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Fermentative capability and aroma compound production by yeast strains isolated from *Agave tequilana* Weber juice

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Abstract

Five yeast strains isolated from agave juice were studied for their fermentative and aromatic capacity. The experiments were performed using agave juice supplemented with ammonium sulphate, as is commonly done in tequila distilleries. Three strains classified as *Saccharomyces cerevisiae* showed high biomass and ethanol production, as well as higher ethanol tolerance than those classified as *Kloeckera africana* and *Kloeckera apiculata*, which showed scarce growth. The results suggest that *Kloeckera* strains were affected by nutritional limitation and/or toxic compounds present in agave juice. Agave juice analyses showed a lower amino acid content than those reported in grape juice. *S. cerevisiae* strains produced predominantly amyl and isoamyl alcohols, *n*-propanol, 2-phenyl ethanol, succinic acid, glycerol, methanol, isoamyl acetate, ethyl hexanoate, acetaldehyde and isobutanol, whereas *Kloeckera* strains showed a high production of acetic acid, 2-phenyl ethyl acetate and ethyl acetate. The methanol concentration was significantly different among the yeasts studied. The diversity between three *S. cerevisiae* strains were higher for the aromatic profile than for genetic level and kinetic parameter. On the other hand, the diversity of *Kloeckera* yeasts were lower than *Saccharomyces* yeasts even when belonging to two different species.

Keywords: Agave; *Saccharomyces*; *Kloeckera*; Alcoholic fermentation; Tequila

1. Introduction

Tequila is a Mexican alcoholic beverage distilled from the fermentated juice of cooked *Agave tequilana* Weber (blue variety). Production is strictly regulated such that only beverages produced from *A. tequilana* Weber (blue variety) cultivated in a protected region of Mexico can be labeled with the guarantee of origin (Norma Oficial Mexicana, 2005). The tequila process involves multiple steps: upon harvest, fermentable sugars are obtained from heads of the agave plant by steaming, milling and pressing. During the steaming process, the polysaccharides (inulin) are hydrolyzed into a mixture of sugars which mainly consist of fructose. Besides the hydrolysis of inulin during the cooking process of agave, many volatile compounds mainly Maillard compounds are produced, principally 5-hydroxymethyl furfural [1]. In some tequila distilleries, fermentation occurs spontaneously while in others, the agave juice is inoculated using commercial or indigenous yeast cultures, often *Saccharomyces cerevisiae*. In spontaneous juice fermentation, Lachance [2] reported that *Torulaspora delbrueckii*, *Hanseniaspora* spp., and *Kluyveromyces marxianus* progressively gave way to *S. cerevisiae*, *Zygosaccharomyces bailii*, *Candida milleri* and *Brettanomyces* spp. After fermentation, the agave juice fermented is distilled twice and then diluted to obtain a final alcohol content of 38% (v/v). The product is aged in white oak barrels for 2–12 months to obtain “reposado” or “añejo” tequila, respectively. A complete description of the production process was reported by Cedeño [3].

During alcoholic fermentation, the yeast cells produce primarily ethanol and CO_2 as well as many flavor compounds as secondary products. Existing studies report the aromatic profile of tequila [4–6]. Benn and Peppard [7] distinguished more than 175 volatile compounds in three types of tequila; high con-

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centration of higher alcohols were observed along with low concentrations of esters, acetals, terpenes, furans, acids, aldehydes, ketones, phenols and sulphurs. Of these compounds, the majority is produced by yeast fermentation; therefore, the microbial community composition is a key factor in the aromatic quality of the fermented beverages. Some yeast, frequently non-*Saccharomyces* such as *Kloeckera* spp. and *Hanseniaspora uvarum*, are known for the quality and amount of volatile compounds produced in wine [8]. Factors which may affect the microbial community in wines, such as the presence of inhibitors (killer toxin, ethanol and high sugar concentration), the availability of nutrients, growth factors and physicochemical conditions, may also prove to be critical in tequila production [9,10].

The tequila industry, however, has very limited knowledge of the parameters that affect agave juice fermentation [11] and yeast characterization [12]. The purpose of this work was to analyze the fermentative and aromatic qualities of a group of selected yeast strains involved in the tequila process.

2. Materials and methods

2.1. Yeast strains

Five strains (S1, S2, S3, K1 and K2) were isolated from *A. tequilana* Weber (blue variety) juice collected from 13 tequila distilleries in the state of Jalisco (the central region, the Tequila Valley and "Los Altos" region). They were selected based on their kinetic parameters and their high production of ethyl acetate (>20 mg/L), as determined by Gschaedler et al. [13].

Strains were identified by biochemical tests (API 20C AUX system) as *S. cerevisiae* (S1, S2 and S3), *Kloeckera africana* (K1) and *Kloeckera apiculata* (K2).

The strains were stored at −70 °C in a 1:1 mixture of the liquid medium used for the inoculum and a 50% glycerol solution.

2.2. Fermentation medium and culture conditions

The *A. tequilana* Weber juice, supplied by a distillery, was filtered and sterilized at 121 °C for 15 min. In the distillery, the agave plants are cooked between 95 and 100 °C for 4 h in a autoclave. The laboratory sterilization step, performed in a autoclave before fermentation, did not significantly modify the sugar juice composition. The sugar concentration of the agave juice was adjusted to 12°Brix (95 ± 5 g/L reducing sugar). The agave juice was then supplemented with ammonium sulphate (1 g/L) and used as fermentation medium. The pH of the unadjusted juice was 4.2. This fermentation medium was similar to the must typically used in industrial distilleries. The agave juice was diluted in water (2:1) and supplemented with ammonium sulphate (1 g/L) to obtain inoculum and pre-inoculum; the chosen yeast was added and the medium was incubated for 12 h at 30 °C, stirring at 250 rpm.

