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Fermentative capabilities and volatile compounds produced by *Kloeckera/Hanseniaspora* and *Saccharomyces* yeast strains in pure and mixed cultures during *Agave tequilana* juice fermentation

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Abstract The fermentative and aromatic capabilities of *Kloeckera africana/Hanseniaspora vineae* K1, *K. apiculata/H. uvarum* K2, and *Saccharomyces cerevisiae* S1 and S2 were studied in pure and mixed culture fermentations using *Agave tequila* juice as the culture medium. In pure and mixed cultures, *Kloeckera/Hanseniaspora* strains showed limited growth and sugar consumption, as well as low ethanol yield and productivity, compared to *S. cerevisiae,* which yielded more biomass, ethanol and viable cell concentrations. In pure and mixed cultures, *S. cerevisiae* presented a similar behaviour reaching high biomass production, completely consuming the sugar, leading to high ethanol production. Furthermore, the presence of *S. cerevisiae* strains in the mixed cultures promoted the production of higher alcohols, acetaldehyde and ethyl esters, whereas *Kloeckera/Hanseniaspora* strains stimulated the production of ethyl acetate and 2-phenyl ethyl acetate compounds.

Keywords *Agave tequilana* · *Kloeckera/Hanseniaspora* · *Saccharomyces cerevisiae* · Mixed cultures · Volatile compounds

Dulce María Díaz-Montaño—Research leader retired.

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Introduction

Tequila is a Mexican alcoholic beverage produced by distilling the fermented juice of the *Agave tequilana* Weber blue variety (*Agave* azul) plant (NOM-006-SCFI-2012 2012). Tequila production involves several steps: harvesting and cooking the *A.* azul plant, milling the cooked *A.* azul, fermenting the sugars, and distilling the must (Cedeño 1995). During tequila fermentation, yeasts convert sugars into ethanol and volatile compounds, which form the bouquet of the alcoholic beverage (Swiegers et al. 2005; Díaz-Montaño et al. 2008). As in wine fermentation, in traditional tequila distilleries spontaneous fermentation is carried out by a succession of different yeast

strains. The first stages of fermentation are characterized by the growth of certain non-*Saccharomyces* species, mainly *Torulaspora delbrueckii, Kluyveromyces marxianus*, and *Hanseniaspora* spp. (Lachance 1995). Their growth is quickly inhibited by the increase in ethanol concentration (Kunkee 1984) or by a nutritional limitation in the culture medium (Díaz-Montaño et al. 2010), progressively ceding the way to more alcohol-tolerant strains, especially *Saccharomyces cerevisiae* (Ciani and Picciotti 1995; Romano et al. 1997; Pérez-Nevado et al. 2006). Despite their limited growth during fermentation, non-*Saccharomyces* species produce a large number of important metabolites such as esters, acetic acid, and acetoin, which can have a significant influence on the sensory properties of the final product (Romano et al. 1997, 2003; Ciani and Picciotti 1995). In addition, non-*Saccharomyces* strains are associated with the synthesis of several extracellular enzymes such as β-glucosidases, which release monoterpenes derived from their glycosylated forms. These compounds give higher fruit-like aromatic characteristics to wine and other alcoholic beverages (Swangkeaw et al. 2010; Díaz-Montaño and Córdova 2009).

Volatile compounds provide taste and odor to alcoholic beverages. Most of these compounds are produced by yeast during fermentation. Many studies have examined tequila's aromatic profile. Benn and Peppard (1996) distinguished more than 175 compounds in three types of tequila (white, rested and aged). Díaz-Montaño et al. (2008) analyzed the volatile composition of agave juice fermented by *Saccharomyces* and *Kloeckera* strains, and determined that *Kloeckera* yeasts lead to higher concentrations of ethyl acetate, acetic acid, and 2-phenyl ethyl acetate than *Saccharomyces* strains, which predominantly produce higher alcohols, acetaldehyde, glycerol, isoamyl acetate, and ethyl hexanoate. Modern distilleries often select specific yeast strains to achieve a specific aroma profile (Ugliano et al. 2010). However, tequila is typically fermented with an inoculum of *S. cerevisiae*; due to its higher fermentative capacity (Hernández-Cortés et al. 2010). Nevertheless, several reports on wine fermentation have revealed the importance of using mixed cultures of non-*Saccharomyces* yeasts in combination with *S. cerevisiae* since they increase the aromatic fraction of the fermented media, therefore improving the sensory quality of the final product (Rojas et al. 2003; Romano et al. 2003; Ciani et al. 2006; Moreira et al. 2005). For example, mixed culture fermentations of non-*Saccharomyces* and *S. cerevisiae* have shown the production of higher concentrations of esters, terpenes, and acetoin than the must fermented by *S. cerevisiae* in monoculture (Mendes-Ferreira et al. 2001; Romano et al. 1997, 2003; Rojas et al. 2003).

To our knowledge, studies using mixed culture fermentations of non-*Saccharomyces* and *S. cerevisiae* native strains have not been reported previously in tequila and their potential use as an alternative to diversify or modify the overall flavour quality of the final product. Therefore, the main purpose of this work was to investigate the fermentative capability, growth, and synthesis of volatile compounds during the fermentation of agave juice, using pure and mixed cultures of *Kloeckera/Hanseniaspora* and *Saccharomyces* native strain

Materials and methods

Yeast strains

Four strains (S1, S2, K1 and K2) were isolated from *A. tequilana* Weber blue variety juice (*A. azul* juice) obtained from a tequila distillery in Jalisco, Mexico, and deposited in the culture collection at the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco (CIATEJ). They were identified by biochemical and molecular tests by Díaz-Montaño et al. (2008). The four strains, *S. cerevisiae* (S1 and S2), *Kloeckera africana/Hanseniaspora vineae* (K1), and *K. apiculata/H. uvarum* (K2) were stored in 1 ml vials at −70 °C in a 1:1 mixture of the propagation medium and a 50 % glycerol solution.

