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ORIGINAL PAPER

Characterization of kinetic parameters and the formation of volatile compounds during the tequila fermentation by wild yeasts isolated from agave juice

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Abstract The production of aroma compounds during tequila fermentation using four native yeast strains isolated from agave juice was quantified at controlled (35 °C) and uncontrolled temperatures (room temperature) by gas chromatography (FID). Three of the four strains were identified as *Saccharomyces cerevisiae* (MTLI 1, MALI 1 and MGLI 1) and one as *Kloeckera apiculata* (MALI 2). Among the aroma compounds produced, acetaldehyde has the highest accumulation at the controlled temperature and before 50% of sugar was consumed. The *S. cerevisiae* strains produced ethyl acetate in almost the same quantity at a concentration of 5 mg/L and the *K. apiculata* produced six-times more (30 mg/L) than the *S. cerevisiae* strains, independent of the fermentation temperature. The rate and amount of 1-propanol, amyl alcohols and isobutanol production were affected by the type of yeast used. The *K. apiculate* strain produced 50% less of the higher alcohols than the *Saccharomyces* strains. The results obtained showed that indigenous isolated yeasts play an important role in the tequila flavor and suggest that mixtures of these yeasts may be used to produce tequila with a unique and desirable aroma.

Keywords Tequila · Fermentation · Indigenous yeast · Aroma compounds

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Introduction

Tequila is a distilled alcoholic beverage derived from the fermented cooked core of the blue agave (*Agave tequilana* Weber blue variety) and produced within a limited region of México. According to the percentage of sugars used in its production, tequila can be classified as either tequila 100% agave or as just tequila. With the former, only agave sugars are used in the fermentation, while the latter may be enriched up to 49% with other sugars, such as sugar cane or corn syrup [16]. Traditionally, the wild yeasts present in agave juice served as the inoculum for fermentation rather than the purified inoculums which are used today, causing variability in the yield and quality of tequila. Moreover, in most of the distilleries today, the fermentation temperature is not controlled. Rather it is carried out under ambient conditions, resulting in variations in aroma and ethanol production during fermentation. In this manner, the sensory quality of tequila is influenced by several factors such as type of yeast, composition and pH of the medium and fermentation temperature [2, 8, 15, 18, 19].

It is well known that yeast type plays an important role in the formation of aroma compounds during the fermentation of alcoholic beverages [18]. The formation of volatile compounds during alcoholic fermentation depends not only on the particular yeast species but also on the particular strain of the species [17]. In addition, the yeasts influence fermentation parameters such as the fermentation speed and are the main factors in the production of diverse types of fermented

beverages [14, 21]. As a result, the selection of yeast type determines the characteristics of the final product [11, 17].

Another important factor in aroma compound production is the fermentation temperature. Higher fermentation temperature results in larger amounts of higher alcohols, esters and fatty acids than at lower temperature [2, 15, 18, 20]. The existing literature concerning the aroma production as a function of temperature during the tequila production is limited. Pinal et al. [18] described the effect of the temperature on the generation of higher alcohols in agave musts.

The purpose of this study was to isolate wild yeast strains from *A. tequilana* Weber blue variety juice obtained from three different tequila distilleries and characterize their production of higher alcohols, ethyl acetate and acetaldehyde in tequila 100% agave must at different temperatures. This information will be useful for the development of starter cultures with predictable characteristics for use in small-scale commercial producers of tequila.

Materials and methods

Strains used and culture conditions

Four wild yeast strains were isolated from the juice of unfermented samples of *A. tequilana* Weber blue variety from tequila factories in the three main regions of tequila production in México: Tequila, Guadalajara and Los Altos, all located in the State of Jalisco. These strains were grown on an agave medium of *A. tequilana* juice cooked in a brick oven, brought to a final concentration of 60 g reducing sugar/L and enriched with ammonium sulphate to 1 g/L with distilled water. The medium pH was adjusted to 4.5 with 1 N NaOH and 1 N HCl solutions and sterilized at 121 °C for 15 min.

