Fermentative capacity of *Saccharomyces* and non-*Saccharomyces* in agave juice and semi-synthetic medium

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A B S T R A C T

Various consortia of yeasts and bacteria involved in the natural fermentation process of tequila have been identified, particularly non-*Saccharomyces* yeasts. This study evaluates the fermentative capacity of two non-*Saccharomyces* yeasts (isolated from traditional mezcal fermentation): *Kluyveromyces marxianus* (DU3) and *Pichia kluyveri* (GRO3), and assesses their production of volatile compounds. The values found are compared with those of the same attributes of a *Saccharomyces cerevisiae* (AR5) isolated from tequila fermentation. The fermentations were performed in two different media, agave juice (JA) and a semi-synthetic medium (M11). The study also compared free and immobilized yeast fermentations in the JA medium in order to evaluate the potential benefits of immobilization on the yeast behaviour. This study demonstrated the potential of non-*Saccharomyces* yeasts, which fermented the agave juice in the same manner as *S. cerevisiae* but with higher ester production. Furthermore, *K. marxianus* produced more higher alcohols than *S. cerevisiae*. This could lead to tequila with different aroma profiles. Results were different in the synthetic medium, thus showing sensitivity to the composition of the medium. No significant differences between yeast fermentations with free and immobilized cells were detected, except for ethanol yield.

1. Introduction

Tequila, the distilled *Agave* beverage traditionally associated with Mexico, is produced exclusively in the territory of the appellation of origin of the beverage, according to Mexican regulations (SECOFI, 2012, NOM-006-SCFI-2012). The classic process of tequila production is divided into five steps: cooking of the *Agave tequilana* Weber var azul stems, milling of the cooked agaves, fermentation, distilling and, in some cases, ageing. In some distilleries, cooking is carried out in brick ovens heated by steam injection; in others, steel autoclaves are used. Agaves contain high concentrations of highly branched fructans, which are hydrolysed during the cooking step so as to obtain simple sugars (fructose, glucose and sucrose). The cooked agave is milled to extract the sweet must containing a high concentration of fructose. In recent years, a diffusion process has been developed using hot water to extract fructans or fructose from crude crushed agave or agave fibres. Hydrolysis is achieved using an acid treatment. The initial sugar concentration in fermentation is between 40 and 160 g/L, depending on which type of tequila is being produced. The lowest sugar concentrations occur when only agave sugars are used (tequila 100%) and higher concentrations result from the addition of other sugars, mainly corn fructose syrup (tequila). Fermentation is mainly carried out with selected *Saccharomyces cerevisiae* inocula, although some factories still work with spontaneous fermentations. Normally, the fermentation lasts for 24–96 h, at a temperature between 30 and 35 °C. The final concentration of ethanol in fermented must lies between 30 and 90 g/L, depending on the initial sugar concentration (Cedeño, 1995).

Non-*Saccharomyces* yeasts have long been considered as contaminants of alcoholic fermentation and, for years, practitioners attempted to avoid their presence during the process. According to the literature, non-*Saccharomyces* yeasts proliferate in early fermentation stages, but their growth is rapidly inhibited due to their low ethanol tolerance (about 50–60 g/L). Thus, the fermentation continues with more tolerant yeasts, some belonging to the genus *Saccharomyces* (Ciani & Picciotti, 1995; Fleet & Heard, 1993). However, although non-*Saccharomyces* yeasts are active for short

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periods in the fermentation, they contribute significantly to the aromatic quality of the final beverage (Romano, Fiore, Paraggio, Caruso, & Capece, 2003).

Interest in the study of non-*Saccharomyces* yeasts for alcoholic fermentation is currently growing, since these are the main component responsible for the production of different flavours (Ciani, Comitini, Mannazzu, & Domizio, 2010). During the production of beverages from *Agave* using spontaneous fermentation, a stable consortium of bacteria and yeasts is present (Lappe-Oliveras et al., 2008). In the process of tequila making, different genera of non-*Saccharomyces* yeasts are detected such as *Brettanomyces*, *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Saccharomycoides*, *Zygosaccharomyces*, *Issatchenkia* and *Torulaspora* (Lachance, 1995). These genera have also been identified in other alcoholic beverages, such as wine.