Inoculum and fermentation media

The *A. tequilana* Weber blue variety juice used was kindly supplied by La Quemada tequila distillery. The agave juice at 20°Bx was filtered and stored at −20 °C. Pre-inoculum and inoculum media were prepared in diluted agave juice adjusted to 3°Bx (31 ± 1.3 g/l of reducing sugars) with distilled water and supplemented with ammonium sulfate (1 g/l). A pre-inoculum was prepared by transferring 1 ml of conserved cells at –70 °C to 50 ml of medium contained in a 200 ml Erlenmeyer flask and incubated

at 30 °C and 250 rpm for 24 h. Then, an inoculum was prepared by adding 20 ml of pre-inoculum in 200 ml of medium contained in a 1 l Erlenmeyer flask and incubated at 30 °C and 250 rpm for 12 h. Fermentation media were prepared with agave juice adjusted to a reducing sugars concentration of 100 ± 13 g/l supplemented with ammonium sulfate (1 g/l) as it is common done in the tequila industry (Cedeño 1995). Fermentation and growth media were sterilized at 121 °C for 15 min.

Fermentation conditions

Pure and mixed culture fermentations were performed under anaerobic growth conditions in 500 ml-Erlenmeyer flasks containing 300 ml of agave juice at 30 °C and 250 rpm. These cultures were protected from exposure to oxygen using stoppers. Pure culture fermentations were inoculated with 3.5×10^6 cells/ml of the selected yeast strain. Meanwhile, mixed culture fermentations were inoculated with 1.75×10^6 cells/ml of each specific yeast. Prior to inoculation, the yeast population was estimated with a Neubauer chamber. All fermentations were stopped after 72 h and performed in duplicate for the concentration of biomass, ethanol, reducing sugars and volatile compounds.

Analytical methods

The yeast growth during fermentation was obtained by viable cell quantification using the classical plate count method. Samples were taken aseptically throughout the fermentations and diluted appropriately with distilled water. The enumeration of the yeasts in pure culture was accomplished in YEPD-agar medium (containing 20 g/l glucose, 20 g/l peptone, 20 g/l agar, and 10 g/l yeast extract at pH 4.7). In mixed culture fermentations, the enumeration of *Kloeckera/Hanseniaspora* yeasts (K1 and K2) cells was performed using YEPD-agar medium supplemented with 0.5 µg/ml of cycloheximide (Fluka analytical) (YEPD + CYH) (Pérez et al. 2000). The number of viable cells of *S. cerevisiae* strains (S1 and S2) in mixed cultures was given as the difference between the total number of colonies on YEPD-agar plates and the total number of colonies on YEPD + -CYH plates. YEPD-agar and YEPD + CYH plates were incubated at 30 °C for 2–6 days. The number of cells for each yeast strain was calculated when no increase in the number of colony forming units (CFU) was observed.

Biomass concentration was determined by measuring the dry weight of 5 mL fermented must centrifuge as reported by Díaz-Montaño et al. (2010). Must supernatants were used to assay sugars, ethanol and volatile compounds. Reducing sugars and ethanol concentrations were measured using the modified DNS method (Díaz-Montaño, 2004) and enzymatic analyzer (YSI model 2700 select, Yellow Springs Instruments) respectively.

Volatile compounds concentrations were determined by duplicated by gas chromatography (GC) following Valle-Rodríguez et al. (2012) conditions. A Hewlett Packard Head-space HP 7694E model connected to a Hewlett-Packard 6890 Series gas chromatograph equipped with a flame ionization detector (FID) and a 60 m × 320 µm × 0.25 µm thickness film HP-Innowax capillary column were used. The oven temperature was held at 35 °C for 10 min, then increased at a rate of 3.5 °C min^{-1} until 175 °C for 2 min, and finally increased at 7 °C min^{-1} until 250 °C were reached. Helium was the carrier gas at 1.5 mL min^{-1}. The detector and injector temperatures were 260 °C and 240 °C, respectively. The Headspace system program temperatures were 80, 110, and 115 °C for vial, loop and *R*-line respectively. The times for the gas chromatograph cycle, vial equilibrium, pressurization, filling loop, equilibrium of loop, injection and agitation were: 41, 10, 0.2, 0.2, 0.5, 1 and 5 min, respectively. Quantification was based on the external standard method using different diluted solutions containing 0.05, 0.2, 0.4, 0.6, 0.8, 1 and 2 mg/l of isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and 2-phenyl ethyl acetate; 1, 4, 8, 12, 16, 20 and 40 mg/l of *n*-butanol, acetaldehyde and ethyl acetate; 2, 8, 16, 24, 32, 40 and 80 mg/l of *n*-propanol, isobutanol and 2-phenyl ethanol, 20, 60, 120, 180, 240, 300 and 600 mg/l of isoamyl alcohol, and 10, 40, 80, 120, 160, 200 and 400 mg/l of methanol. Calibration curves gave a correlation coefficient (R^2) greater than or equal to 0.999 for each compound as determined using the HP Chemstation software Rev. A.05.04.

Data treatment and statistical analysis

The response variables data (biomass, ethanol and reducing sugar) of the two fermentations for each

yeast strain in pure and mixed culture were compared using the Student's *t* test for means comparison of paired samples at a 95 % probability. When significant differences were found in response variable data between replicates, the experiment was performed again two times in order to obtain more reliable data. Experimental data were adjusted by using the Curve Expert 1.3 program to determine the kinetic parameters (EBT Comm, Columbus, USA). The statistical method used for comparing yeast strain performance was the one-way variance analysis (ANOVA). The response variables measured were as follows: final concentration of different volatile compounds, biomass, ethanol, and consumed substrate, as well as maximal value of the specific growth rate, ethanol production rate, and sugar consumption rate. The differences in the amounts of volatile compounds were analyzed by principal component analysis (PCA). The ANOVA analysis employed Statgraphics plus software (Manugistics Inc; Rockville, USA). PCAwas performed using Simca software P7.01.

Results and discussion

Kinetic analysis of *Kloeckera/Hanseniaspora* and *Saccharomyces* strains in pure and mixed cultures.