Yeast identification

Identification of yeasts was carried out using an API 20 C AUX yeast identification system (BioMérieux, Marcy l'Etoile, France). The standard taxonomic description was obtained by electrophoretic karyotype according to Giudici et al. [8]. For electrophoretic protocol, a CHEFF Mapper System was used (Bio-Rad, Richmond, CA, USA). Yeast chromosomes were separated by electrophoresis at 14 °C on 1% agarose gel in 0.5× TBE buffer. A constant voltage of 200 V was applied for 24 h. The gel was stained with ethidium bromide solution with DNA chromosomal from *S. cerevisiae* and *S. pombe* (BIO-RAD) as markers.

Inoculum

Samples of the different strains taken with a loop were inoculated separately into the agave medium (50 mL) and incubated at 30 °C for 24 h with a rotary shaker. A 10 mL aliquot of each inoculum sample was added to the agave medium (200 mL) and incubated at 30 °C, for 10 h (MALI 1 and MGLI), 12 h (MTLI 1) and 14 h (MALI 2), according to the time needed for each to grow.

Yeast growth

Yeast strains were grown in a 1.5 L bioreactor, containing 1.1 L of sterilized agave medium. The initial yeast concentration in the medium was 20×10^6 cells/mL. The operating temperature was maintained at 30 °C for 72 h and air was supplied at 1 v/v per min into the reactor.

The experiments were carried out in duplicate. The kinetic parameters including maximum specific growth rate (μ_{max}), carbon substrate consumption rate (r_s), yield coefficient for cells on the carbon substrate ($Y_{x/s}$) and biomass production (X) were evaluated by non-linear regression using a C++ developed program.

Fermentation

Batch fermentations were carried out using 12 L bioreactors, containing 9 L of agave media under anaerobic conditions. The experiment was a two way analysis of variance (4 × 2), complete and balanced with two replications. The factors were: strain of yeast (MALI 1, MALI 2, MGLI 1 and MTLI) and temperature. Two temperature levels were used: 35 °C (controlled temperature) and room temperature (uncontrolled) which fluctuated between 16 and 30 °C. The response variables were ethanol production (g/L), fermentation time (h), fermentation yield (g ethanol/g reducing sugar), production rate (g ethanol/L/h) and sugar consumption (g reducing sugar/L/h) and all were measured at the end of the fermentation. Bioreactors were inoculated with 20×10^6 cells/mL using a Neubauer chamber (100% viability). Fermentation time was 72 h. The kinetic parameters were calculated by a non-linear regression program.

Tequila distillation

Each fermentation sample was distilled using a microdistillator with a vigreaux column. Five mL of must and 5 mL of distillated water were added to a bowl and boiled until 5 mL of distillate remained.

Analytical methods

Reducing sugars were determined by the dinitrosalicylic acid method [13]. Biomass was determined by gravimetry. Dry weight was obtained by first centrifuging at 5,000 rpm for 15 min and then drying to a constant weight. Alcohol

concentration was determined by the potassium dichromate method [6].

Gas chromatography (GC)

Volatile compounds were analyzed by direct injection of 0.2 μL of the distillate samples in a HP6890 GC gas chromatograph coupled with a flame ionization detector. A DB-Wax column (30 m × 0.25 mm × 0.25 μm) was used to separate the volatile compounds with helium as the carrier gas at a flow-rate of 2 mL/min. The oven temperature was programmed from an initial temperature of 32 °C for 5 min and then raised to 80 °C at a rate of 4 °C/min. The injector and detector temperatures were both 250 °C. Quantification was performed using commercially available standards (Sigma Aldrich). Calibration factors were determined using the standard addition method and creating linear regression models.

Statistical analysis

The variance in the kinetic parameters of growth and fermentation was calculated using Statgraphics Plus for Windows version 4 (Statpoint, Inc.).

Results and discussion

Yeast characterization

Four wild yeasts were isolated from samples of non-fermented *A. tequilana* juice: two strains from a tequila factory in Arandas (strain MALI 1 and MALI 2), a third from a tequila factory in Tequila (strain MTLI 1) and the fourth from a tequila factory in Guadalajara (strain MGLI 1).

