culture media: synthetic medium, maguey (*Agave angustifolia*) and sotol (*Dasylirion* spp.) juice. The synthetic medium was prepared as described by Segura-García et al. (2015), but with some modifications. It contained 100 g/L fructose, 1 g/L $(NH_4)_2SO_4$, 3.27 g/L $MgSO_4$, 2.23 g/L K_2HPO_4 and the pH value was adjusted to 4.7. A maguey pineapple was provided by a producer from Tepozatlán, Morelos, Mexico; sotol pineapple was provided by a producer from Aldama, Chihuahua, Mexico. Sotol pineapple was cut into small pieces, placed in a 4 L glass flask where a H_2SO_4 (0.05%) solution was added at 2:1 and then hydrolized at 105 °C for 6 h using an autoclave. The juice of the hydrolyzed sotol was extracted using a kitchen juice extractor. The concentrated juice was stored at -70ºC until use. The same procedure was applied to hydrolyze and extract the maguey juice.

Concentrated juice obtained from cooked maguey and sotol was filtered, diluted with distilled water, and adjusted at 10°Brix using a refractometer (Atago, Tokyo, Japan). A total of 300 mL of culture medium was dispensed in 500 mL flasks. Culture media were sterilized at 121 °C for 15 min.

Fermentations conditions

Mixed culture of *S. cerevisiae* with *P. kudriavzevii* were inoculated concurrently at the cell ratio of 1:1 on the culture media. Each yeast strain was inoculated at 6 x 10^6 CFU/mL, approximately. Pre-culture consisted of yeast grown in YEPD liquid

medium overnight and its cell concentration was determined using a Neubauer chamber.

All the fermentations were performed by duplicate at ambient temperature under static conditions.

Enumeration of viable yeast cells during fermentations

Samples were taken during fermentation every 24 h. To enumerate the viable yeast cells, ten-fold serial dilutions in 0.1 % w/v peptone water were place onto Wallerstein nutrient agar (WL) plate, then incubated at 30 °C for 3 days. WL is designed to differentiate yeasts according to their color and morphology of the colonies (Domizio et al., 2011) and was used to differentiate *P. kudriavzevii* (colored colonies) to *S. cerevisiae* (white colonies).

Determination of sugar and ethanol concentration

Samples were centrifugated at 14 000 rpm for 20 min and supernatants were filtered through a 0.45 µm Millipore membrane filter (EMD Millipore, MA, USA). Sugar (glucose and fructose) and ethanol were determined using a liquid chromatography system (Waters Corporation, MA, USA) equipped with a refractive index detector (Waters 2414, MA, USA) and ion-exclusion aminex HPX-87H column (300 x 7.8 mm) (BIO-RAD, USA). The analysis conditions were: 5 mM of sulfuric acid as mobile phase, at a flow rate of 0.6 mL/min, and a column compartment temperature of 45ºC. The concentrations of these compounds were determined by using the calibration curves of the corresponding standards compound. All samples were analyzed by duplicate.

Ethanol yield (Yp/s) was calculated as grams of ethanol produced per gram of utilized sugar. Ethanol productivity was calculated by ratio of ethanol production and fermentation time (72 h). The efficiency of sugar conversion was calculated by ratio of the experimental yield (Yp/s) and the theoretical yield (0.511 g/g) multiplied by 100.

Determination of higher alcohols

Concentrations of higher alcohols (isobutyl alcohol, isoamyl alcohol, 1-propanol, 2-phenylethanol, 2-butanol) and methanol were determined using a gas chromatography system (6890N, Agilent Technologies) equipped with a flame ionization detector, and an Optima Wax column (60 m, 0.250 mm of internal diameter, and 0.25 µm of film thickness: Macherey-Nagel). Samples were centrifuged at 14000 rpm for 20 minutes, subsequently, the supernatant was filtered with a 0.45 µm millipore membrane. Hexanol (10 mg / L) was added as an internal standard.