The fermentations were carried out under anaerobic conditions at 35 °C and 250 rpm in a 3 L bioreactor (Applikon, The Netherlands). The temperature conditions were similar to those used in tequila distilleries. The inoculation level was 20 million cells/mL. Prior to inoculation, the yeast population was estimated with a Neubauer counting chamber. Methylene blue staining was used to determine yeast cell viability. Two fermentations were performed with each yeast. Each must was fermented for 72 h, as is common in industrial production, and sampling was performed every 2 h during the first 12 h of fermentation, then every 4 h during the following 48 h, until the last sampling event at 72 h.

2.3. Analytical methods

Biomass concentration was obtained by dry weight measurement. A 5 mL sample of the fermented must was filtered through a cellulose acetate membrane (0.45 μm), prior to drying and weighing. It was washed twice with 5 mL of distillated water and dried for 24 h at 80 °C. The membrane was removed from the oven and placed in a desiccator until attaining constant weight.

The fermented samples were centrifuged for 15 min at 5554 × *g* and 4 mL of the supernatant was spiked with 0.5 mL of $ZnSO_4$ (5%, w/v) and 0.5 mL of $Ba(OH)_2$ (0.3N) solutions. After 10 min, the mixture was centrifuged again for 15 min at 5554 × *g* and the supernatant was used to determine the reducing sugar concentration by the DNS method [14]. Glycerol and succinic acid concentration was determined using a ThermoSeparation Products^{TM} Inc. (California, USA). HPLC coupled to a Spectra Physics Refract Monitor IV SP 8430 Refractive Index detector. The fermented samples were filtered and separation was performed an Animex HPX-87H Bio-Rad column. The operating conditions were as follows: temperature, 40 °C; mobile phase, 0.005 M sulphuric acid at 0.4 mL/min; injection volume, 20 μL. Quantification was based on five-point calibration curves (1, 2, 3, 4 and 5 mg/mL) of glycerol and succinic acid.

Samples were micro-distilled according to the Official Mexican Standard NOM-006-SCFI-2005 [15]. Ethanol concentration was determined in distillates by using the potassium dichromate method [16].

Distillates obtained from musts after 72 h of fermentation were also used to determinate the major volatile compounds by gas chromatography. GC analysis was carried out with a Hewlett-Packard 6890 Series gas chromatograph equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm i.d. × 0.30 μm film thickness HP-Innowax capillary column. Direct injections (1 μL) were performed in splitless mode at 220 °C. The column temperature was held at 50 °C for 2 min, then increased to 240 °C at a rate of 5 °C/min and held for 15 min. Helium was the carrier gas at 1 mL/min and the FID detector was operated at 260 °C. Quantification was based on the external standard method by using five diluted solutions containing 5, 25, 50, 100 and 200 mg/L of acetaldehyde, ethyl acetate, acetic acid, methanol, *n*-propanol, isoamyl and amyl alcohols, isobutanol, *n*-butanol and 2-phenyl ethanol. Calibration curves reported a correlation coefficient (r^2) greater than or equal to 0.99 for each compound as determined using the HP Chem Station software Rev A.05.04. Sample analysis was then performed by injecting each sample in duplicate, so that four data points were obtained for each fermentation condition and used to determine the average concentration and standard deviation of selected compounds. The lowest point in the calibration curve was used as the method reporting limit.

Analysis of minor esters was performed by GC–MS using a Hewlett-Packard 5890 Series II, gas chromatograph, coupled with a HP 5972 mass spectrometer. Volatile compounds were isolated by liquid–liquid extraction. The procedure used was as follows: 35 mL of 3:1 (v/v) pentane:dichloromethane solution was added to 160 mL of must (sample obtained at 72 h of fermentation). The mixture was then centrifuged at 5 °C and 7552 × *g* for 5 min. The organic extracts were dried with sodium sulphate and reduced to 0.4 mL using a Kuderna-Danish apparatus. A sample volume of 0.5 μL was injected into the chromatograph in split mode (60:1). Separations were performed using a HP-Innowax capillary column (60 m × 0.25 mm i.d. × 0.25 μm film thickness). Operating conditions were as follows: carrier gas (helium) at 1 mL/min; initial oven temperature 40 °C for 5 min, then ramped at 2.5 °C/min to 220 °C and held for 35 min. Injector and detector temperatures were 220 and 260 °C, respectively. The mass spectrometer was operated at an ionization voltage of 70 eV, scanning between 30 and 350 *m/z* at 1.39 scans/s.

Peak identification was performed by comparing the mass spectra to that provided in the library (5972 MS ChemStation G1034 C version C.01.05 Wiley 175) and by retention time, previously determined by injecting an esters standard solution. Quantification was based on the external standard method by using seven diluted solutions containing 0.01, 0.2, 1.25, 3.5, 7, 14 and 25 mg/L of each of the following compounds: isobutyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and 2-phenyl ethyl acetate. Calibration curves reported a correlation coefficient (r^2) greater than or equal to 0.99 for each compound. Each sample was injected in duplicate, so that four data points for each fermentation condition were used to determine the average concentration of the compounds and its standard deviation. The lowest point in the calibration curve was used as the method detection limit.

Amino acid analysis was performed on agave juice with and without protein hydrolyzed. Amino acid analyses were obtained from the Centro de Investigación en Alimentación y Desarrollo (CIAD), Sonora (Mexico) and the

contents were determined by high performance liquid chromatographic analysis as reported by Vázquez-Ortiz et al. [17]. The acid hydrolysis of agave juice was performed as reported by Umagath et al. [18].