Pure cultures

Fermentations by pure cultures of *S. cerevisiae* (S1 and S2), *K. africana/H. vineae* (K1) and *K. apiculata/ H. uvarum* (K2) were performed under anaerobic growth at 30 °C and 250 rpm. Viable cell (CFU), biomass, reducing sugar consumption, and ethanol production profiles for each yeast strain in pure cultures are plotted in Fig. 1, and the kinetic parameters are shown in Table 1. The behaviour of *Saccharomyces* and *Kloeckera/Hanseniaspora* strains in pure culture was different. Both strains of *S. cerevisiae* grew faster than *Kloeckera/Hanseniaspora* yeasts reaching 4.22 and 3.68 g/l of biomass (S1 and S2) in 24 and 16 h of fermentation respectively. On the one hand, *Kloeckera/Hanseniaspora* (K1 and K2) strains grew slowly; reaching maximal concentrations of 1.83 and 1.93 g/l at the end of fermentation (Fig. 1). On the other hand, the viable cells (CFU) of *S. cerevisiae* (S1 and S2) strains remained constant for a much longer time, reaching 98.5 and 147 $\times$ 10^6 CFU/ml at 16 h of fermentation. *Kloeckera/Hanseniaspora* strains (K1 and K2) showed a maximum level at 16 and 20 h of fermentation, reaching 74 and 62.5 $\times$ 10^6 CFU/ml respectively (Fig. 1). However, the viable cell number for *Kloeckera/Hanseniaspora* yeasts declined gradually to 30 $\times$ 10^6 CFU/ml at the end of fermentation (Fig. 1).

Significant differences were observed for sugar consumption and ethanol production among the yeast strains in pure culture (95 % LSD). Both *Saccharomyces* strains (S1 and S2) completely consumed all the fermentable sugars from agave juice after 24 and 36 h of fermentation and produced high ethanol concentration >36 g/l (Fig. 1). By contrast, *Kloeckera/Hanseniaspora* species showed low fermentative capacity compared to *Saccharomyces*, leaving around 40 g/l of residual sugar in the culture medium at 72 h of fermentation and producing lower ethanol concentrations (≥10 g/l) (Fig. 1).

Growth yields were also different: *Saccharomyces* strains converted sugars into ethanol more efficiently than *Kloeckera/Hanseniaspora* strains, with ethanol/-sugar yields reaching 0.33 and 0.34 g/g. These values are close to the theoretical ethanol/sugars yield (0.51). In contrast, the *Kloeckera/Hanseniaspora* strains presented a lower ethanol/sugar yield of 0.10 and 0.18 g/g (Table 1). No significant differences were observed in biomass/sugar yields ($Y_{x/s}$) among strains. Moreover, statistical analysis (95 % LSD) showed significant differences among yeast strains in all kinetic parameters. As expected, the higher values of specific growth rate (μ_{max}), sugar consumption rate (q_{pmax}), and ethanol production rate (q_{pmax}) were reached by *Saccharomyces* strains (Table 1). Previous tequila studies have reported similar results. Díaz-Montaño et al. (2008) observed that *Kloeckera/Hanseniaspora* strains presented a lower fermentative capability than *Saccharomyces,* probably due to a nutritional limitation and/or to the presence of toxic compounds in agave juice. However, *K. africana/H. vineae* in pure culture in the presence of specific growth factors such as amino acids and vitamins presents a better growth and fermentative efficiency (Díaz-Montaño et al. 2010; Valle-Rodríguez et al. 2012). This can be explained since the presence of amino acids in the medium may increase the ability for rapid synthesis of degraded proteins as glucose transporters, which allow the yeast to achieve complete fermentations in the particular case of non-*Saccharomyces* strains (Díaz-Montaño