Three yeast strains (MALI 1, MTLI 1 and MGLI 1) were identified as *S. cerevisiae*. MALI 1 and MTLI 1 showed 11 bands in the karyotype technique, and the strain MGLI 1 showed 12 bands, in a pattern similar to that reported for *S. cerevisiae* (11–14 chromosomes). The electrophoretic pattern of the fourth strain (MALI 2) showed 4 bands, identical to these of the *Kloeckera* spp.

Yeast growth

Table 1 shows the kinetic parameters of isolated yeast in a 1.5 L reactor. The maximum specific growth (μ_{max}) was very similar among the four strains ($P = 0.6511$). On the contrary, the highest value for the carbon substrate consumption rate (r_s) was observed in the MGLI 1 strain, whereas the lowest r_S value was observed in MALI 2. The yield coefficient for cells on the carbon substrate ($Y_{x/s}$) was also similar among the strains (Table 1). In addition, maximum biomass formation (X) was obtained in the MALI 1 and MGLI 1 strains (Table 1). The kinetic parameters are the most important signs for the adaptation of the microorganism to the medium [10]. These results were similar to those obtained by Gschaedler et al. [9] who isolated 12 yeast strains and measured the specific growth rates. The growth rates obtained in the current study are close to 6 of their yeast strains.

The maximum specific growth rate (μ_{max}) and yield coefficient on the carbon substrate ($Y_{x/s}$, Table 1), were smaller than those obtained by Di Serio et al. [7] who grew baker's yeast in a yeast extract peptone dextrose medium (YEPD) and an industrial medium prepared with molasses and salts, with a 5 g/L initial sugar concentration. These results suggest that agave juice may not supply sufficient nutrients for the growth of yeasts as compared to the YEPD medium. Another probability is that agave juice may have compounds which inhibit the specific growth.

Fermentation characteristics of the isolated yeast strain

Isolated yeasts were used to carry out tequila must fermentation for "tequila 100% agave" at the laboratory scale. Table 2 shows the changes in fermentation variables during tequila fermentation at controlled (35 °C) and ambient tem-

Table 1 Comparison of the growth kinetic parameters of isolated yeast in a 1.5 L reactor

Parameter	*S. cerevisiae* (MALI 1)	*Kloeckera spp.* (MALI 2)	*S. cerevisiae* (MTLI 1)	*S. cerevisiae* (MGLI 1)	Statistical difference ($P \leq 0.05$)
μ_{max} (per h)	0.455 ± 0.018^{a}	0.480 ± 0.120^{a}	0.412 ± 0.017^{a}	0.492 ± 0.049^{a}	0.6511
$r_{s\ max}$(g/L/h)	6.52 ± 0.320^{a}	2.59 ± 0.491^{b}	6.01 ± 2.502^{ab}	10.43 ± 1.006^{c}	0.0207*
$Y_{x/s}$ (g/g)	0.082 ± 0.008^{a}	0.176 ± 0.090^{a}	0.079 ± 0.024^{a}	0.102 ± 0.016^{a}	0.2839
X (biomass) (g/L)	3.34 ± 0.084^{a}	1.17 ± 0.132^{b}	2.43 ± 0.238^{c}	3.04 ± 0.290^{a}	0.0047*

[a, b, c] significantly different at $P < 0.05$

μ_{max} maximum specific rate of growth

$r_{s\ max}$ maximum specific rate of sugar consumed

$Y_{x/s}$ yield of mass cell produced by mass substrate consumed

peratures. Almost all the strains showed an increase in ethanol concentration (20–25%) at 35 °C except MALI 1. This yeast strain produced less ethanol at 35 °C. In addition, the fermentation time was influenced by operating temperature. At room temperature, MALI 1, MTLI and MGLI 1 required two to three times longer than at 35 °C. According to Berovic et al. [5], temperature is one of the most significant parameters affecting the rate of the fermentation process. In the current study, the maximum rate production of ethanol (r_{pmax}) increased almost four times at 35 °C with MALI 2 yeast, while the MTLI 1 increased only 0.3 times. These results were similar to the results of Arrizon and Gschaedler [3]. The increased ethanol production could have been due to the fact that the temperature (35 °C) was near the optimum temperature for the yeasts and the sugars are consumed and assimilated faster at this temperature (Table 2).

The yield of ethanol production is an important parameter to the tequila industry, since it is the direct measurement of the relationship between ethanol production and sugar consumption.