2.4. Data analysis

The response variables data (biomass, ethanol and reducing sugar) of the two fermentations for each yeast, 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 two more 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 methods used for comparing yeast strain performance were the one-way variance analysis (ANOVA) and Cluster Analysis (Nearest Neighbor method). The response variables measured were as follows: final concentration of different aroma 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 the volatile compounds were analyzed by cluster analysis (Nearest Neighbor method) and the principal component analysis (PCA).

The ANOVA and cluster analyses were performed by Statgraphics plus 4 software (Manugistics Inc., Rockville, USA). Principal component analysis was performed by Simca software-P7.01.

2.5. RAPD analysis

The strains genomic DNA was isolated according to the Leach et al. [19] method with some modifications. DNA quality and concentration were determined spectrophotometrically at 260/280 nm. RAPD analysis was performed by using the Ready-To-Go RAPD Analysis Kit (Amersham Pharmacia Biotech). These commercial beads had been optimized for PCR reactions and contain buffer, nucleotides and *Taq* DNA polymerase. The only reagents which were added were template DNA and primers, also supplied in the kit. Six random 10-mer primers (Amersham Pharmacia Biotech) were included in this study: 1-(5′-GGTGCGGGAA-3′), 2-(5′-GTTTCGCTCC-3′), 3-(5′-GTAGACCCGT-3′), 4-(5′-AAGAGCCCGT-3′), 5-(5′-AACGCGCAAC-3′), 6-(5′-CCCGTCAGCA-3′). Each RAPD bead was resuspended in 19 μL of sterile water and 25 ng of total nuclear DNA and 25 pmol of each respective primer was added. Amplifications were performed for 1 cycle at 95 °C for 5 min followed by 45 cycles at 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min. Amplification products were analyzed by electrophoresis on 2% agarose gel and stained with ethidium bromide. A matrix was created based on the presence or absence of amplicons observed after electrophoretic separation. From these data, similarity matrices of Jaccard coefficients were calculated and used with the UPGMA (unweighted pair-group method using arithmetic averages) linkage [20] to produce a dendogram.

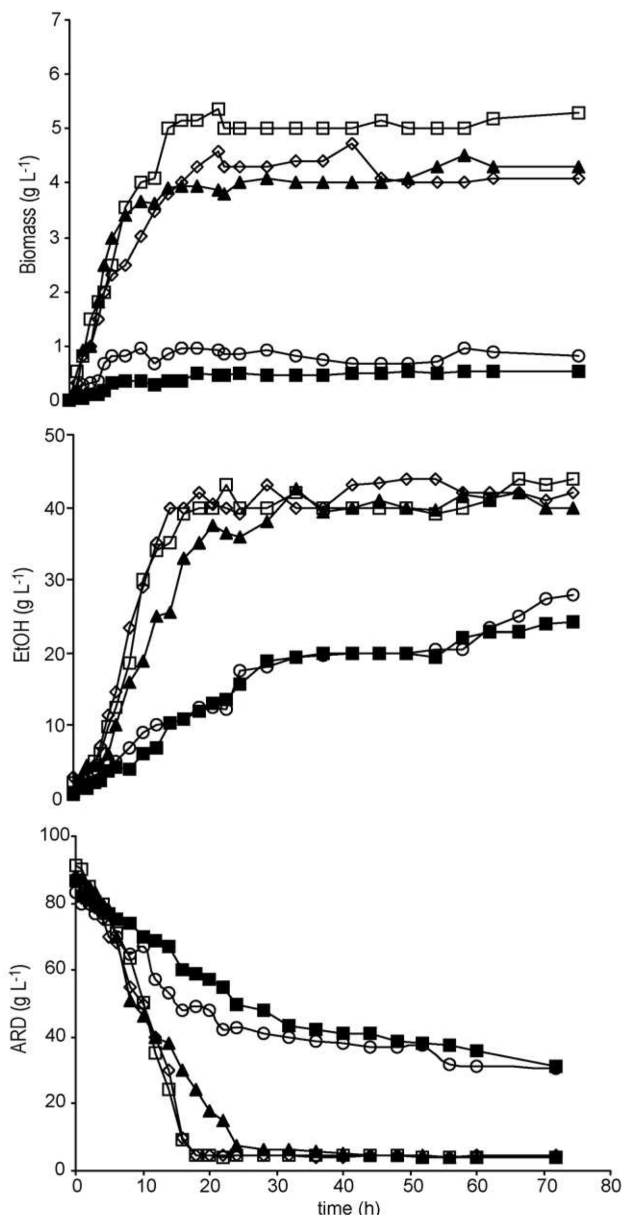

Fig. 1. Kinetic profiles of the fermentation of S1 (◇), S2 (□), S3 (▲), K1 (◯) and K2 (■) strains in a *Agave tequilana* Weber (blue variety) juice medium at 12°Brix, supplemented with ammonium sulphate (1 g/L). Biomass: biomass concentration profile; ARD: reduction sugar concentration profile; ETOH: ethanol concentration profile.

3. Results

3.1. Fermentation kinetic analysis

The evolution of biomass, sugar consumption and ethanol production versus time were plotted in Fig. 1 and Table 1, showing the kinetic parameters of each strain.

The behavior of the *Saccharomyces* and *Kloeckera* strains were different. For example, all *Saccharomyces* strains grew faster than *Kloeckera*, where the biomass level reached 4–5.3 g/L by approximately 12 h and sugar was completely depleted by 18–24 h of the fermentation (Fig. 1). On the other hand, *Kloeckera* strains grew slowly, reaching a maximal biomass level of <1.2 g/L early (8 h) and the sugar was not completely consumed (Fig. 1; Table 1). Also growth yields were different: 0.046–0.059 g/g for *Saccharomyces* strains and 0.015–0.031 g/g for *Kloeckera* strains (Table 1). Thus, the *Saccharomyces* strains were more efficient than *Kloeckera* for ethanol production; however, conversion yields were the same (Table 1). Statistical analysis (95% LSD) showed significant differences between yeast strains in all kinetic parameters (Table 1). The cluster analysis (Fig. 2) confirmed that the *Saccharomyces* group was different from the *Kloeckera* group. *Saccharomyces* species were separated into two subgroups, and S1 and S3 were nearer neighbors than S2. In the *Kloeckera* subgroup, K1 and K2 appear separated.