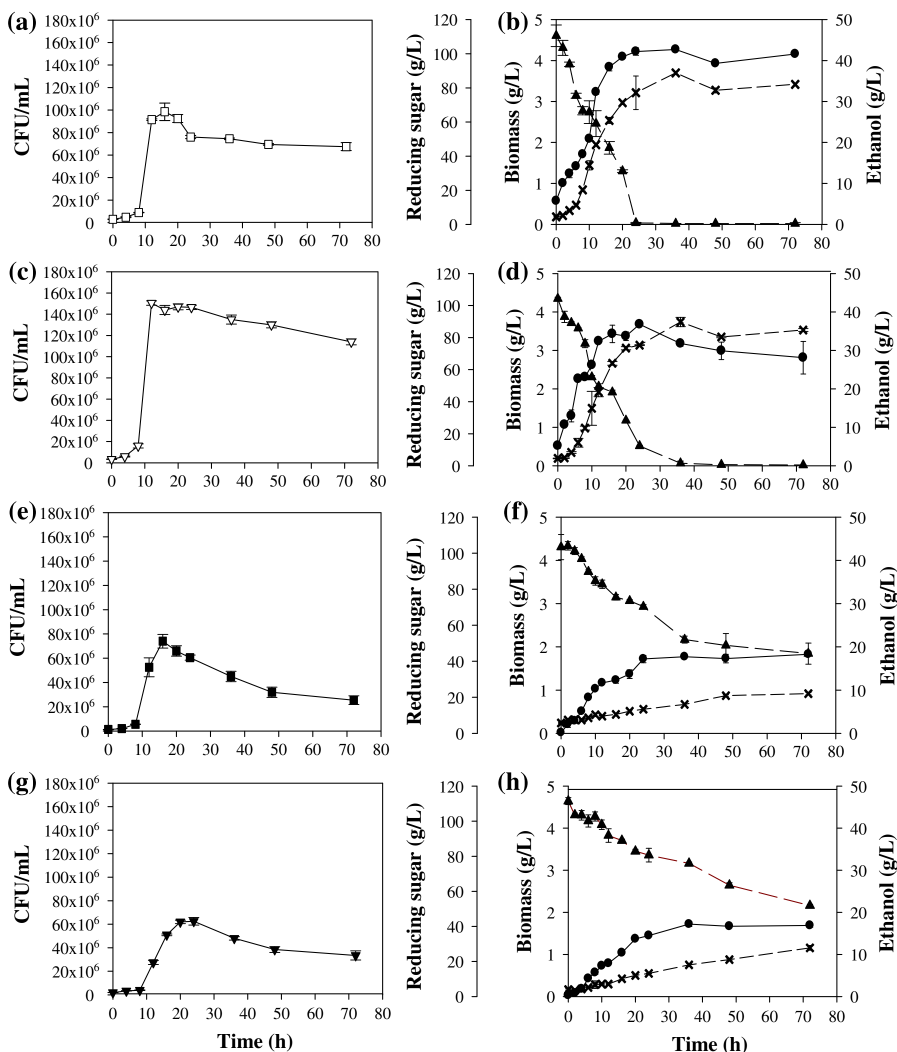

Fig. 1 Kinetic profiles of fermentations by pure culture of *S. cerevisiae* S1 (-□-), *S. cerevisiae* S2 (-▽-), *K. africana*/*H. vineae* K1 (*filled square*) and *K. apiculata*/*H. uvarum* K2 (*filled inverted triangle*) strains in *Agave tequilana* juice media at 12°Brix supplemented with ammonium sulphate (1 g/l). CFU: viable cell (**a**, **c**, **e**, **g**); biomass: biomass concentration profile (*filled circle*); reducing sugar: reducing sugar concentration profile (*filled triangle*) and ethanol: ethanol concentration profile (-✱-). Each value represents the average ± SD of two fermentations. *Vertical bars* represent SD

Table 1 Comparison of kinetic parameters for different stains in pure cultures

Strain	μ_{max} (h^{-1})	q_{smax} (g/g h^{-1})	q_{pmax} (g/g h^{-1})	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)
K1	0.11 ± 0.002	2.94 ± 0.64	0.14 ± 0.01	0.027 ± 0.002	0.10 ± 0.016
K2	0.08 ± 0.005	2.79 ± 0.02	0.15 ± 0.02	0.034 ± 0.003	0.18 ± 0.038
S1	0.18 ± 0.03	4.82 ± 0.50	1.12 ± 0.03	0.031 ± 0.006	0.33 ± 0.005
S2	0.27 ± 0.03	3.64 ± 0.25	1.45 ± 0.44	0.028 ± 0.02	0.34 ± 0.01

μ_{max} maximum specific growth rate, q_{smax} maximum specific sugar consumption rate, q_{pmax} maximum specific ethanol production rate, $Y_{x/s}$ and $Y_{p/s}$ yields of biomass and ethanol. Each value represents the average ± SD of two fermentations

et al. 2008, 2009). With regard to the selectivity of sugar transport in *S. cerevisiae*, it has been considered that this yeast is mostly glucosophilic and its high fermentative capacity is mainly attributed to its ability to metabolize both inorganic and organic N-sources from the culture medium (Díaz-Montaño et al. 2010). Nevertheless, other mechanisms could be implicated during the fermentation of agave using mixed cultures.

Mixed cultures

Development of biomass, viable cell (CFU), sugar consumption, and ethanol production versus time were plotted in Fig. 2 and Table 2 shows the kinetic parameters obtained from mixed culture fermentations of *Kloeckera/Hanseniaspora* and *S. cerevisiae*. Three different yeast groups were tested during the fermentation of agave juice; Group 1, a mixed culture of *K. africana/H. vineae* and *S. cerevisiae* (K1 + S1 − G1), Group 2, a mixed culture of *K. apiculata/H.uvarum* and *S. cerevisiae* (K2 + S1 − G2) and Group 3, a mixed culture of *K. apiculata/H. uvarum* and *S. cerevisiae* (K2 + S2 − G3). During the first eight hours of culture, *K. africana/H. vineae* (K1) and *K. apiculata/H. uvarum* (K2) presented similar growth rates than *S. cerevisiae* (S1 and S2). However, the proportion of *S. cerevisiae* strains in the mixed cultures increased during fermentation, while the proportion of *Kloeckera/Hanseniaspora* decreased (Fig. 2a, c, e). *S. cerevisiae* strains reached maximum growth of 16.8 (K1 + S1 − G1), 10.9 (K2 + S1 − G2), and 17.1 × 10^6 CFU/ml (K2 + S2 − G3) at 24, 36 and 16 h of fermentation (Fig. 2). The maximum growth of *Kloeckera/Hanseniaspora* strains in mixed cultures with *S. cerevisiae* reached 9.95 (K1 + S1 − G1), 7.5 (K2 + S1 − G2), and 10.7 × 10^6 CFU/ml (K2 + S2 − G3) at 24, 20 and 16 h of fermentation respectively. In all cases, the population of *Kloeckera/Hanseniaspora* dropped below 6.5 × 10^6 CFU/ml (Fig. 2). Although