In tequila, recent studies have shown that fermentation with non-*Saccharomyces* yeasts (*Hanseniaspora*) is able to produce ethanol levels as high as those given by *S. cerevisiae* in an agave juice enriched with yeast extract. The failure of non-*Saccharomyces* yeasts to survive under traditional agave juice conditions is attributed to a deficiency of nutrients and not to their inability to tolerate ethanol (Diaz-Montaño, Favela-Torres, & Córdova, 2010). López-Alvarez, Díaz-Pérez, Sosa-Aguirre, Macías-Rodríguez, & Campos-García (2012) demonstrated that a strain of *Kluyveromyces marxianus* was also able to ferment the juice of *A. tequilana*, and that the concentrations of volatile compounds and the ethanol yield were higher than those obtained with *S. cerevisiae*.

Yeast immobilization is also an alternative for the alcoholic beverage industry. For example, this is used in wine production to obtain a higher yeast concentration during the fermentation stage, thus increasing productivity, in order to favour the production of esters (Viana, Taillandier, Vallés, Strehaiano, & Manzanares, 2011) and to allow recycling of the yeasts, which results in decreased costs (Zhao & Xia, 2010). In mixed fermentations, this strategy helps to obtain and maintain equal concentrations of non-*Saccharomyces* and *Saccharomyces* yeasts during the fermentation step. However, immobilization can generate physiological and biochemical changes with respect to free yeasts. In particular, these changes can lead to an increased ethanol yield (Ciesarová, Dömény, Smogrovicová, Pátková, & Sturdík, 1998) or to the limited transfer of nutrients into the immobilization matrix, which decreases their availability (Verbelen, De Schutter, Delvaux, Verstrepen, & Delvaux, 2006).

In this study, in order to produce tequila with an aromatic profile rich in esters and with acceptable ethanol yields, the use of two non-*Saccharomyces* yeasts isolated from spontaneous mezcal fermentations was explored and compared with a fermentation performed with *S. cerevisiae* isolated from tequila fermentation. Also, the influence of yeast immobilization on the productions of ethanol and volatile compounds was considered in order to evaluate the potential use of immobilized yeast during tequila fermentation. As *Agave* juice is a medium that presents differences in each batch, the fermentation experiments were performed in parallel in a semi-synthetic medium (M11), as an internal control having a chemical composition that is easy to reproduce everywhere.

2. Methodology

2.1. Yeast strains

Three yeasts from the CIATEJ (Centro de Investigación y Asistencia en Tecnologia y Diseño del Estado de Jalisco) collection were used: AR5 *Saccharomyces cerevisiae*, isolated from tequila fermentation, and DU3 *K. marxianus* and GRO3 *Pichia kluyveri*, isolated from mezcal fermentation (Amaya-Delgado, Herrera-Lopez, Arrizon, Arellano-Plaza, & Gschaedler, 2013).

2.2. Fermentation and culture medium

Two fermentation media were used: a semi-synthetic medium M11 (100 g/L fructose, 1 g/L yeast extract, 1 g/L $(NH_4)_2SO_4$, 3.27 g/L $MgSO_4 \cdot 7H_2O$, 2.23 g/L K_2HPO_4, 0.98 g/L $Ca(NO_3)_2 \cdot 4H_2O$) and an agave juice medium (*A. tequilana* Weber var. azul with 100 g/L of sugar enriched with 0.5 g/L $(NH_4)_2SO_4$).

YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) was used for the preparation of the inocula.

2.3. Yeast immobilization

The immobilized yeast was prepared using alginic acid sodium salt from brown algae (Sigma–Aldrich®) mixed with a suspension of cells in water (2E+09 cell/mL). Equal volumes of the two solutions were used so as to obtain approximately 1E+09 cell/g bead as a final concentration in a 20 g/L alginic sodium yeast cells suspension, which was dripped using a pump and a 29G needle into a 170 g/L $CaCl_2$ solution mixed with a magnetic stirring bar. After the beads had been incubated for 30 min in the $CaCl_2$ solution, they were removed and washed with distilled sterile water to remove the excess calcium. The beads had an average diameter of 2 mm and were stored in a 40 g/L glucose solution at 4 °C until use.