Table 1
Comparison of kinetic parameters and final concentration of biomass, consumed substrate and ethanol for the different strains

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)	X_f (g/L)	S_c (g/L)	$ETOH_f$ (g/L)
K2	0.13 ± .007	3.09 ± .16	1.04 ± .06	0.016 ± .001	0.46 ± .030	0.75 ± .05	45.3 ± .70	21.0 ± 1.2
K1	0.29 ± .040	3.19 ± .20	1.16 ± .04	0.029 ± .002	0.50 ± .006	0.95 ± .17	50.0 ± 2.0	25.5 ± 1.3
S1	0.43 ± .016	4.28 ± .27	1.56 ± .12	0.050 ± .004	0.49 ± .027	4.34 ± .26	86.7 ± 2.0	42.6 ± 1.0
S2	0.33 ± .030	2.85 ± .15	1.34 ± .06	0.055 ± .004	0.49 ± .001	4.86 ± .44	87.4 ± 1.2	43.5 ± .55
S3	0.35 ± .020	3.74 ± .27	1.52 ± .06	0.052 ± .001	0.47 ± .015	4.35 ± .10	83.9 ± .30	39.9 ± 1.4

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; S_c: consumed substrate concentration; X_f: final biomass concentration; $ETOH_f$: final ethanol concentration. Each value represents the average ± standard deviation of duplicate determinations of two fermentations.

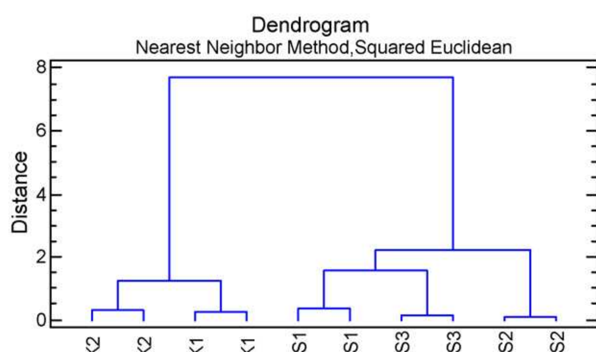

Fig. 2. Result of cluster analysis on the basis of the kinetic parameters for S1, S2, S3, K1 and K2.

3.2. Aromatic compounds

Higher alcohols, esters, acids and acetaldehyde constitute the main group of compounds that produce the "fermentation bouquet" [21]. *S. cerevisiae* strains showed higher production of amyl and isoamyl alcohols, *n*-propanol, 2-phenyl ethanol, acetaldehyde, isobutanol, isoamyl acetate, ethyl hexanoate, ethyl octanoate and ethyl decanoate than *Kloeckera* strains (Table 2). On the other hand, a higher production of ethyl acetate, acetic acid and 2-phenyl ethyl acetate was observed in the *Kloeckera* strains. No significant differences were found between strains with isobutyl acetate concentration (95% LSD). The only strain that *n*-butanol was detected was the K1 yeast. Methanol concentration was significantly different among the five yeast strains (95% LSD). Fermentation with the S1 strain reported the highest

Table 2
Concentrations of aroma compounds produced by *Saccharomyces* and *Kloeckera* yeasts

Products	Strain				
	S1	S2	S3	K1	K2
Aldehyde					
Acetaldehyde[a]	24.3 ± 1.3	5.0 ± 0.7	8.8 ± 0.2	5.0 ± 0.8	9.0 ± 0.3
Organic acid					
Acetic acid[a]	nd	nd	nd	75.2 ± 0.7	110.0 ± 0.3
Succinic acid[b]	0.21 ± 0.01	0.29 ± 0.05	0.60 ± 0.02	0.18 ± 0.04	0.13 ± 0.01
Alcohol					
Methanol[a]	109.5 ± 1.6	70.6 ± 0.5	51.3 ± 1.3	80.9 ± 0.9	46.7 ± 1.0
n-Propanol[a]	30.9 ± 1.1	22.8 ± 0.3	15.0 ± 1.0	19.9 ± 2.9	12.9 ± 0.2
n-Butanol[a]	nd	nd	nd	6.0 ± 0.2	nd
Isobutanol[a]	26.9 ± 1.5	26.0 ± 1.0	14.0 ± 0.6	6.5 ± 0.2	8.1 ± 0.9
Isoamyl and amyl alcohols[a]	75.6 ± 2.6	82.9 ± 0.9	45.1 ± 0.9	23 ± 2.0	11.3 ± 0.2
2-Phenyl ethanol[a]	26.6 ± 0.6	20.3 ± 0.3	18.3 ± 0.7	4.9 ± 0.9	3.3 ± 0.3
Glycerol[b]	4.3 ± 0.2	4.6 ± 0.5	4.7 ± 0.2	2.7 ± 0.1	2.5 ± 0.3
Ester					
Ethyl acetate[a]	8.7 ± 1.2	4.0 ± 0.5	6.5 ± 1.2	22.0 ± 0.3	24.9 ± 1.5
Isobutyl acetate[c,d]	28.2 ± 1.3	25.8 ± 0.26	28.3 ± 2.7	28.0 ± 6.1	33.4 ± 1.6
Isoamyl acetate[c]	199.3 ± 16.6	259.8 ± 3.6	265.4 ± 4.9	12.2 ± 2.6	18.1 ± 3.5
Ethyl hexanoate[c]	72.5 ± 0.58	61.5 ± 1.30	131.0 ± 4.5	7.6 ± 0.80	10.1 ± 0.88
Ethyl octanoate[c]	71.9 ± 10.5	74.6 ± 9.5	105.4 ± 9.6	4.9 ± 0.88	4.7 ± 0.79
Ethyl decanoate[c]	72.1 ± 10.6	80.5 ± 6.5	118.6 ± 19.2	9.9 ± 0.50	11.5 ± 2.3
2-Phenyl ethyl acetate[c]	58.9 ± 7.3	60.2 ± 2.0	51.6 ± 0.76	327.3 ± 6.1	421.9 ± 15.2