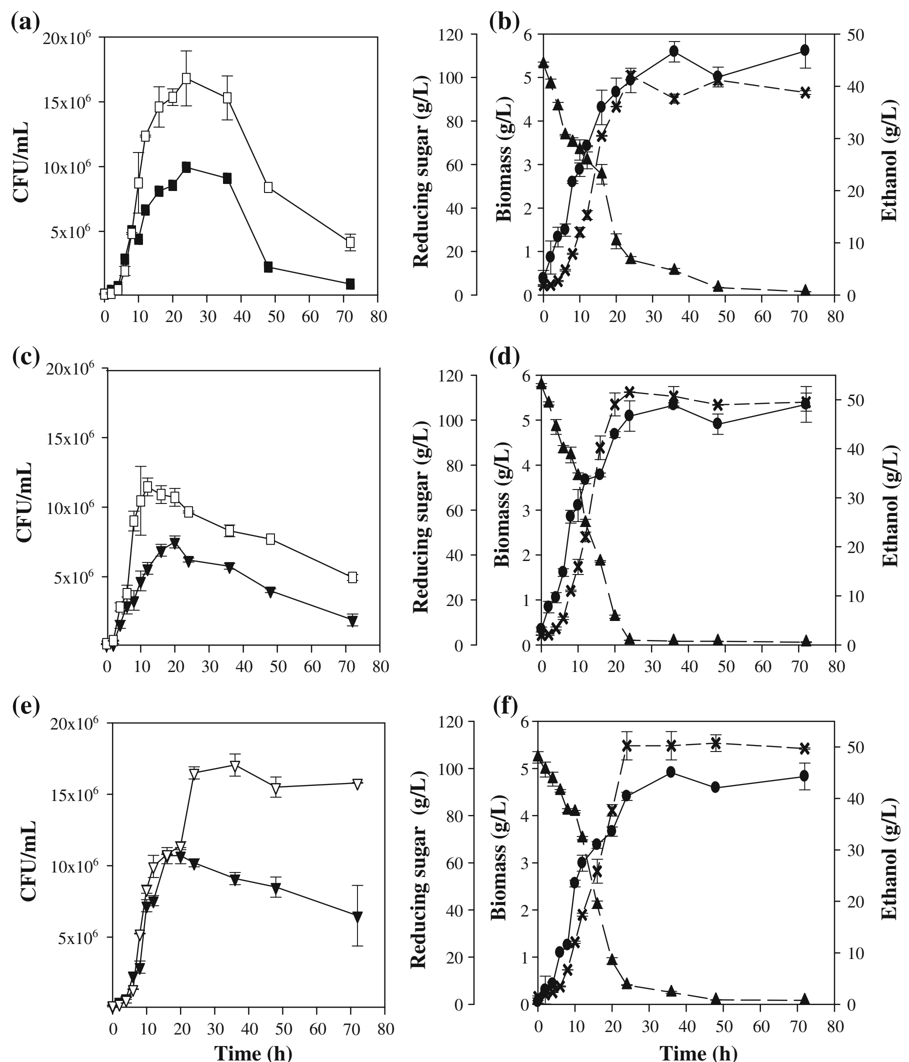

Fig. 2 Kinetic profiles of fermentations by mixed culture of *S. cerevisiae* S1 (-□-), *S. cerevisiae* S2 (-▽-), *K. africana/H. vineae* K1 (*filled square*) and *K. apiculata/H. uvarum* K2 (*filled inverted triangle*) strains in *Agave tequilana* juice media at 12°Brix supplemented with ammonium sulphate (1 g/l). Group 1: K1 + S1; Group 2: K2 + S1 and Group 3: K2 + S2. CFU: viable cell (**a**, **c**, **e**) biomass: biomass concentration profile (*filled circle*); reducing sugar: reducing sugar concentration profile (*filled triangle*) and ethanol: ethanol concentration profile (-✱-). Each value represents the average ± SD of two fermentations. *Vertical bars* represent SD

Table 2 Comparison of kinetic parameters for different stains in mixed cultures

Mixed cultures	μ_{max} (h^{-1})	q_{smax} (g/g h^{-1})	q_{pmax} (g/g h^{-1})	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)
Group 1[a]	0.22 ± 0.06	5.24 ± 0.34	0.91 ± 0.10	0.049 ± 0.005	0.37 ± 0.015
Group 2[b]	0.27 ± 0.06	16.6 ± 4.38	0.78 ± 0.24	0.037 ± 0.05	0.38 ± 0.01
Group 3[c]	0.31 ± 0.16	8.65 ± 6.97	2.12 ± 1.94	0.042 ± 0.01	0.48 ± 0.04

μ_{max} maximum specific growth rate, q_{smax} maximum specific sugar consumption rate, q_{pmax} maximum specific ethanol production rate, $Y_{x/s}$ and $Y_{p/s}$ yields of biomass and ethanol

[a] Mixed culture of *K. africana/H. vineae* (K1) and *S.cerevisiae* (S1)

[b] Mixed culture of *K. apiculata/H. uvarum* (K2) and *S. cerevisiae* (S1)

[c] Mixed culture of *K. apiculata* (K2) and *S. cerevisiae* (S2). Each value represents the average ± SD of duplicate determinations of two fermentations

Saccharomyces strains remained active for a longer period than *Kloeckera/Hanseniaspora* strains, their growth in Group 1 and Group 2 declined at 36 h and 24 h to cell concentrations of 4.15 and 4.95 × 10^6 CFU/ml at the end of fermentation (Fig. 2a, b). However, the viable cells (CFU) of *S. cerevisiae* S2 (group 3) showed higher values until the end of fermentation (Fig. 2e).

No significant differences were observed (95 % LSD) among the mixed cultures regarding biomass concentration (values ≥5 g/l at 36 h of fermentation), ethanol production (>42 g/l in 24 h of fermentation) and sugar consumption (Fig. 2b, c, f). Although fermentable sugars were completely consumed in all mixed cultures, there were significative differences in maximum specific rate of sugar consumtion (q_{smax}) and ethanol production. Mixed culture group 2 presented a higher rate of maximum specific sugar consumption (16.6 g/g h^{-1}) than the other two mixed cultures (5.24 and 8.65 g/g h^{-1} for G1 and G3 respectively), depleting the sugar in 24 h of fermentation, while the other two groups consumed it in approximately 50 h. Furthermore, mixed culture group 2 presented a higher rate of maximum specific ethanol production (2.12 g/g h^{-1}) than the G1 and G3 mixed cultures (0.91 and 0.78 g/g h^{-1} respectively). Otherwise, no significant differences were observed (95 % LSD) in the maximum specific growth rate (μ_{max}) among the three mixed cultures (G1, G2, G3), which reached 0.22, 0.27 and 0.31 h^{-1} respectively. Moreover, no significant differences were observed in either ethanol/sugar ($Y_{p/s}$) or biomass/sugar ($Y_{x/s}$) yields among three groups (95 % LSD).