2.4. Fermentation conditions

All the fermentations were carried out in 250 mL Erlenmeyer flasks with 50 mL of medium, at a temperature of 30 °C and 100 rpm stirring for 72 h. Fermentation time was selected according to the standard industrial process. The 100 rpm stirring was chosen on the basis of a previous study (not published) which showed that this condition allowed yeast growth and ethanol production to be obtained simultaneously, as occurs in classical fermentation in the tequila industry (Cedeño, 1995). The inocula were incubated at 30 °C and 250 rpm for 18 h. The fermentation medium was inoculated with liquid inoculum grown in YPD medium overnight to start at a concentration of 1E+06 cell/mL or with beads to start at a concentration of 1E+07 cell/mL. Every 24 h, some beads were removed and the number of cells in the beads were counted. The fermentations were carried out in duplicate.

2.5. Cell counting

The cell concentration in the liquid medium was determined by microscopic counting. The beads were dissolved in a 20 g/L citric acid solution and stirred in a vortex, then the cells were counted in a Neubauer chamber.

2.6. Sugar concentration

The reducing sugar concentration (fructose and glucose) was measured with DNS (Dinitrosalicylic acid) reagent (Miller, 1959).

2.7. Ethanol and volatile compounds determination

Volatile compound profiles were obtained using a dynamic Head Space Sampler (HSS Model 7694 E, Hewlett Packard, Agilent Technologies, Palo Alto, CA, USA) coupled to a gas chromatograph (GC Hewlett Packard 6890, Agilent Technologies, Palo Alto, CA, USA) with a flame ionization detector (FID). A 2 mL sample of must was introduced into a 20 mL vial, which was immediately sealed and stored at −20 °C until analysis. Each vial was equilibrated at

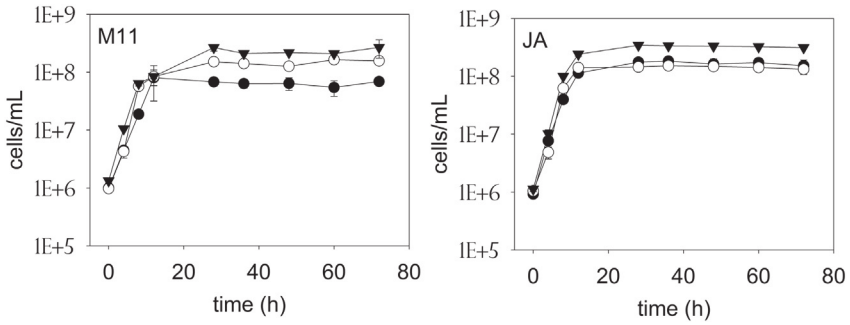

Fig. 1. Growth kinetics of free yeasts in semi-synthetic medium (M11) and agave juice medium (JA) for the yeasts AR5 (*Saccharomyces cerevisiae*) ●, DU3 (*Kluyveromyces marxianus*) ○ and GRO3 (*Pichia kluyveri*) ▼.

80 °C for 5 min in the HSS and shaken for 1 min, pressurized with carrier gas for 12 s, loaded into the sample loop for 12 s, equilibrated for 30 s, and finally injected into the column for 1 min. Loop temperature was 110 °C and the transfer line temperature was 115 °C.

Substances were separated on a polyethylene glycol capillary column (HP-Innowax, 60 m × 0.32 mm i.d.; film thickness, 0.25 μm) and analysed by GC under the following conditions: 45 °C for 7 min, heated at 10 °C/min up to 160 °C, heated at 20 °C/min to 220 °C, and maintained at 220 °C for 8 min. Injector and detector temperatures were set at 250 °C. The carrier gas was helium at a flow rate of 1.8 mL/min. Data acquisition and analysis were performed using software supplied by the manufacturer (Arellano, Gschaedler, & Alcazar, 2011).