nd: not detected. Each value represents the average ± standard deviation of four determinations.
[a] Concentration are in mg/L of must micro-distillated (GC-FID).
[b] Concentration are in g/L of must (HPLC).
[c] Concentration are in μg/L of must (GC-MS).
[d] No significantly different among the yeasts.

Table 3
PCA factor loadings of the volatile compounds and explained variance of each component

	PC1		PC2
Variance explained (%)	66.0	Variance explained (%)	18.9
2-Phenyl ethyl acetate	−0.295	Succinic acid	−0.395
Acetic acid	−0.294	Ethyl hexanoate	−0.246
Ethyl acetate	−0.288	Ethyl decacoanoate	−0.209
Isobutyl acetate	−0.190	Ethyl octanoate	−0.165
n-Butanol	−0.160	Isoamyl acetate	−0.120
Methanol	0.104	Isobutyl acetate	−0.104
Acetaldehyde	0.117	Glycerol	−0.087
n-Propanol	0.159	Acetic acid	−0.014
Succinic acid	0.182	2-Phenyl ethyl acetate	0.001
Isobutanol	0.250	Ethyl acetate	0.043
Ethyl hexanoate	0.251	*n*-Butanol	0.136
Isoamyl + amyl alcohols	0.267	2-Phenyl ethanol	0.138
Ethyl decacoanoate	0.269	Isoamyl + amyl alcohols	0.189
Ethyl octanoate	0.281	Isobutanol	0.222
Glycerol	0.285	Acetaldehyde	0.312
2-Phenyl ethanol	0.286	*n*-Propanol	0.457
Isoamyl acetate	0.289	Methanol	0.496

methanol concentration. Both glycerol and succinic acid formation were higher in *Saccharomyces* than in the *Kloeckera* strains. Table 2 shows strains of *S. cerevisiae* that exhibit different abilities to produce these aromatic compounds. As was expected, the genus of the yeasts and the aromatic compounds produced was grouped by the PCA into two principal components with an 84.9% explained variance (Fig. 3; Table 3). The aromatic compounds presenting a greater variation between yeast were, 2-pheny ethyl acetate, acetic acid, ethyl acetate, succinic acid, ethyl hexanoate, isoamyl acetate, 2-phenyl ethanol, ethyl octanoate, glycerol, methanol, *n*-propanol and acetaldehyde (Fig. 3; Table 3).

The cluster analysis for aromatic compounds (Fig. 4) presented two main groups (*Kloeckera* versus *Saccharomyces*) where as in the *Saccharomyces* subgroup, strain S1 was largely different but the S2 and S3 strains were similar. Kinetic parameter analysis confirmed that strain S2 was different in comparison with S1 and S3 (Fig. 2). It must also be observed that the *Kloeckera* strains, even if they belong to two different species, seem to be more alike than *Saccharomyces* strains belonging to same species (*cerevisiae*). The diversity between *Saccahromyces* strains was more important for aromatic characteristics than for kinetic parameters.

3.3. RAPD analysis

RAPD analysis of the strains tested gave distinctive patterns that permitted a clear differentiation of the considered species. Nine to 15 bands of amplified DNA, with sizes ranging from 350 to 2300 base pairs (bp) was typically obtained in PCR reactions with the different primers. The use of a combination of two primers in the same reaction did not produce additional separation of the strains. A total of 152 scorable markers were analyzed (an average of 11.5 bands per primer) of which 56 (34%) were polymorphic. The dendrogram of the genetic distance data is shown in Fig. 5. Two groups were identified and separated by genetic distances between 0.1 and 0.2.