The operating mode was direct automatic injection, and the operating conditions were hydrogen flow at 40 mL/min, air at 400 mL/min and nitrogen at 28.3 mL/min. The oven temperature was programmed at 40 °C for 5 minutes, followed by a gradual increase of temperature at a rate of 5 °C/min up to 140 °C; and then raised to 240 °C at a rate of 10 °C/min. The identification of the compounds was carried out by comparing the retention times obtained from standards. The concentration of the analytes was expressed by means of the areas obtained normalized with the area of the hexanol for each compound.

The percentage peak areas were calculated by dividing the peak area for a peak by the total peak area expressed as a percentage.

Statistical analysis

The normality of the data obtained (peak area of higher alcohols and methanol; initial and residual sugar, ethanol production, ethanol yield and productivity, and efficiency) was analyzed using the Kolmogorov-Smirnov

normality test. Then, they were transformed using the expression $((x) +1)^{1/2}$ that allowed obtaining a better fit in the normality of the data, subsequently an analysis of variance (ANOVA) was performed using a completely random design. The comparison of means between treatments (culture media) was made with a Tukey test ($p \leq 0.05$). The analyzes were carried out with the statistical package Minitab Statistical Software V.19.

III. Results and discussion

Identification of isolates from natural fermentations

A total of 10 different yeast morphologies were obtained based on their morphotype (color and morphology of the colonies) onto WL nutrient agar. These yeasts were identified by 5.8S-ITS-RFLP analysis as *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Pichia kodamaea*, *Schizosaccharomyces pombe* and *Candida glabrata*. In a recent study (Zavala-Díaz de la Serna et al., 2020), *Pichia*, *Candida* and *Saccharomyces* genera were also identified during the natural fermentation process of sotol, where the *Pichia* genus was the most abundant, including *P. kudriavzevii, P. guilliermondii,* and *P. fermentans*. Similarly, Casas-Acevedo et al. (2021) also identified *P. kudriavzevii* during the sotol production process. Therefore, it could be suggested that *P. kudriavzevii* is a common yeast associated with the sotol fermentation and it could play an important role in the sotol fermentation.

The presence of *S. cerevisiae* and *C. glabrata* in sotol fermentation has been previously detected (De La Garza-Toledo et al., 2008). From these yeasts, *S. cerevisiae* was predominant at the end of the fermentation (De La Garza-Toledo et al., 2008). This is probably because *S. cerevisiae* is a competitive species (Andorrà et al., 2012). *S. cerevisiae* and *P. kudriavzevii* have also been reported as predominant yeasts in natural fermentation of maguey to produce mezcal (Nolasco-Cancino et al., 2018). Several authors (Lachance, 1995; Páez-Lerma et al., 2013; Kirchmayr et al., 2017; Nolasco-Cancino et al., 2018; Aldrete-Tapia et al., 2020;) indicate that *S. cerevisiae* is a common yeast in maguey fermentation to produce mezcal and tequila. While *P. kodamaea* and *S. pombe* yeast species have not been reported in others sotol producing regions. It is the first report of the presence of these yeast species in sotol fermentation, however, in the maguey fermentation *S. pombe* has already been reported (Kirchmayr et al., 2017). Other authors have reported a broader species diversity during the natural fermentation of sotol, including *Kluyveromyces marxianus*, *Rhodosporidium fluviae*, *Candida inconspicua*, *C. humillis*, *Issatchenkia occidentalis* (Casas-Acevedo et al., 2021) and *C. cellae*, *C. parapsilopsis*, *C. tropicalis*, *Dipodascus australiensis*, *Galactomyces geotrichum*, *Kodamaea ohmeri*, *P. fermentans*, *P. guilliermondii*, *P. kudriavzevii* and *Wickerhamamyces anomalus* (Zavala-Díaz de la Serna et al., 2020). Therefore, these yeasts could be exclusive of the distillery studied. *S. pombe* has also been reported in maguey juice fermentation to produce mezcal (Kirchmayr et al., 2017), a Mexican alcoholic beverage made in a similar way to sotol.