Volatile compounds production

Figure 1 shows the changes in the acetaldehyde levels during fermentation. It was noted that the formation and concentration of acetaldehyde was a function of the operating conditions and the yeast strain. At the controlled temperature, it was observed that the level of acetaldehyde produced by MALI 1 (*Kloeckera* spp. 24 mg/L) was higher than that produced by the *S. cerevisiae* strains during fermentation but was at a similar level at the end of fermentation. In addition, it was also noted that the fermentation temperatures influenced the final concentration of acetaldehyde; concentration of 5–10 mg/L were observed at room temperature in comparison to 10–15 mg/L at 35 °C. The maximum concentration of this compound was found at 40% fermentable sugar and 35 °C (1.07 mg/L) by MALI 2. Moumeni et al. [15] reported that acetaldehyde dehydrogenases, which converts the acetaldehyde to ethanol, was inhibited at temperatures higher than 35 °C. This result suggests that fermentation temperature is a critical factor in acetaldehyde accumulation. The official Mexican standard for tequila [16] establishes that the maximum permissible level for acetaldehyde is 40 mg/L. Controlling the presence of this compound is important in defining the quality of tequila. This compound gives a fruity character at low levels, however, at higher levels a pungent odor and irritant effect is produced [12].

Ethyl acetate formation during fermentation is shown in Fig. 1c, d. The evolution of ethyl acetate was extremely low and constant for both fermentation temperatures and for the *Saccharomyces* strains. The production at room temperature reached the maximum level when 25% of the reducing sugars were consumed and that level was maintained throughout the process. At 35 °C, the production increased gradually until the end of fermentation, reaching the same level as in the room temperature fermentation (35 mg/L). MALI 2, however, produced a significantly higher amount (6-fold of ethyl acetate) than the *S. cerevisiae* strains.

The ethyl acetate, which imparts another fruity flavor in tequila [22] is the main ester that occurs in distilled tequila at a concentration around 176 mg/L [4]. Its production

Table 2 Fermentation parameters for the isolated tequila yeasts

Fermentation parameters	Temp	*S. cerevisiae* (MALI 1)	*Kloeckera* ssp (MALI 2)	*S. cerevisiae* (MTLI 1)	*S. cerevisiae* (MGLI 1)
Alcohol produced (g/L ± SE)	Room	32 ± 2.2^{ad}	24.3 ± 3.8^{ad}	27.9 ± 4.1^{ad}	24.1 ± 2.9^{ad}
Alcohol produced (g/L ± SE)	35 °C	29.3 ± 1.5^{ad}	33.4 ± 2.3^{ad}	30.0 ± 1.1^{ad}	30.1 ± 1.2^{ad}
Fermentation time (h)	Room	32.0	72.00	56.00	40.00
Fermentation time (h)	35 °C	18.00	40.00	24.00	20.00
$Y_{p/s}$ (g alcohol/g substrate)	Room	0.37 ± 0.07^{ad}	0.36 ± 0.04^{ad}	0.33 ± 0.09^{ad}	0.35 ± 0.07^{ad}
$Y_{p/s}$ (g alcohol/g substrate)	35 °C	0.37 ± 0.05^{ad}	0.45 ± 0.01^{ad}	0.43 ± 0.10^{ad}	0.43 ± 0.01^{ad}
r_p(g alcohol/Lh)	Room	1.77 ± 0.37^{ad}	0.49 ± 0.15^{bd}	1.39 ± 0.21^{bcd}	1.54 ± 0.32^{acd}
r_p(g alcohol/Lh)	35 °C	2.82 ± 0.28^{ac}	2.08 ± 0.07^{bc}	1.83 ± 0.02^{bcc}	2.63 ± 0.37^{acc}
r_s (g substrate/Lh)	Room	5.59 ± 2.30^{ac}	1.65 ± 0.39^{ac}	4.92 ± 0.65^{ac}	4.32 ± 0.57^{ac}
r_s (g substrate/Lh)	35 °C	6.31 ± 1.97^{ad}	4.872 ± 1.02^{ad}	5.85 ± 0.13^{ad}	7.12 ± 0.14^{ad}