4. Discussion

4.1. Fermentation kinetic analysis

The *Kloeckera* genus is well known as a less efficient fermentative yeast than *Saccharomyces* strains. In wine for example, these yeast strains exhibit very little growth within the first hours of fermentation. This fact is attributed to their weak tolerance to ethanol (5–6%, v/v) [22]. In the present study, however, when *Kloeckera* yeasts growth was inhibited, ethanol concentration was very low (2.9 ± 0.2%, v/v). Thus it is concluded that ethanol may not be the only factor for growth arrested of *Kloeckera* yeasts. An experiment was done with the K1 strain by adding 9% (v/v) ethanol in the culture medium and growth was only reduced to half of the control value (data not shown). Similar results were obtained with *Saccharomyces* S1 yeast at the same ethanol concentration. Perez-Nevado et al. [23] suggested that *S. cerevisiae* produces compounds toxic to the *Kloeckera* genus, other than ethanol and killer toxins. In the present study, however the fermentations were performed in a pure culture. Alternatively, it has been reported that the two macronutrients frequently implied in the causes of stuck fermentation when present in small quantities are nitrogen and phosphate (see the reviews by Bisson [9]). In grape juice, the supplement of some amino acid in the medium may increase the ability for rapid synthesis of degraded proteins as glucose transporters [24]. The analysis of amino acids of agave juice and of its hydrolyzate was performed and compared to grape juice (Table 4). These results show that agave juice is naturally amino acid poor, even when hydrolyzed, and so it can be concluded that a nutritional limitation may act in agave juice fermentations. In the present work, agave juice fermentations were run without protein hydrolysis. However, in these experiments, a sufficient concentration of nitrogen was added to the agave juice prior to fermentation in order to maintain a good performance of the yeast. Albergaria et al. [25] mentions that *Hanseniaspora* (a sexual form of *Kloeckera*) needs complex nutrients to grow. It is possible than a least one essential nutrient was lacking in the agave juice medium. In wine, it has been shown that the low concentration of vitamins limit fermentation kinetics [9]. In this case, the agave juice are cooked and then sterilized by heat treatment, vitamin degradation and consequent vitamin deficiencies can be the cause to the poor fermentation performance of the *Kloeckera* strains. Indeed, it is possible that some inhibitory substances formed in the cooking step of tequila production (Maillard compounds) can act on *Kloeckera* strain activity. For example, furfural has been shown to be a toxic compound to yeast [26], and the concentration of this compound in agave juice may affect the growth of this yeast. In order to understand the exact cause of the low fermentative capacity of *Kloeckera* strains in agave juice, further investigation in to the relation between nutrition limitation and the toxic components of the medium is necessary. In contrast, *Saccharomyces* strains appear to have less limitation.

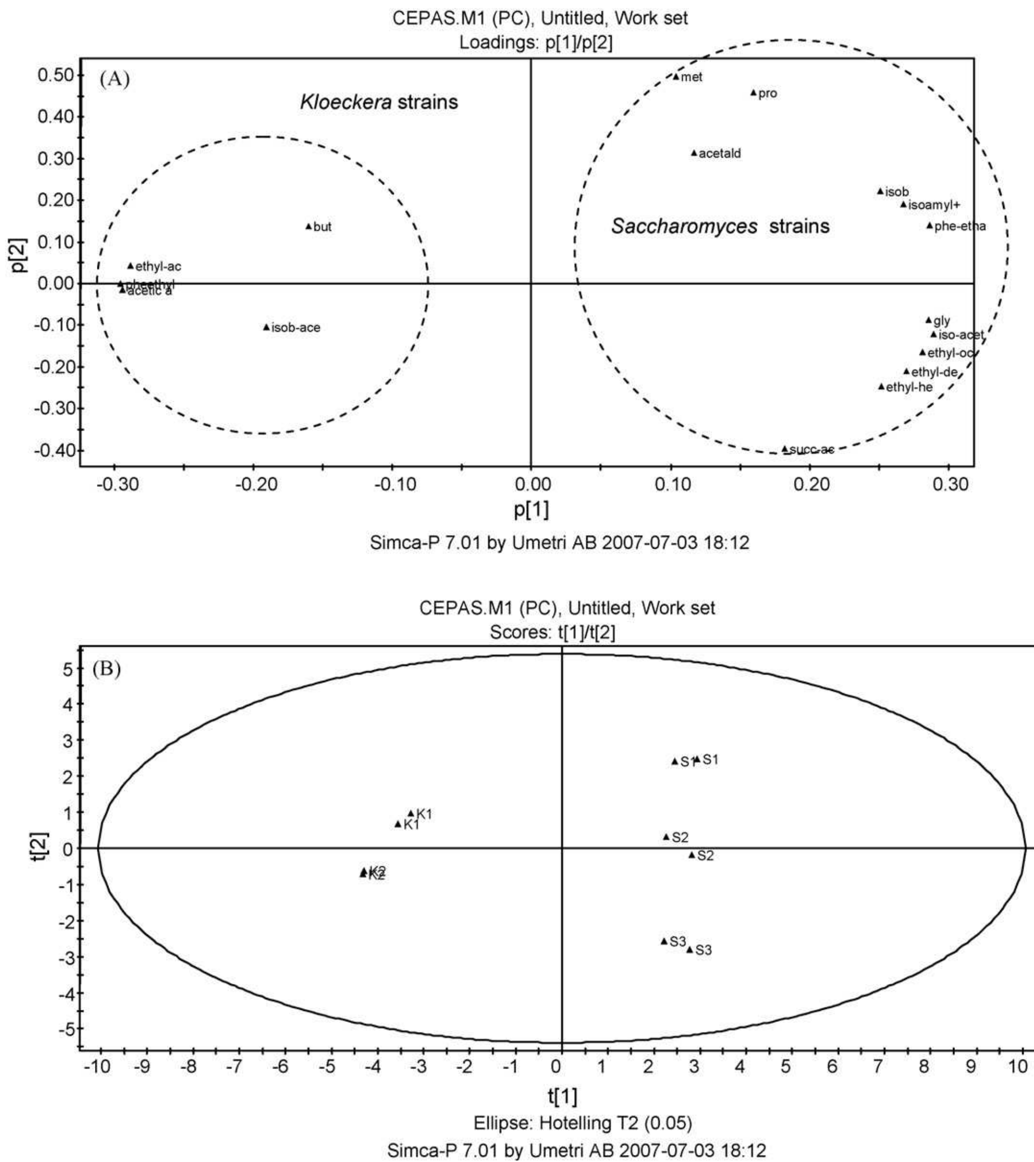

Fig. 3. PCA of (A) the aroma compounds and (B) the yeast strains. Acetald: acetaldehyde; met: methanol; acetic ac: acetic acid; pro: *n*-propanol; but: *n*-butanol; isob: isobutanol; isoamyl+: isoamyl and amyl alcohols; phe-ethanol: phenyl ethanol; ethyl-acet: ethyl acetate; succ-ac: succinic acid; gly: glycerol; iso-acet: isoamyl acetate; ethyl-hexa: ethyl hexanoate; ethyl-octa: ethyl octanoate; ethyl-deca: ethyl decacoanoate; pheethyl-acet: 2-phenyl ethyl acetate; isob-acet: isobutyl acetate.