Growth, sugar consumption, and ethanol kinetics during fermentations

According to Martínez-Avila et al., 2020, *P. kudriavzevii* has been characterized by the ability for producing 2-phenylethanol, an alcohol that contributes positively to the aroma of alcoholic beverages. In addition, this yeast species is known as thermotolerant (> 40 °C) and resistant to low pH (3) (Dhaliwal et al., 2011; Martínez-Avila et al., 2020), and several strains are considered producers of high ethanol concentrations (70 g/L) at high temperatures (40 ºC) (Dhaliwal et al., 2011; Chamnipa et al., 2018). These

technological characteristics were considered to select *P. kudrivzevii* LTS8 and evaluate its fermentative characteristics in mixed cultures with *S. cerevisiae* LTS12 using three different culture media: sotol and maguey juice and a synthetic medium as control. *S. cerevisiae* was selected because it is a predominant species in the fermentations of sotol and maguey (De La Garza-Toledo et al., 2008: Nolasco-Cancino et al., 2018), and because several studies have demonstrated that fermentations with mixed cultures between *Saccharomyces* and non-*Saccharomyces* improve the fermentative and aromatic profile of the final product (Luan et al., 2018; Chagas-Junior et al., 2021; Larroque et al., 2021).

Maguey and sotol juices consisted mainly of fructose (91.46 and 93.85%, respectively). All culture media were adjusted at 10ºBrix; but the synthetic medium was prepared using only fructose, the major carbohydrate in these plants (Willems and Low, 2012; Sánchez-Madrigal et al., 2017). Figure 1 shows the growth dynamics of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8 in mixed culture in the synthetic, maguey, and sotol culture media.

In synthetic medium, *S. cerevisiae* LTS12 presented counts that were higher than that of *P. kudriavzevii* LTS8 (Figure 1a). The cell counts of *S. cerevisiae* LTS12 reached 7.05 Log CFU/mL, that is, it increased by about 0.36 log CFU/mL with respect to the initial inoculum (Figure 1a), while *P. kudriavzevii* LTS8 only increased 0.09 log CFU/mL within the 192 h, then, its population decreased (Figure 1a). This fermentation can be considered stuck because after 384 h (16 days) only less than 50% of the sugar was consumed (57.58±0.25 g/L of residual sugar), which is related with the low ethanol concentration (9.98±0.36 g/L). This result had already been observed by Nolasco Cancino et al. (2018), who evaluated the fermentative capacity of *P. kudriavzevii* (JA10) and *S. cerevisiae* (DI14), yeast strains isolated from mezcal. The stuck fermentation in synthetic medium could be attributed to the fermentation temperature (26-28 °C) used in this experiment, considering that several authors have reported that *P. kudriavzevii* has an optimal fermentation temperature around of 40 °C (Dhaliwal et al., 2011; Gallardo et al., 2011; Chamnipa et al., 2018). In addition, results suggest that the composition of the culture medium significantly influences on the fermentative capacity of the co-culture. For example, it has been shown that *A. angustifolia* juice has a low C/N ratio (137.5) when it was compared with *A. tequilana* (276.9) (Alcazar-Valle et al., 2019). The low C/N ratio enhances the higher alcohols production (Alcazar-Valle et al., 2019). Saponins are another important factor in the fermentation process of maguey sugars, they are present in different concentration (14.05 g/L for *A. durangensis* and 26.53 g/L for *A. salmiana*) and types depending on maguey species (Alcázar et al., 2017). This metabolite inhibits the growth of yeast during fermentation process (Alcázar et al., 2017).