Mixed cultures of *Kloeckera/Hanseniaspora* and *S. cerevisiae* presented similar fermentative capacity with respect sugar consumption and ethanol production than *S. cerevisiae* in pure culture, which in mixed cultures was the dominant yeast. However, significant differences were observed regarding CFU profiles. Pure cultures of *Saccharomyces* and *Kloeckera/Hanseniaspora* presented higher culture stability and cell concentration than mixed cultures, which exhibited a different performance in each culture (Figs. 1, 2). Our results are in agreement with previous studies conducted in wine fermentation. Mendoza et al. (2007) reported that *K. apiculata* and *S. cerevisiae* in pure cultures reached higher cell concentrations than mixed cultures. The maximum cell density in both cultures decrease; possible due to mechanisms related to ethanol concentration, killer toxins, and competition for assimilable nitrogen compounds. However, the persistence of non-*Saccharomyces* yeasts during fermentation may depend upon many other factors, such as the presence of toxic compounds other than ethanol, availability of nutrients, fermentation temperature, oxygen availability, and the strength of the *Saccharomyces* inoculum (Díaz-Montaño et al. 2010; Mancilla-Margalli and López 2002; Erten 2002; Holm Hansen et al. 2001; Nissen et al. 2003). The main cause reported for the early death of non-*Saccharomyces* yeasts during mixed culture fermentation with *S. cerevisiae* is usually the lower ethanol tolerance of non-*Saccharomyces* in comparison to *S. cerevisiae*. In spite of that, it has been reported in tequila fermentation that *K. africana* K1 requires N-organic sources and certain growth factors such as asparagine to stimulate and improve its growth and fermentative

Table 3 Concentration of volatile compounds produced by pure and mixed cultures of *Kloeckera africana/H. vineae* (K1), *Kloeckera apiculata/H. uvarum* (K2), *Saccharomyces cerevisiae* (S1) and *Saccharomyces cerevisiae* (S2)

Volatile compounds (mg/l)	Pure cultures				Mixed cultures		
	S1	S2	K1	K2	Group 1[a]	Group 2[b]	Group 3[c]
Σ Aldehydes	157.94 ± 2.71	170.72 ± 3.04	47.89 ± 5.06	46.73 ± 5.94	89.46 ± 2.31	101.04 ± 1.64	144.11 ± 1.45
Acetaldehyde	157.94 ± 2.71	170.72 ± 3.04	47.89 ± 5.06	46.73 ± 5.94	89.46 ± 2.31	101.04 ± 1.64	144.11 ± 1.45
Alcohols	64.18 ± 0.58	63.28 ± 1.15	58.94 ± 0.37	46.05 ± 2.73	66.82 ± 1.6	63.67 ± 1.58	64.99 ± 0.18
Methanol[d]	64.18 ± 0.58	63.28 ± 1.15	58.94 ± 0.37	46.05 ± 2.73	66.82 ± 1.6	63.67 ± 1.58	64.99 ± 0.18
Σ Higher alcohols	284.92 ± 14.25	318 ± 4.99	122.51 ± 6.69	114.69 ± 7.88	247.3 ± 3.50	254.36 ± 9.40	307.28 ± 0.91
n-Propanol[d]	25.28 ± 2.03	32.18 ± 0.37	27.60 ± 1.35	29.38 ± 2.53	25.55 ± 0.23	23.97 ± 0.5	30.65 ± 0.02
n-Butanol	0.38 ± 0.02	0.77 ± 0.04	4.47 ± 0.18	1.03 ± 0.13	1.31 ± 0.01	0.75 ± 0.014	0.75 ± 0.02
Isobutanol	53.95 ± 3.34	56.74 ± 0.99	23.04 ± 0.28	29.08 ± 2.53	51.69 ± 0.56	54.64 ± 5.03	57.28 ± 0.2
Isoamyl alcohol	156.36 ± 7.15	162.44 ± 2.76	56.91 ± 3.57	42.98 ± 0.65	129.75 ± 1.68	127.91 ± 2.32	148.21 ± 0.25
2-Phenyl ethanol	48.95 ± 1.71	65.87 ± 0.83	10.485 ± 1.31	12.22 ± 2.04	39.00 ± 1.01	47.09 ± 1.53	70.39 ± 0.67
Σ Esters	13.45 ± 2.29	14.37 ± 0.44	79.193 ± 1.78	67.74 ± 1.04	34.58 ± 0.91	47.91 ± 2.01	69.124 ± 1.74
Ethyl acetate	13.19 ± 2.26	14.11 ± 0.43	74.22 ± 1.42	65.37 ± 0.80	33.13 ± 1.33	46.44 ± 1.99	67.94 ± 1.64
Isoamyl acetate	0.13 ± 0.03	0.08 ± 0.003	2.97 ± 0.28	1.43 ± 0.096	0.09 ± 0.001	0.28 ± 0.005	0.14 ± 0.001
Ethyl hexanoate	0.025 ± 0.002	0.030 ± 0.001	0.018 ± 0.001	0.031 ± 0.001	0.022 ± 0.003	0.014 ± 0.001	0.029 ± 0.003
Ethyl octanoate	0.041 ± 0.001	0.052 ± 0.002	nd	0.059 ± 0.006	0.050 ± 0.003	0.053 ± 0.001	0.052 ± 0.002
2-Phenyl ethyl acetate	nd	nd	1.92 ± 0.08	1.79 ± 0.07	1.25 ± 0.01	1.05 ± 0.01	0.91 ± 0.085
Ethyl decanoate	0.064 ± 0.003	0.096 ± 0.003	0.065 ± 0.001	0.061 ± 0.005	0.041 ± 0.001	0.069 ± 0.002	0.053 ± 0.001

nd not detected. Each value represents the average ± SD of two determinations

[a] Mixed culture of *Kloeckera africana/H. vineae* (K1) and *Saccharomyces cerevisiae* (S1)

[b] Mixed culture of *Kloeckera apiculata/H. uvarum* (K2) and *Saccharomyces cerevisiae* (S1)

[c] Mixed culture of *Kloeckera apiculata//H. uvarum* (K2) and *Saccharomyces cerevisiae* (S2)