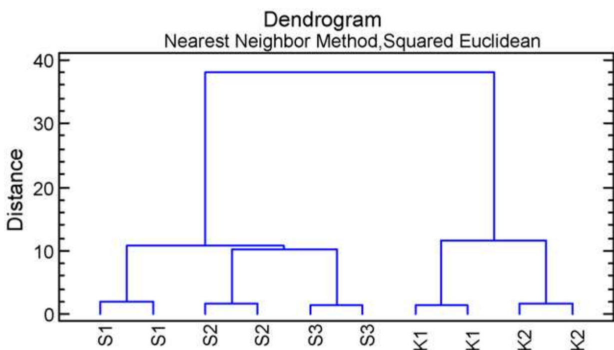

Fig. 4. Result of cluster analysis on the basis of the aromatic compounds for S1, S2, S3, K1 and K2.

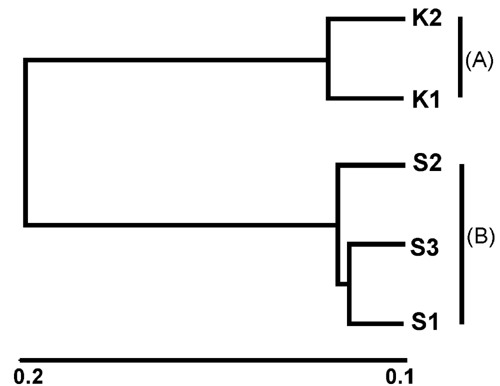

Fig. 5. Dendogram obtained by UPGMA linkage with clustering of Jaccard coefficients calculated from RAPD data. The scale represents dissimilarity (squared distance). The cophenetic correlation coefficient of the similarity matrix and the resulting dendogram was 0.9825.

Table 4
Amino acid composition of grape and agave juices

Amino acid (mg/L)	Grape juice[a]	Agave juice[b]	Hydrolyzate agave juice[b]
L-Alanine	58.5[c]	0.72 ± 0.005	20.98 ± 0.153
L-Arginine	255.9 ± 182.3	5.76 ± 0.030	38.68 ± 0.676
L-Aspartate	46.4 ± 22.9	0.41 ± 0.018	25.51 ± 0.322
L-Glutamate	91.2 ± 37.7	0.12 ± 0.001	42.12 ± 0.117
L-Glutamine	122.9 ± 93.9	nq	nq
L-Glycine	4.1 ± 3.1	0.44 ± 0.016	21.75 ± 0.526
L-Histidine	103.9 ± 85.9	0.19 ± 0.008	10.09 ± 0.301
L-Isoleucine	13.4[c]	0.06 ± 0.003	11.70 ± 0.196
L-Leucine	13.4[c]	0.14 ± 0.003	21.28 ± 0.524
L-Lysine	7.6 ± 6.67	0.06 ± 0.002	6.59 ± 0.150
L-Metionine	24.2 ± 13.9	nd	4.10 ± 0.126
L-Phenylalanine	16.9 ± 11.3	0.06 ± 0.003	12.44 ± 0.100
L-Serine	53.1 ± 23.4	1.34 ± 0.024	32.52 ± 0.306
L-Threonine	51.6 ± 25.1	0.32 ± 0.014	18.54 ± 0.270
L-Tyrosine	13.3[c]	0.22 ± 0.010	13.97 ± 0.109
L-Valine	17.7[c]	0.14 ± 0.004	21.49 ± 1.058

nd: not detected; nq: not quantified.
[a] Amino acid concentration of 11 grape varieties must [29].
[b] Each value represents the average ± standard deviation of duplicate determinations, the method limited detection is 1 pmol/mL.
[c] Amino acid concentration constant in the 11 varieties of grape [29].

4.2. Aromatic compounds

The production of selected aromatic compounds in the fermentative step was estimated with the five different yeast strains (Table 2). The main volatile compounds produced during agave juice fermentation are as follows: higher alcohols, ester, aldehyde and methanol, with the higher alcohols (isoamyl and amyl alcohols, isobutanol, *n*-propanol, *n*-butanol and 2-phenyl ethanol) being the most abundantly produced [27]. These results are in accord with those produced in the present study regarding the fermentation of agave juice by *S. cerevisiae* strains. The major concentration of volatile compounds produced by *Kloeckera* strains, however, was acetic acid, followed by methanol and higher alcohols. As well, in the present study a similar behavior between *Saccharomyces* and *Kloeckera* yeasts was observed in the production of the major volatile compounds to that of wine [8]. Romano et al. [8] reported that the *Hanseniaspora* strain (*Kloeckera*) produced a lower quantity of isoamyl alcohol (40 mg/L) and a higher concentration of acetic acid (2100 mg/L) and ethyl acetate (100 mg/L) than the *Saccharomyces* strain (250, 400 and <10 mg/L, respectively), whereas the *S. cerevisiae* strain produced a higher concentration of higher alcohols. Additionally, Romano et al. [8] reported significant differences related to yeast strains of the same species acting on the same must, as well as differences related to the nature of the must used. As well in tequila, a different behavior was found in the production of isoamyl alcohol by different *S. cerevisiae* strain during alcoholic fermentation stage with the same must [11]. On the other hand, Regodón Mateos et al. [28] reported that the variability of major volatile compound production (acetaldehyde, ethyl acetate and some fusel alcohols) with different *S. cerevisiae* yeast strains in white and red musts, depends mostly on fermentation conditions and must composition. In fact, a close relationship was found between must amino acid composition and volatile compounds in wine, as most of these compounds are related to the metabolism of branched-chain amino acids in the yeast cell [29]. Isobutyl alcohol, isoamyl and amyl alcohols are produced from leucine, valine and isoleucine, respectively and *n*-propyl alcohol is produced from threonine [30]. It can be deduced then that the low concentrations of higher alcohols and other by-products analyzed in this study may be linked to the very poor amino acid concentration in agave juice. The precursors of higher alcohols (e.g. leucine, isoleucine and valine) were almost negligible in the amino acid analysis of juice without protein hydrolysis prior to fermentation (Table 3).