Temp temperature used in the assays

Yp/s yield of ethanol produced by substrate consumed

r_p ethanol production rate

r_s substrate consumed rate

[a, b, c] significantly different between strains at $P < 0.05$

[d, e] significantly different between temperatures at $P < 0.05$

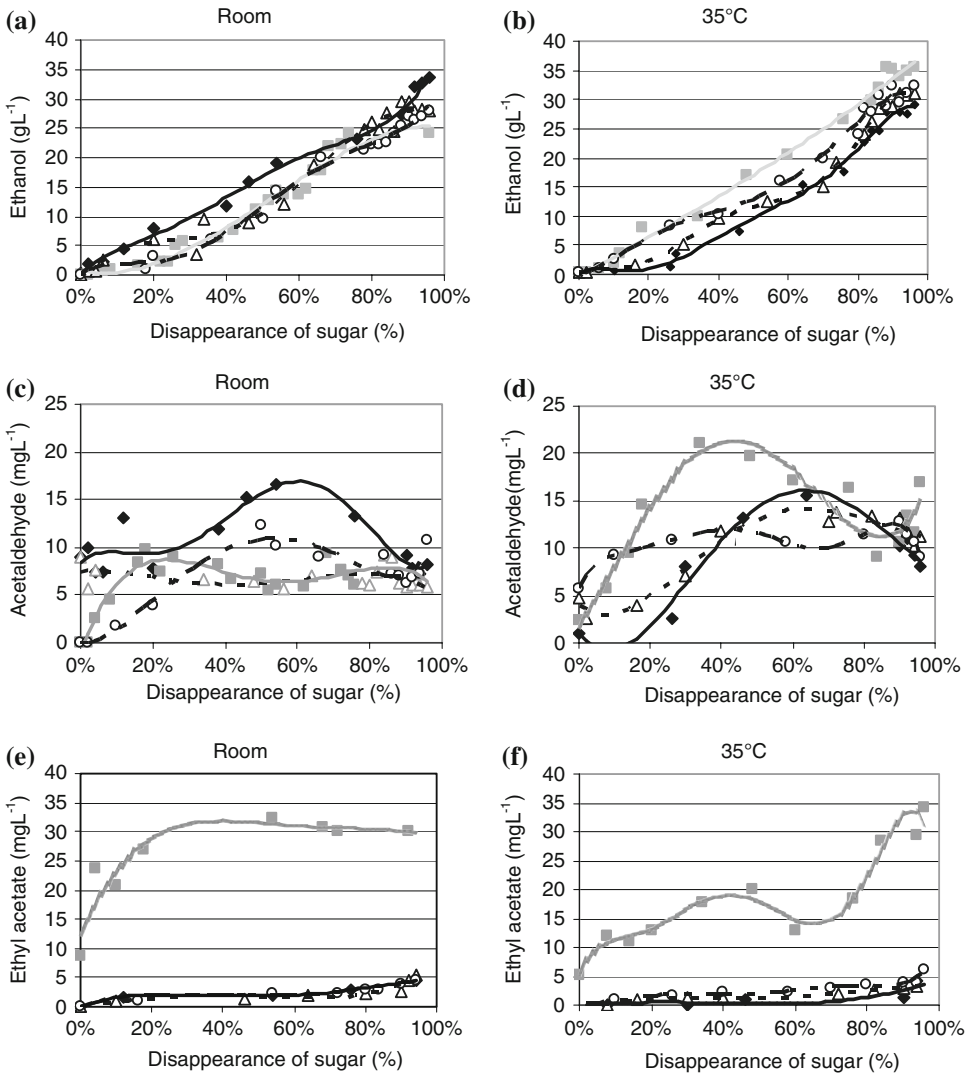

Fig. 1 Evolution of ethanol at **a** room temperature and **b** 35 °C, acetaldehyde at **c** room temperature and **d** 35 °C, and ethyl acetate at **e** room temperature and **f** 35 °C, during tequila 100% agave fermentation by the yeast isolated from agave juice MALI 1 (*filled diamond*), MALI 2 (*filled square*), MTLI 1 (*open triangle*) and MGLI 1 (*open circle*)

takes place through esterification of acetyl CoA and ethanol at high concentrations. Mexican regulations limit its quantity in tequila. It was demonstrated that at a concentration higher than 150 mg/L, the ethyl acetate imparted undesirable characteristics to the alcoholic beverages [12].