[d] No significantly different among the yeasts

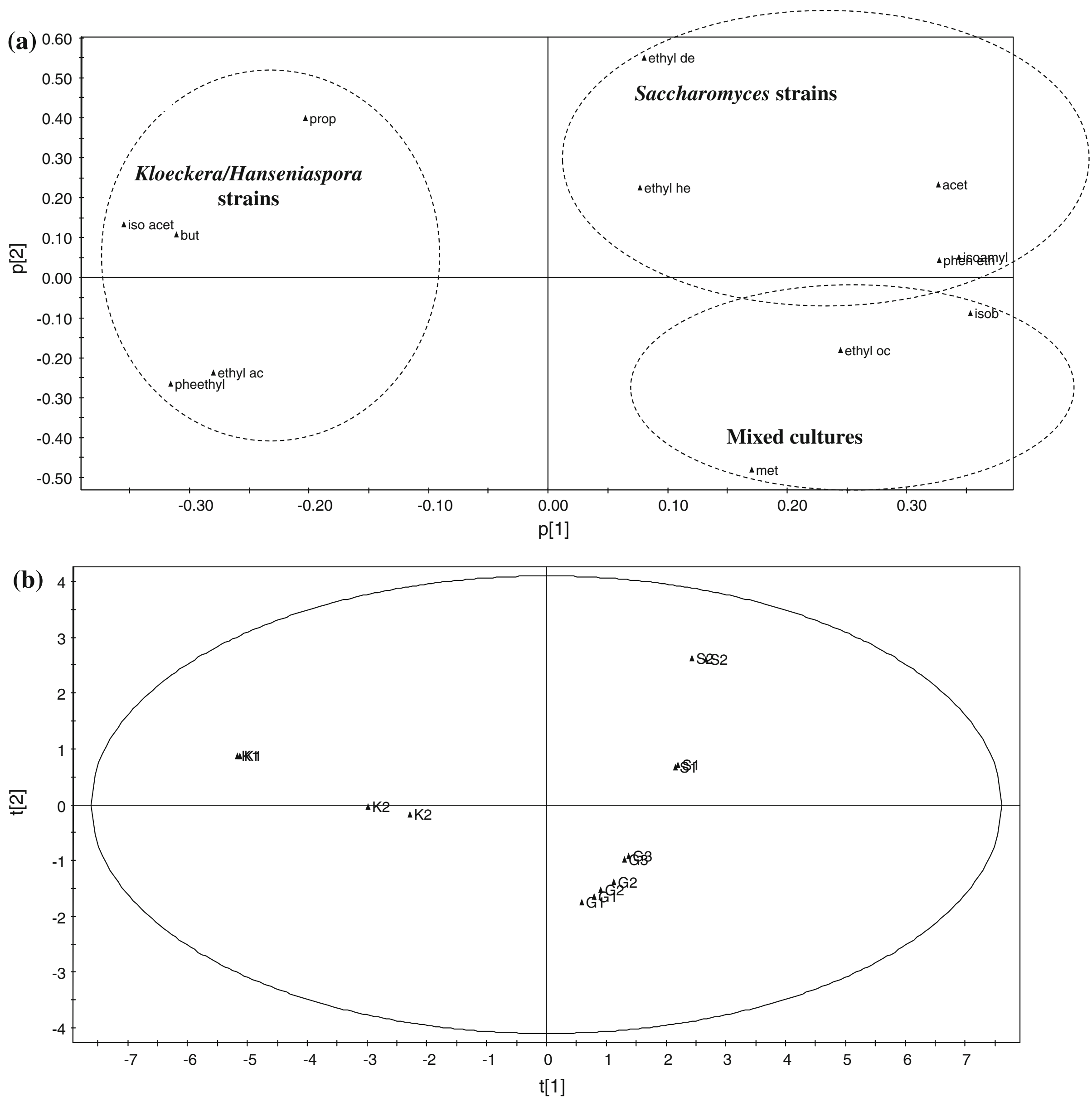

Fig. 3 PCA of **a** the volatile compounds and **b** the yeast strains in pure and mixed cultures. *acet* acetaldehyde, *met* methanol, *ethyl-acet* ethyl acetate, *iso-acet* isoamyl acetate, *phenethyl-acet* 2-phenyl ethyl acetate, *ethyl-hex* ethyl hexanoate, *ethyl-oct* ethyl octanoate, *ethyl-dec* ethyl decanoate, *prop n*-propanol, *but n*-butanol, *isob* isobutanol, *isoamyl* isoamyl alcohol, *phen-eth* 2-phenyl ethanol. K1: *K. africana/H. vineae*; K2: *K. apiculata/H. uvarum*; S1: *S. cerevisiae;* S2: *S. cerevisiae;* G1: mixed culture of K1 and S1; G2: mixed culture of K2 and S1; G3: mixed culture of K2 and S2

efficiency (Díaz-Montaño et al. 2010; Valle-Rodríguez et al. 2012).

Since *K. africana* K1 is one of the strains used in this study, the low availability of N-organic sources and vitamins in the agave juice could be the main cause of the limited growth of *Kloeckera* yeasts in mixed culture fermentation with *Saccharomyces*. Although the present study provides a better understanding regarding the fermentative capabilities of non-*Saccharomyces* strains during agave juice fermentation in mixed culture with *S. cerevisiae* under similar conditions to those used in the tequila industry,

the effect of supplementing the agave juice with specific amounts of growth factors and nitrogen sources have been performed and an increase in growth and fermentative capability of *K. africana/H. vineae* in co-culture with *S. cerevisiae* has been observed (González-Robles 2012).