Compounds were identified according to their retention times, obtained with ethanol, methanol, ethyl acetate, 3-methylbut-1-yl ethanoate (isoamyl acetate), ethanal (acetaldehyde), 2-methylpropan-1-ol (isobutanol), propan-1-ol (propanol), 2-methylbutan-1-ol and 3-methylbutan-1-ol (amylic alcohols), which were purchased from Sigma–Aldrich Canada (Oakville, ON, Canada). Volatile compounds were quantified with ChemStation software (Agilent), by comparing retention indices with those of pure standard compounds and using calibration curves obtained in hydroalcoholic solution covering the normal concentration range of the substances in tequila.

2.8. Consumable nitrogen by Sörensen method

The formol-titration method was used to determine the amino-acid nitrogen in the medium. A 100 mL sample was mixed with 40 mL of neutralized formaldehyde. The solution was titrated with NaOH 0.1 mol/L (Brown, 1922; Sörensen, 1907).

2.9. Statistical analyses

The statistical analyses used to compare fermentative performances were one-way Analysis of variance (ANOVA) to test for significant differences between the media, the strains, and the immobilized yeasts. Principal component analysis (PCA) was also carried out on the concentration of volatile compounds, ethanol yield, and yeast population in order to visualize relationships between variables. Statistical analyses were performed using Statgraphics Centurion XVI statistical software.

3. Results and discussion

Each strain was cultivated as free cells in both agave juice (JA) and semi-synthetic medium (M11). Because the behaviour of free cells was better in agave juice, this medium was selected for the study of the influence of immobilization. Figs. 1 and 2 show the growth evolution of the different strains, both free and immobilized, and Fig. 3 depicts sugar concentration throughout the fermentations. In Tables 1 and 2, the final concentrations of ethanol, sugar, yeast population and the main volatile compounds are shown for both media. Table 3 presents the *p-value* results of the ANOVA analysis.

3.1. Yeast population

3.1.1. Free cells

Fig. 1 shows the kinetics of growth of the free yeasts. Final populations were found to range from 6.90E+07 cells/mL (AR5 M11) to 3.13E+08 cells/mL (GRO3 JA). In both media, the GRO3 (*P. kluyveri*) strain showed the highest population. For AR5 and GRO3, the final concentration of yeast was higher in JA than in medium M11. These results point out that non-*Saccharomyces* yeast growth is similar to or greater than that of *S. cerevisiae* (AR5). The differences in final population could be statistically attributed to the strain factor (Table 3).

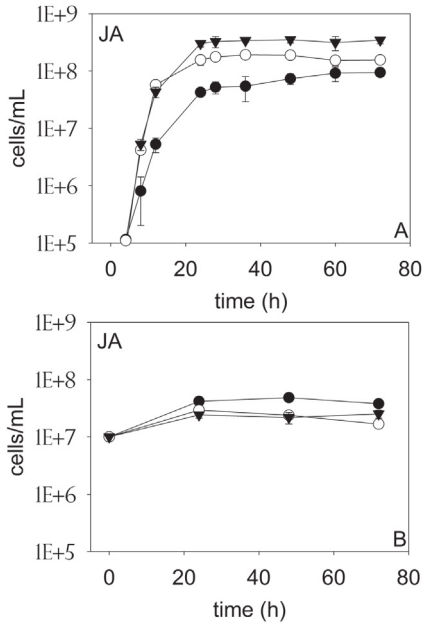

Fig. 2. Growth kinetics of immobilized yeasts in agave juice medium (JA) for AR5 (*Saccharomyces cerevisiae*) ●, DU3 (*Kluyveromyces marxianus*) ○ and GRO3 (*Pichia kluyveri*) ▼ (2A: yeast cells in liquid medium; 2B: yeast cells immobilized in the beads).