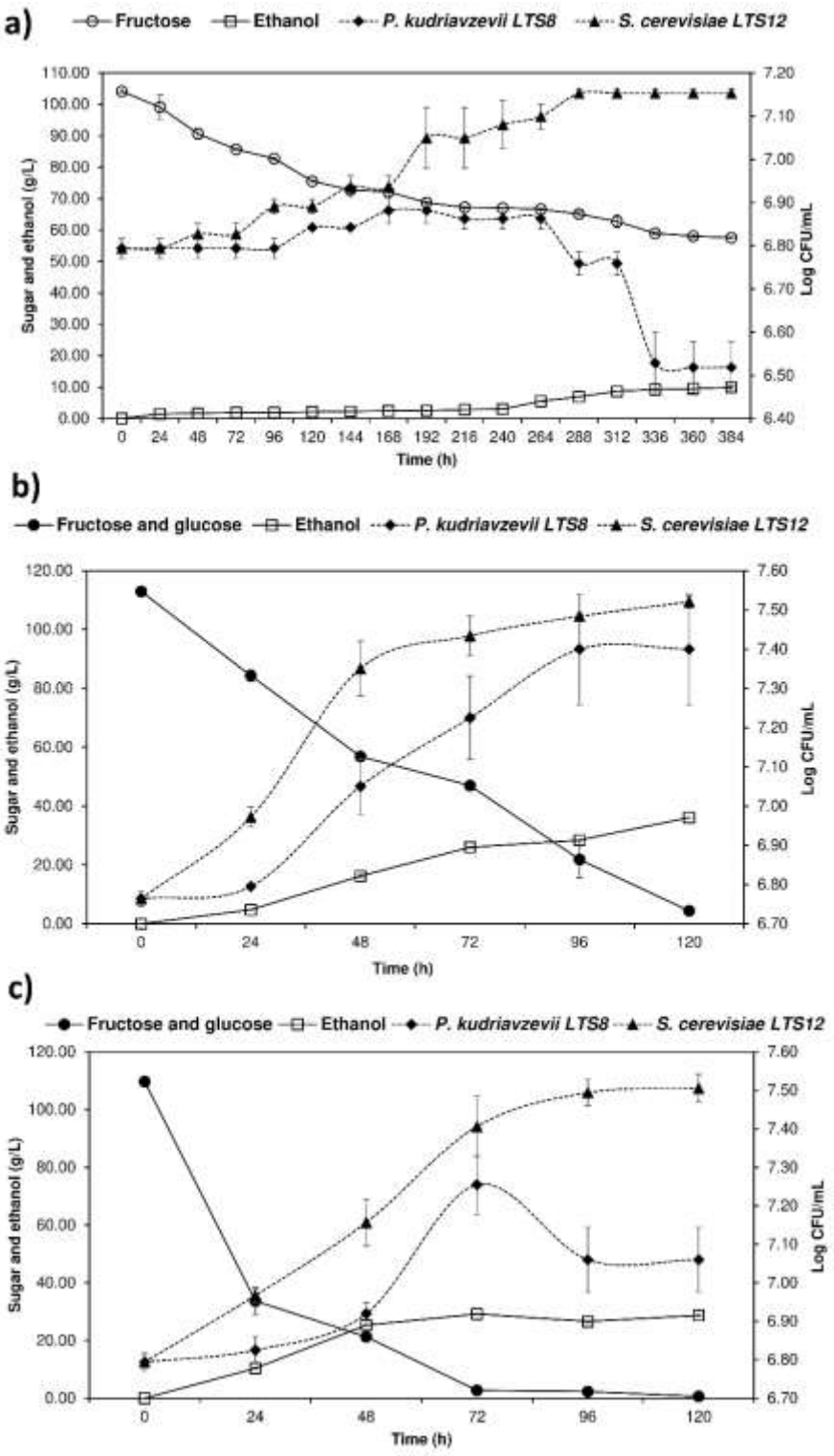

Figure 1. Growth, sugar consumption and ethanol production kinetics during fermentations of a) synthetic medium, b) maguey juice, and c) sotol juice with a mixed culture of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8.

According to the results of microbial growth by pure culture (see Figure S1), it can be concluded that *P. kudriavzevii* LTS8 decreased the growth of *S. cerevisiae* LTS12

when they were co-cultured in synthetic medium. Also, the sugar consumption and ethanol production were lower in mixed culture than in pure culture (see Figure S1).