Figure 2 shows the production of the higher alcohols during the fermentation. 1-Propanol production was proportional to the consumption of reducing sugars reaching an average of 15 mg/L at the end of fermentation (Fig. 2a, b) for all the strains, except MGLI 1, which produced 23 mg/L at the end of the fermentation at 35 °C. The biosynthesis of this compound involves two possible mechanisms: the first through the α-ketobutyrate followed by decarboxylation and reduction [12] and the second via an enzymatic pathway involving the amino acid synthesis from sugars. It is unknown exactly which pathway is used by the yeasts, however, according to the current results the production of 1-propanol was proportional to the disappearance of reducing sugars indicating the possibility of the latter.

The production rate of amyl alcohols among *Saccharomyces* strains was similar up to a concentration of 30 mg/L, where a 50% disappearance of the reducing sugar was observed at 35 °C. After that point, the accumulation started at a higher rate reaching 100 mg/L at 35 °C and 140 mg/L at room temperature. An interesting observation was that the production of amyl alcohols by *Kloeckera spp.* was 2.3 times less than that of *Saccharomyces* at 35 °C and the final concentration was 6.5 times less at room temperature. It was also shown that *Kloeckera* spp. produced two times more amyl alcohol at 35 °C than at room temperature. On the other hand, *S. cerevisiae* strains showed a contrary result: the final amount of amyl alcohol at room temperature was larger than at 35 °C. Pinal et al. [18] reported an increased amount of amyl alcohol at 35 °C compared to 30 °C in tequila production (agave juice enriched with sugar cane). Here the final amyl alcohol concentrations of 21 mg/L at 30 °C and 39 mg/L at 35 °C are lower than those obtained in the current study, but were similar to the