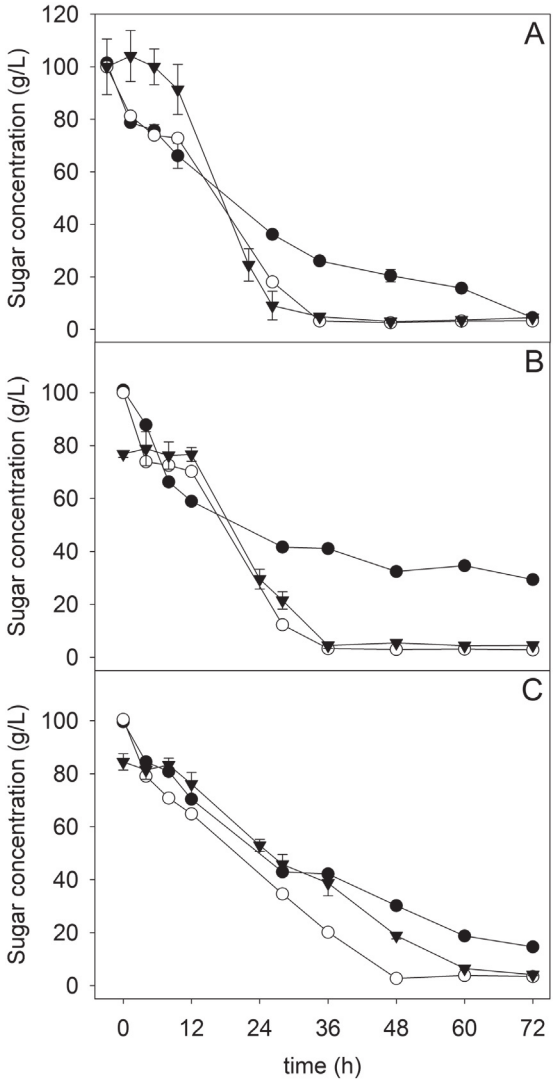

Fig. 3. Sugar consumption kinetics in agave juice medium (JA) and semi-synthetic medium (M11) medium with free and immobilized yeasts. Fig. 3A (AR5, *Saccharomyces cerevisiae*): ●M11 AR5 free, ○JA AR5 free, ▼JA AR5 immobilized. Fig. 3B (DU3, *Kluyveromyces marxianus*): ●M11 DU3 free, ○JA DU3 free, ▼JA DU3 immobilized. Fig. 3C (GRO3 *Pichia kluyveri*): ●M11 GRO3, ○JA GRO3, ▼JA GRO3 immobilized.

Similar behaviour was observed by Sadoudi et al. (2012) for different non-*Saccharomyces* in wine fermentations, with a sugar concentration of 200 g/L. Under this condition, the maximal number of cells obtained with *S. cerevisiae* was 1.48E+08 (on day 5), while with non-*Saccharomyces C. zemplinina* achieved 2E+08 (on day 6). As in our study with free cells, the highest population was obtained with non-*Saccharomyces*.

3.1.2. Immobilized cells

Fig. 2 illustrates the kinetics of fermentations inoculated with immobilized cells, and presents the growth of free cells in the medium after the inoculation (Fig 2A) and of immobilized yeast included in the beads (Fig 2B). The non-*Saccharomyces* tended to grow more in the free medium, while *S. cerevisiae* (AR5) grew more inside the beads. As in the free cell culture (Fig. 1), GRO3 presented the highest final population. No significant differences were observed between the final populations with free or immobilized yeast for a given strain.

The initial cell concentration included in the beads of each strain was 10E+06 cells/mL and, at the end of the fermentation, the AR5 concentration was 38E+06 cells/mL, for DU3 16.7E+06 cells/mL and for GRO3 25E+06 cells/mL (cell concentrations inside the beads). These results are evidence of yeast cell growth inside the beads, the highest population being detected with *S. cerevisiae* (AR5). Growth was also observed in the liquid medium because the beads were not made with a double layer and the yeast cells at the surface of the beads could thus become free in the medium. This work is, to our knowledge, the first to report the behaviour of non-*Saccharomyces* yeasts immobilized in pure culture, and highlights behaviour very different from that observed with *S. cerevisiae*.