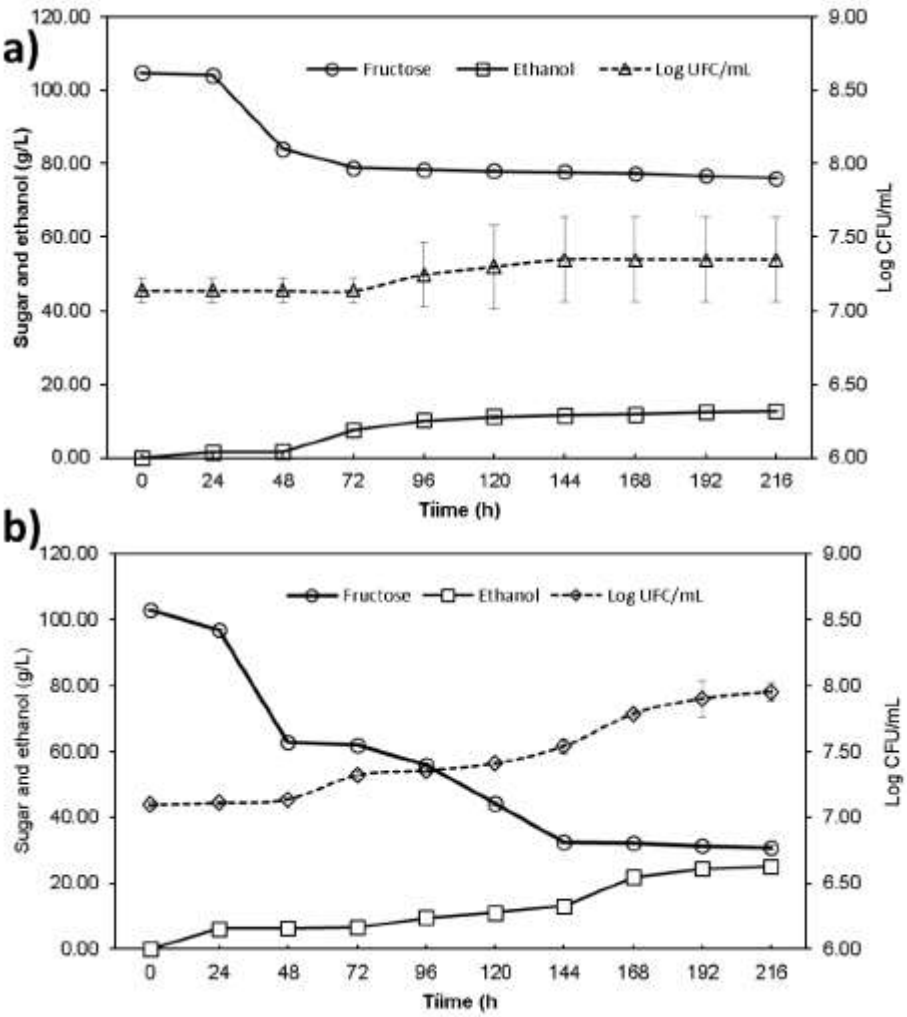

Figure S1. Growth, sugar consumption and ethanol production kinetics during fermentations of synthetic medium using pure cultures of a) *P. kudriavzevii* LTS8 and b) *S. cerevisiae* LTS12.

The growth of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8 in co-fermentation in maguey (Figure 1b) and sotol (Figure 1c) juices was favored as compared with that in synthetic medium. In these natural musts, fermentations finished after 120 h (5 days), like occur in natural fermentation of sotol in the distilleries (Zavala-Díaz de la Serna et al., 2020). In fermented maguey juice, the residual sugars were 4.39±1.31 g/L, while in sotol fermentation they were 0.67±0.15 g/L. These residual sugars concentrations were lower than that (14.45 g/L) reported in sotol fermentation at the distillery level (Zavala-Díaz de la Serna et al., 2020). Zavala-Díaz de la Serna et al. (2020) reported that sotol must had a sugar concentration of 118 g/L, approximately, like the concentrations used in our experiments (109.7 g/L). Sotol juice used in these trials contained 94% fructose and 6% glucose, while maguey juice had 91.5% fructose and 8.5% glucose.

Findings suggest that the mixed culture of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8 can successfully complete the alcoholic

fermentation. Results highlighted the role of the nutrients in maguey and sotol juice on the growth of yeasts and sugar consumption.

Fermented maguey and sotol juices had a final ethanol concentration of 36.08 g/L and 28.71 g/L, respectively (Table 1). These concentrations were higher than that reached (25.53 g/L) in the sotol fermentation at the distillery level (Zavala-Díaz de la Serna et al., 2020). Mixed culture of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8 had a great fermenting potential in maguey juice with an ethanol yield of 0.33 g ethanol/g substrate and a higher efficiency (65%) than that of sotol (51.54%) and synthetic medium (41.89%) (Table 1). The fermentation efficiency of sotol in these trials was higher than that reported in sotol fermentation (42.3%) at the distillery level (Zavala-Díaz de la Serna et al., 2020). The results show the fermentative potential that these yeasts in co-culture could have in the production of sotol or mezcal.