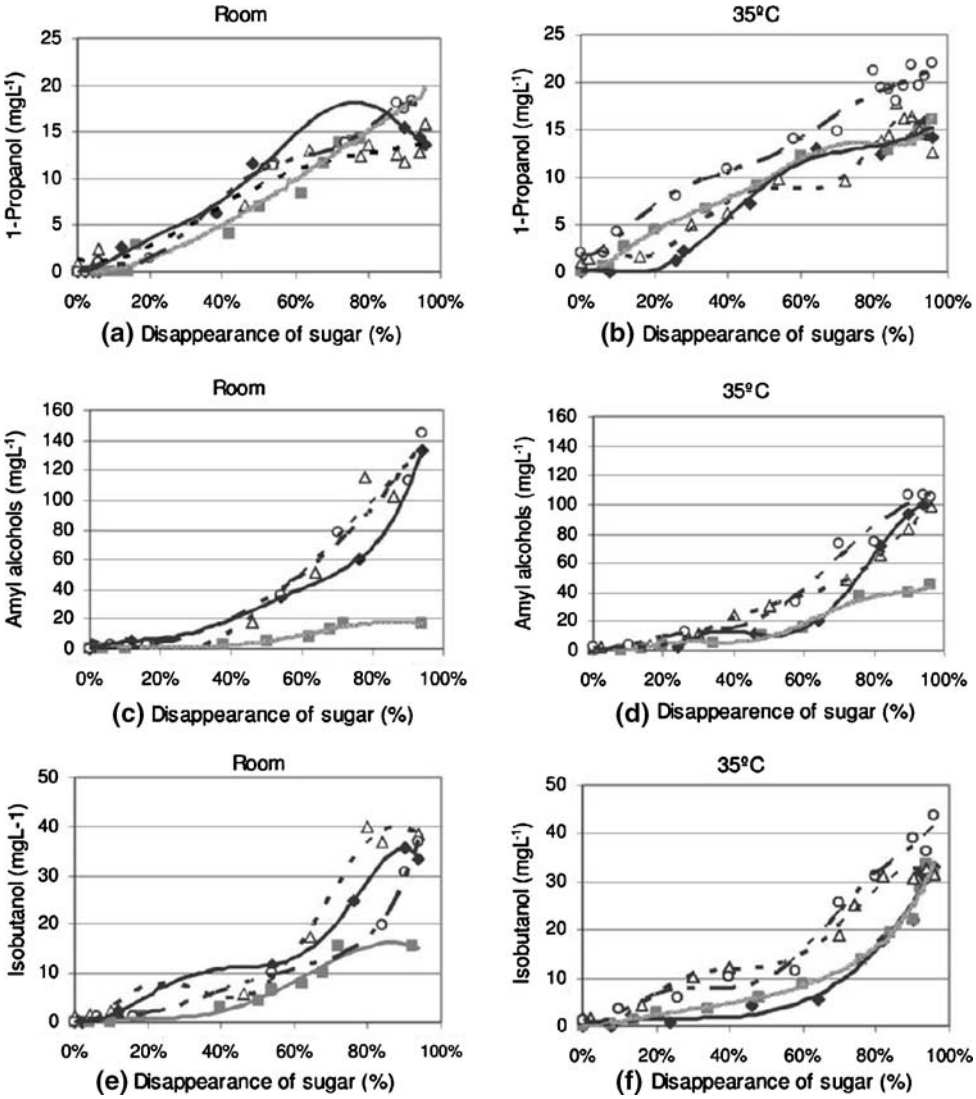

Fig. 2 Evolution of 1-propanol at **a** room temperature and **b** 35 °C, amyl alcohols at **c** room temperature and **d** 35 °C, and Isobutanol at **e** room temperature and **f** 35 °C, during tequila 100% agave fermentation by the yeast isolated from agave juice**.** MALI 1 (*filled diamond*), MALI 2 (*filled square*), MTLI 1 (*open triangle*) and MGLI 1 (*open circle*)

accumulation by the *Kloeckera* strain. These differences may be due to the system and the types of substrates.

The formation of amyl alcohols is through two possible pathways; the first by the deamination of leucine and isoleucine and the second by the intermediates in biosynthesis of these amino acids. The latter may be the main pathway of the amyl alcohol production, since the agave juice has no more than 0.2 mg/L of leucine and isoleucine combined and the formation of amyl alcohol occurred after the yeast started to grow, when the amino acids were actively needed.

The production of isobutanol at room temperature and 35 °C was similar for the *S. cerevisiae* yeasts (MALI 1, MTLI 1 and MGLI 1). *Kloeckera* spp. (MALI 2) produced 2.5 times less isobutanol than the *S. cerevisiae* and produced two times more at 35 °C than at room temperature. The difference in isobutanol production at the different fermentation temperatures evaluated may be due to the fact that the yeast needs valine when the temperature is higher than 30 °C and the isobutanol maybe formed by deamination of valine or during the biosynthesis of this amino acid. Similar results were shown by Moumeni et al. [15], where the production of isobutanol was less than 8 mg/L at temperatures less than 30 °C, while almost 50 mg/L at 35 °C.

Undoubtedly the higher alcohols are the most important aroma components in fermented alcoholic beverages. Benn and Peppard [4] found higher alcohols in distilled tequila which were the main components (in quantity) among other aroma components including amyl alcohol (491 mg/L), 1-propanol (232 mg/L) and isobutanol (228 mg/L).

Conclusions

Three *S. cerevisae* strains (MALI 1, MTLI 1 and MGLI 1) and one *Kloeckera* strain (MTLI 2) were isolated from *Agave tequilana* Weber blue variety juice from three tequila distilleries. These yeasts were allowed to grow in

agave juice to study the biomass production and the maximum rate of sugar consumption as an indication of adaptation to the agave juice.

The fermentation temperatures and type of yeast used in tequila production are two of the principal factors influencing the productivity of ethanol and the production of acetaldehyde, ethyl acetate and higher alcohols. The characteristics of fermentation by the three *S. cerevisiae* strains isolated were not significantly different, while the difference between these yeast species and *Kloeckera* was pronounced.

Observed differences in aroma compound production by the isolated yeasts support the idea that yeast strains used in fermentation play an important role in the flavor characteristics of tequila and suggest that mixtures of these yeasts may be used to produce tequila with a unique and desirable aroma. The information presented in the current study may be utilized in the development of starter cultures with predictable characteristics for use in the small-scale commercial production of tequila.

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