3.2. Sugar consumption

Fig. 3 compares for each strain the kinetics of sugar concentration during the free and immobilized fermentations in medium M11 and JA. The sugar consumption after 72 h was always faster in JA with free cells than in M11 medium. After 36 h of fermentation, AR5 and DU3 had consumed nearly all the sugar present in the JA medium, while GRO3 took a further 12 h to achieve this. In contrast, the residual sugar concentrations in M11 were higher, particularly in the case of *Kluyveromyces* DU3 (29.4 g/L).

The observations generally reported in the literature for wine yeasts show that *S. cerevisiae* strains cause faster fermentation than non-*Saccharomyces* strains (Romano et al., 2003). Sadoudi et al.

Table 1
Residual sugar, ethanol, total yeasts and major volatile compounds final concentrations in fermentations with free yeasts, AR5 (*Saccharomyces cerevisiae*), DU3 (*Kluyveromyces marxianus*), GRO3 (*Pichia kluyveri*) in JA and M11.

	M11 AR5	M11 DU3	M11 GRO3	JA AR5	JA DU3	JA GRO3
Acetaldehyde (mg/L)	161 ± 122.2	7 ± 4.3	25 ± 2.79	86 ± 56.19	28 ± 6.63	33 ± 1.8
Ethanol (g/L)	41 ± 2.47	16.6 ± 0.91	33.4 ± 0.48	30 ± 3.38	30.21 ± 0.34	30 ± 2.32
Methanol (mg/L)	5.83 ± 0.5	5.94 ± 0.52	4 ± 5.51	42.74 ± 0.85	45 ± 3.68	47 ± 4
Ethylacetate (mg/L)	13 ± 2.61	138 ± 8.36	658 ± 48.27	2.08 ± 0.87	33.05 ± 1.31	257 ± 29.92
Isoamylacetate (mg/L)	ND	ND	6.67 ± 1.28	ND	ND	3.39 ± 0.48
1-Propanol (mg/L)	20 ± 4.98	18.18 ± 0.54	7.02 ± 0.8	14 ± 2.55	11.34 ± 0.24	5.67 ± 0.53
Isobutanol (mg/L)	20.46 ± 0.32	70.31 ± 0.06	172 ± 5.82	19 ± 4.05	107 ± 14.42	123 ± 4.16
1-Butanol (mg/L)	0.21 ± 0.23	ND	ND	0.49 ± 0.04	0.07 ± 0.1	ND
Amylicalcohols (mg/L)	31 ± 5.06	70.62 ± 1.46	6.27 ± 1.27	72 ± 16.06	188 ± 7.85	15.87 ± 0.54
Initial sugar (g/L)	101.46 ± 0.74	100.94 ± 0.74	99.63 ± 0.37	103.06 ± 0.89	104.53 ± 1.19	100.55 ± 0.89
Final sugar (g/L)	4.48 ± 1.15	29.37 ± 1.4	14.64 ± 0.07	3.25 ± 0.98	2.81 ± 0.18	3.46 ± 0.03
Sugarconsumed (g/L)	96.99 ± 0.41	71 ± 2.14	84.99 ± 0.44	99.81 ± 0.09	102 ± 1.37	97.08 ± 0.92
Final population yeast (cell/mL)	6.90E+07 ± 1.41E+06	1.58E+08 ± 7.78E+06	2.69E+08 ± 9.40E+07	1.53E+08 ± 3.89E+07	1.33E+08 ± 2.47E+07	3.13E+08 ± 5.66E+06
Ethanol yield	0.426 ± 0.02	0.232 ± 0.01	0.392 ± 0.01	0.297 ± 0.03	0.297 ± 0	0.308 ± 0.02

ND = Non detected. JA = agave juice. M11 = semi-synthetic medium.

Table 2
Residual sugar, ethanol, total yeasts and major volatile compounds final concentrations in fermentations with the immobilized yeasts, AR5 (*Saccharomyces cerevisiae*), DU3 (*Kluyveromyces marxianus*), GRO3 (*Pichia kluyveri*) in JA.