Table 1. Fermentative capacity of the of mixed culture of *S. cerevisiae* LTS12 with *P. kudriavzevii* LTS8 in synthetic medium, maguey and sotol juices.

Parameters	Media		
	Synthetic	Maguey	Sotol
Initial sugar (g/L)	104.20±0.035^{c}	112.95±0.065^{a}	109.71±0.44^{b}
Residual sugar (g/L)	57.58±0.25^{a}	4.39±1.31^{b}	0.67±0.15^{c}
Ethanol production (g/L)	9.98±0.37^{c}	36.08±0.04^{a}	28.72±0.23^{b}
Ethanol yield (Yp/s)	0.21±0.01^{c}	0.33±.0.00^{a}	0.26±0.00^{b}
Ethanol productivity (g/Lh)	0.03±0.00^{c}	0.30±0.00^{a}	0.24±0.00^{b}
Efficiency (%)	41.89±1.41^{c}	65.04±0.66^{a}	51.54±0.25^{b}

These values are the mean ± standard deviation of two independent experiments. Different lowercase letters in the same row show significant differences according to the analysis of variance at $p \leq 0.05$ (Tukey test). Ethanol yield (Yp/s) was calculated as grams of ethanol produced per gram of utilized sugar. Ethanol productivity was calculated by ratio of ethanol production and fermentation time (72 h). The efficiency of sugar conversion was calculated by ratio of the experimental yield (Yp/s) and the theoretical yield (0.511 g/g) multiplied by 100.

Higher alcohols production

Isobutyl alcohol, isoamyl alcohol, 2-butanol, 1-propanol, and 2-phenylethanol were the higher alcohols determined at the end of the fermentation trials. Table 2 shows the peak area of each higher alcohol detected in the fermented culture media. The sum of the higher alcohols reported in the Table 2 shows that the total peak area ranged of 71.67 (synthetic medium) to 453.92 (sotol juice). These metabolites production was favored in natural musts. Findings indicate that the higher alcohols production depends on the culture medium composition. It has been demonstrated that yeast assimilable nitrogen sources play an important role in the formation of higher alcohols (Liu et al., 2021). Ammonium salts reduce the higher alcohols content in the following order NH_4Cl > $(NH_4)_2HPO_4$ > NH_4HCO_3 > $(NH_4)_2SO_4$ (Liu et al., 2021). In the present study, ammonium sulphate (1 g/L $(NH_4)_2SO_4$) was used as a nitrogen source in the synthetic medium, which could explain the lowest percentage of peak areas (PA) in the synthetic medium than that found in natural musts (Table 2).

Table 2. Concentration of higher alcohols and methanol produced in synthetic, maguey and sotol juices inoculated with mixed culture of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8.

Compounds	Synthetic medium		Maguey juice		Sotol juice	
	Peak area	PA (%)	Peak area	PA (%)	Peak area	PA (%)
Methanol	0.00±0.00^{c}	0.00	3.82±0.67^{b}	2.88	27.82±2.62^{a}	5.77
Isobutyl alcohol	36.80±1.13^{a}	51.35	13.00±0.29^{b}	9.81	4.99±1.01^{c}	1.03
Isoamyl alcohol	4.76±0.28^{b}	6.64	12.59±1.08^{b}	9.51	212.70±33.10^{a}	44.15
2-phenylethanol	27.39±1.65^{c}	38.21	92.72±7.84^{b}	70.00	203.10±30.20^{a}	42.16
2-Butanol	2.72±0.31^{c}	3.80	10.33±1.52^{b}	7.80	14.41±1.45^{a}	3.00
1-Propanol	0.00±0.00^{b}	0.00	0.00±0.00^{b}	0.00	18.72±2.62^{a}	3.89

These values are the mean ± standard deviation of two independent experiments. Different lowercase letters in the same row show significant differences according to the analysis of variance at $p \leq 0.05$ (Tukey test). All compounds were quantified at the end of fermentation.