Methanol is generated by agave pectin demethylation at the high temperature and low pH found in cooking, and from agave pectin hydrolysis by enzyme pectin methyl esterase [31], which was produced by some yeast strains present in the fermentation stage [3]. In the present study, all the yeasts produced methanol and showed significant differences between each other (95% LSD). A study of eight tequila distilleries found that the methanol concentration in agave juice is typically 60–70 mg/L and increases during alcoholic fermentation to 117–120 mg/L in the must [27]. In the present study, the concentration of methanol with the five different strains were smaller than the industry report, even when the methanol concentration in the agave juice was similar to that reported to the industry.

Six minor ester compounds considered odor-active, found in wine, tequila, brandy and whisky [7,32–34], were selected for this study to determine the production capacity of these aromatic compounds in tequila genus *Saccharomyces* and *Kloeckera* (Table 2). These compounds were as follows: isobutyl acetate, isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and 2-phenyl ethyl acetate. *Saccharomyces* yeast showed a higher concentration than *Kloeckera* in four of six ester compounds: isoamyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate. No significant effect was found between different yeast strains on the concentration of isobutyl acetate (95% LSD). *Saccharomyces* has been reported to produce higher ester

concentration in wine than *Kloeckera* [35]. In tequila, the most abundant ester found at the end of agave juice fermentation was ethyl acetate [36]. This behavior was shown in each of the five strains tested. The abundances of the other esters present in the must of industrial origin can vary widely. For example, in the CIATEJ laboratory, a number of musts obtained from agave juice inoculated with *S. cerevisiae* yeast (main strain) from industry were analyzed to determine the concentrations of minor esters, using the determinative method followed in this study. Here, the following concentration ranges were determined: 80.7–475.7 μg/L isoamyl acetate, 117.4–497.3 μg/L ethyl octanoate, 122.6–411.6 μg/L 2-phenyl ethyl acetate, 110.9–325.3 μg/L ethyl decanoate, 24.6–161.2 μg/L ethyl hexanoate and 9.8–172.9 μg/L isobutyl acetate. In the present study with *S. crerevisiae* strains, the concentration of isoamyl acetate and ethyl hexanoate were within rage of the results of the tequila distillery, however, the concentration of ethyl octanoate, ethyl decanoate and 2-phenyl ethyl acetate were lower than in industry. On the other hand, the concentration of ester essayed with *Kloeckera* strains, was generally lower than in the tequila distillery with the exception of 2-phenyl ethyl acetate, which was within range of the industry. For isobutyl acetate, the five strains tested showed concentrations within industry range.

The glycerol productions in our study of two genus yeasts were comparable to the wine reports [37,38]. In wines, the glycerol production of *Saccharomyces* strains is reported ranging from 4.8 to 8 g/L and 1.36 to 4.44 g/L for *Kloeckera* yeasts. Furthermore, the succinic acid concentration in fermentation with *Saccharomyces* was similar to wine, but in the case of *Kloeckera* strains, the succinic acid was much lower than the values reported in wine [37].

The aromatic concentrations of all yeasts studied in this paper were in compliance with the Official Mexican Standard for aromatic compounds in tequila production (NOM-006-SCFI-2005 [15]), thus demonstrating that these yeasts are safe and could be used in tequila process.

4.3. RAPD analysis

All six primers used efficiently amplified diverse regions of the investigated yeast genomes. They revealed different levels of variability but in general, a substantial level of polymorphism was detected among the five strains. The dendrogram (Fig. 5) revealed two clusters (A and B). Cluster A contained K1 and K2 strains (*Kloeckera*); their RAPD patterns displayed characteristic differences similar to those reported by Flores Berrios et al. [12]. In contrast, Cluster B involves *Saccharomyces* strains; although strain S2 was situated separately from S1 and S3, this strain is still identified as *Saccharomyces*.

Genetic relationships between strains shown on the dendrogram (Fig. 5) are very similar to results revealed by the kinetic profile (Fig. 2), that suggest that this phenotypic trait should be considered as a variable in the taxonomic description of the studied strains. Nevertheless, the variability shown in the aromatic profiles of *Saccharomyces* strains (Fig. 4), are different from the variability presented at a genetic level (Fig. 5) and the kinetic parameters (Fig. 2). The diversity of the *S. cerevisiae* strains were higher for the aromatic profiles than for kinetic parameters and genetic levels.

To summarized, the tequila fermentation process involves a large variety of yeast strains, belonging to different genera and species; among which, *Saccharomyces* and *Kloeckera* (or *Hanseniaspora*) are the most common. The fermentative and aromatic abilities of the strains, as confirmed by molecular analysis (RAPD), make it possible to clearly distinguish the two genera (*Saccharomyces* and *Hanseniaspora*). The low aromatic concentration in agave juice fermentation may be due to the low weak content of amino acids. *Kloeckera* strains were more susceptible by nutritional limitation and/or toxic compounds present in agave juice than were the *Saccharomyces* strains, which results in a stronger production of acetic acid and ethyl acetate. The diversity of the two species of *Kloeckera* strains, were lower than the three *S. cerevisiae* strains.

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