	JA AR5[a]	JA DU3[a]	JA GRO3[a]
Acetaldehyde (mg/L)	180 ± 38.21	59 ± 5.6	40.08 ± 1
Ethanol (g/L)	33 ± 6.39	31 ± 3.35	31.91 ± 1.18
Methanol (mg/L)	9 ± 2.28	8.62 ± 1.23	9.19 ± 0.44
Ethyl acetate (mg/L)	0.88 ± 1.09	17 ± 3.19	251 ± 71.59
Isoamyl acetate (mg/L)	ND	ND	2.3 ± 1.19
1-Propanol (mg/L)	15 ± 3.78	15.56 ± 1.4	7.59 ± 0.49
Isobutanol (mg/L)	14 ± 5.08	101 ± 22.38	120.64 ± 1.04
1-Butanol (mg/L)	0.55 ± 0.06	ND	ND
Amylic alcohols (mg/L)	64 ± 20.06	148 ± 15.92	10.89 ± 0.26
Initial sugar (g/L)	83 ± 10.59	76.79 ± 1.32	84 ± 3.12
Final sugar (g/L)	4.47 ± 0.2	4.55 ± 0.15	4.13 ± 0.37
Sugar consumed (g/L)	79 ± 12.91	72.25 ± 0.99	80 ± 2.15
Final population yeast (cell/mL)	1.32E+08 ± 1.65E+07	1.73E+08 ± 1.34E+07	3.72E+08 ± 4.19E+07
Ethanol yield	0.412 ± 0.01	0.436 ± 0.04	0.397 ± 0

[a] Immobilized yeast cells. ND = Non detected. JA = agave juice. M11 = semi-synthetic medium.

(2012) also showed that sugar consumption by *S. cerevisiae* was faster than that of non-*Saccharomyces*.

Immobilization of the DU3 and AR5 strains did not affect the consumption of sugar, giving identical sugar consumption profiles on JA. GRO3 immobilization affected the sugar consumption more: it was slightly slower when cells were immobilized but these differences were not statistically significant (Table 3).

In all the strains, the sugar consumption rate proved to be affected by immobilization during the first 12 h and presented a latency stage at the beginning of the fermentation. This may have been due to the limitation of nutrient transfers into the immobilization matrix as reported by Converti, Casagrande, De Giovanni, Rovatti, and Del Borghi (1996) and Verbelen et al. (2006). However, the diameter of the alginate beads played an important role in the transfer of nutrients when immobilized yeast was used. According to the literature, a diameter between 1.5 mm (Abraham & Surender, 1993) and 4 mm (Shiotani & Yamané, 1981) is recommended. In order to avoid nutrient transfer limitation in this study, beads with an average diameter of 2 mm were used.

3.3. Ethanol production and yield of sugar conversion to ethanol

Final ethanol concentrations ranged between 16.6 g/L with free DU3 in medium M11 and 41.3 g/L with free AR5 in medium M11 (Table 1). In the M11 medium, each strain had a different final ethanol concentration. In contrast, all the strains presented similar ethanol production in JA (30 g/L).

Because the amount of sugar consumed differed among experiments, the ethanol yield was calculated. The highest yield obtained was 0.426 g/g, given by free *S. cerevisiae* (AR5), and the lowest yield, 0.232 g/g, was for free *K. marxianus* (DU3) in M11 medium (Table 1). Better yields were obtained with immobilized yeasts (Table 2). Non-*Saccharomyces* DU3 and GRO3 showed low yields in M11 medium with free yeast. Thus, if the medium is adequate, non-*Saccharomyces* yeasts are able to exhaust sugar and produce satisfactory levels of ethanol (similar to the concentration obtained in the industry). The significant differences found in the ethanol yield were due to the immobilization effect only (Table 3).

Similarly, Mariam, Manzoor, Ali, and Haq (2009) reported that the use of immobilized *Saccharomyces* for bioethanol production gave better results and Babu, Satyanarayana, Balakrichnan, Rao, and Rao (2012) found high fermentation efficiencies (from 91 to 95%) in repeated batch experiments with immobilized *Saccharomyces* (both authors used sugar cane molasses, and Babu et al. sugar cane juice too). Norton, Watsin, and D'amore (1995) suggested that the matrix structure could protect the immobilized *Saccharomyces* against ethanol.