Volatile compounds produced by *Kloeckera/Hanseniaspora* and *Saccharomyces* yeasts in pure and mixed cultures

This study determined secondary metabolites such as higher alcohols, esters and aldehydes, obtained from pure and mixed cultures of *Saccharomyces* and *Kloeckera/Hanseniaspora* strains (Table 3). *Saccharomyces* strains (S1 and S2) in pure and mixed cultures produced higher concentrations of acetaldehyde, isobutanol, isoamyl alcohol and 2-phenyl ethanol than pure cultures of *Kloeckera/Hanseniaspora* (K1 and K2), which generated higher amounts of ethyl acetate, isoamyl acetate and 2-phenyl ethyl acetate (Table 3). *K. africana/H. vineae* K1 in pure culture produced the highest concentration of *n*-butanol (Table 3). No significant differences were observed in *n*-propanol or methanol concentrations among pure and mixed cultures (95 % LSD) (Table 3). Methanol concentration was similar in all fermentations since it is produced mainly during the agave-cooking step (Cedeño 1995).

Other authors reported similar results. Zironi et al. (1993) reported that *S. cerevisiae* in pure and mixed cultures produced higher concentrations of higher alcohols than *K. apiculata* and *H. guillermondii* in pure cultures, which only produced greater amounts of 2,3-butanediol and acetoin. Díaz-Montaño et al. (2008) reported that *Saccharomyces* strains produced higher concentrations of higher alcohols and acetaldehyde than *Kloeckera,* which produced high concentrations of ethyl acetate, 2-phenetyl acetate, and acetic acid. Higher alcohols confer a strong pungent taste and odor to alcoholic beverages. In concentrations below 300 mg/l, they contribute to desired complexity but in concentrations greater than 400 mg/l, there is a negative effect on the aroma (Swiegers et al. 2005; Díaz-Montaño and Córdova 2009). The concentration of higher alcohols depends on several factors, including the type of yeast strain, fermentation temperature, pH, and amino acid composition of the culture medium (Swiegers et al. 2005; Pinal et al. 1997). Acetaldehyde at low concentrations (<100 mg/l) contributes to the sensory properties of the beverage by conferring apple-like, citrus-like and nutty descriptors. However, high concentrations result in an irritating, spicy scent (Schreier and Jennings 1979). Moreover, a high concentration of ethyl acetate (>200 mg/l) does not improve the aroma of the fermented beverage it generates an off-flavour instead. Yet, at low concentrations (50–80 mg/l), it contributes to the quality of beverage by conferring fruity notes (Zohre and Erten 2002; Moreira et al. 2005). Our results are in accordance with those reports since the *Kloeckera/Hanseniaspora* strains (K1 and K2) in pure and mixed cultures produced greater amounts of ethyl

Table 4 PCA factor loadings of the volatile compounds and explained variance of each component

	PC1		PC2
Varianza explicada (%)	58.8	Varianza explicada (%)	15.0
Isoamyl acetate	−0.3552	Methanol	−0.4792
2-Phenyl ethyl acetate	−0.3158	2-Phenyl ethyl acetate	−0.2649
n-Butanol	−0.3106	Ethyl acetate	−0.2359
Ethyl acetate	−0.2801	Ethyl octanoate	−0.1812
n-Propanol	−0.2031	Isobutanol	−0.0888
Ethyl hexanoate	0.0768	2-Phenyl ethanol	0.0438
Ethyl decanoate	0.0801	Isoamyl alcohol	0.0534
Methanol	0.1703	*n*-Butanol	0.1089
Ethyl octanoate	0.2447	Isoamyl acetate	0.1350
Acetaldehyde	0.3265	Ethyl hexanoate	0.2270
2-Phenyl ethanol	0.3272	Acetaldehyde	0.2339
Isoamyl alcohol	0.3436	*n*-Propanol	0.3997
Isobutanol	0.3536	Ethyl decanoate	0.5504

acetate, in the order of 33.13–74.22 mg/l (Table 3). Besides ethyl acetate, other acetate esters that have a positive effect on the overall flavour of the beverage are isoamyl acetate and 2-phenyl ethyl acetate. These were produced in greater amounts by *Kloeckera/Hanseniaspora* yeasts in pure cultures, providing sweet, rose-like, fruity and banana-like descriptors (Díaz-Montaño and Córdova 2009; Swiegers et al. 2005). Nevertheless ethyl hexanoate, ethyl octanoate, and ethyl decanoate were mainly produced by *Saccharomyces* yeast strains in pure and mixed cultures (Table 3; Fig. 3).

PCA grouped volatile compounds produced by *Kloeckera/Hanseniaspora* and *Saccharomyces* yeasts in pure and mixed cultures into two principal components with a 73.8 % explained variance (Fig. 3; Table 4). Volatile compounds with a greater weight in the first component (PC-1) were isoamyl acetate, 2-phenyl ethyl acetate, and *n*-butanol with negative weights, and acetaldehyde, 2-phenyl ethanol, isoamyl alcohol, and isobutanol with positive weights. Meanwhile, the volatile compounds for PC-2 were methanol and 2-phenyl ethyl acetate with negative weights; and *n*-propanol and ethyl decanoate with positive weights (Fig. 3; Table 4).

Conclusions

Kloeckera africana/H. vineae, K. apiculata/H. uvarum, S. cerevisiae S1 and S2 were examined in pure and mixed culture fermentations of agave juice. Results showed that *Kloeckera/Hanseniaspora* strains grew poorly in the presence of *S. cerevisiae.* Possible hypotheses to explain the growth inhibition include a nutritional limitation in agave juice or the presence of toxic compounds. On the other hand, *Saccharomyces* yeast strains showed high fermentative efficiency, remaining active for much longer time during fermentation. They also exhibited higher ethanol and biomass concentrations than fermentations with *Kloeckera/Hanseniaspora* strains. In addition, the presence of *Kloeckera/Hanseniaspora* yeast strains in mixed cultures had a significant influence on the sensorial profile; since high ester concentration like ethyl acetate, isoamyl acetate and 2-phenyl ethyl acetate were present at the end of fermentation. Indeed, lower concentrations of the volatile compounds regulated under the Official Mexican Standard (NOM-006-SCFI-2012 2012) were observed from the mixed culture than from the pure culture of *Saccharomyces*.

These results suggest the potential and beneficial use of using mixed cultures during tequila fermentation because could produce beverages with distinctive sensory properties and low concentrations of regulated volatile compounds. However, further studies are needed to assure all the implications of their application during tequila production.

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