In synthetic medium, isobutyl alcohol and 2-phenylethanol were the main higher alcohols produced with 51.35% and 38.21% (PA, Table 2). It could be said that isobutyl alcohol is only produced by *S. cerevisiae*, as demonstrated in fermentation with pure cultures (Figure S2). The production of this alcohol in a synthetic medium (20.46 mg/L) has been demonstrated by Segura-García et al. (2015). Isobutyl alcohol formation results from valine metabolism via the Ehrlich pathway (Chem et al., 2011). However, valine is synthetized from pyruvate in mitochondria. Pyruvate decarboxylases and alcohol dehydrogenases catalyze the pathway from L-valine to isobutyl alcohol (Chem et al., 2011).

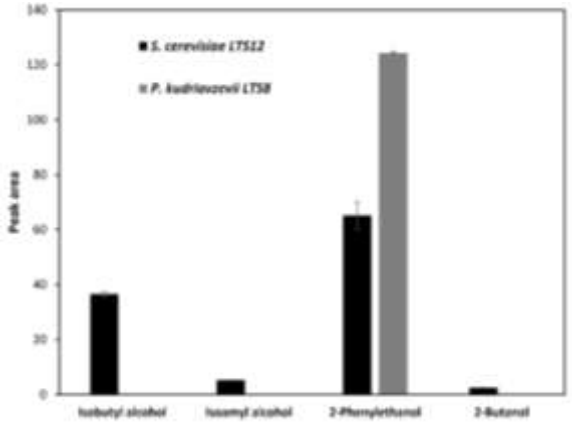

Figure S2. Higher alcohols production by pure culture of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8 in synthetic medium.

P. kudriavzevii LTS8 is the main 2-phenylethanol producer. It was demonstrated by means of the pure culture of *S. cerevisiae* LTS12 and *P. kudriavzevii* LTS8 in synthetic medium, where *P. kudriavzevii* LTS8 proved to be a strong producer of 2-phenylethanol (peak area of 123.8; supplementary material, see Figure S2). Therefore, 2-phenylethanol was a predominant alcohol in all fermented culture media, thanks at the presence of *P. kudriavzevii* LTS8. It reached values of PA of 70% and 42% in maguey and sotol fermentations, respectively. This higher alcohol has been recently reported during the natural fermentation of sotol (Casas-Acevedo et al., 2021). It is a higher alcohol with the most pleasant aroma to roses, honey and floral (Pei-Tong et al., 2016). In addition, this alcohol is a precursor to obtain 2-phenetyl acetate, an ester that also has a floral like fragrance (Martínez Avila et al., 2020). Thus, this indigenous *P. kudriavzevii* LTS8 could possess attractive characteristics to produce 2-phenylethanol.

The content of isoamyl alcohol was higher in sotol juice (44.15% PA) than that in synthetic medium and maguey juice (6.64% and 9.51% PA). This higher alcohol has been detected in sotol fermentation (Casas-Acevedo et al., 2021). The production of isoamyl alcohol

depends on the presence of the leucine in the culture medium (Stribny et al., 2015), so that, this amino acid could be predominant in sotol plants. Findings suggest that the higher alcohols concentrations depend on the culture medium composition and the yeast strain.

Methanol was detected in maguey and sotol fermentation (Table 2), which indicates that this compound was produced from the vegetal material. Methanol is produced from the pectin of the maguey plants. During the maguey cooking, methoxylated pectins are hydrolyzed and converted in this alcohol (Pinal et al., 2009).

IV. Conclusions

The results of this study revealed that *S. cerevisiae* and non-*Saccharomyces* yeasts coexist in the natural fermentation of sotol. From these non-*Saccharomyces*, the isolated *P. kudriavzevii* LTS8 exhibited potential to produce 2-phenylethanol, an important alcohol in the aroma of sotol. The fermentative efficiency, alcoholic yield, productivity of ethanol production, and the higher alcohols concentration depend on the culture medium composition. *S. cerevisiae* LTS12 co-cultured with *P. kudriavzevii* LTS8 displays a high efficiency to ferment maguey juice, so that, this mixed culture can be used in both sotol and maguey fermentation.

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