3.4. Production of acetaldehyde

Acetaldehyde is the last intermediate compound in the production of ethanol. At high concentrations (more than 40 mg/mL of anhidric alcohol in the final product), it is considered to have a negative impact on aroma (Escalona Buendía et al., 2004). The maximum concentration of acetaldehyde was obtained in JA with immobilized AR5 (180 mg/L). The minimal concentration was 7 mg/L, obtained in M11 with free DU3 (Tables 1 and 2). No significant differences in the acetaldehyde production where detected between immobilized and free cells or between JA and M11 media; significant differences were observed only between strains (Table 3).

3.5. Production of higher alcohols

The production of higher alcohols, considering 1-propanol, isobutanol, 1-butanol and amylic alcohols, showed differences in concentration due to the strain factor but no influence of immobilization was detected (Table 3). Production of 1-propanol was also dependent on the medium factor (Table 3). In general, the highest concentrations were observed with the strain AR5 (Tables 1 and 2).

Isobutanol concentrations, in comparison with 1-propanol concentrations, had opposite production profiles, in which the GRO3 strain produced the highest concentration, followed by DU3, and the lowest isobutanol concentration was observed with AR5 strain (Table 1). Isobutanol production was influenced only by the

Table 3
ANOVA *p-value* (*$p < 0.05$).

Main effects	Residual sugar	Total yeast	Ethanol yield Y p/s	Ethyl acetate	Isoamyl acetate	1-Propanol	Isobutanol	Amylic alcohols	Acetaldehyde
A:strain	0.0806	**0.0000***	0.1251	**0.0000***	**0.0000***	**0.0000***	**0.0000***	**0.0000***	**0.0011***
B:immobilization	0.7218	0.9920	**0.0148***	0.8896	0.5931	0.1137	0.7217	0.2201	0.1165
C:medium	**0.0018***	0.1821	0.0631	**0.0081***	0.1222	**0.0044***	0.7077	**0.0013***	0.5647

strain factor (Table 3). In this study, large differences between yeast species were highlighted.

Amylic alcohols were produced at a higher concentration than the other higher alcohols studied. In this case, the DU3 strain provided the highest concentration, followed by AR5, and the GRO3 strain gave the lowest concentration (Table 1). The production of amyl alcohols was always higher in JA than in M11. Statistical significant differences in the amyl alcohols production were found for the factors medium and strain (Table 3).

1-butanol was the higher alcohol that was produced at the lowest concentration in comparison with the other higher alcohols studied. Non-*Saccharomyces* did not produce 1-butanol. It was produced only by the AR5 strain and at a higher concentration in JA (0.49 mg/L) (Table 1).

Higher or fusel alcohol formation takes place by the anabolic and catabolic route. In the anabolic route, the 2-oxo acids are formed via de-novo biosynthesis of amino acids. They are transformed to aldehydes by a decarboxylation, which is termed the catabolic (Ehrlich) route to higher alcohol formation. In the catabolic route, higher alcohol concentration is determined by the amino acid concentration in the medium and the sugar consumption rate. In addition, some higher alcohols could be generated from the reduction of aldehydes and ketones present in the fermentation medium. So the production of higher alcohols is generally correlated with the nitrogen concentration (Brányik, Vicente, Dostálek, & Teixeira, 2008; Carrau et al., 2008; Pires, Teixeira, Brányik, & Vicente, 2014). Assimilable nitrogen concentration in the agave juice, basically in the form of amino acids, was 47.9 mg/L. In M11 medium, it was 40.9 mg/L, brought by the yeast extract (1 g/L). The concentration of inorganic nitrogen, added as $(NH_4)_2\ SO_4$, was 100 mg/L in agave juice and 200 mg/L in M11 medium. Arrizon and Gschaedler (2007) reported that the ratio of organic/inorganic nitrogen in the medium affected the production of higher alcohols, showing that, when the inorganic nitrogen concentration was higher in relation to the organic nitrogen concentration, the amylic alcohol concentration decreased and the propanol concentration increased. In the present study, the same behaviour was observed, principally in JA. For example, AR5 amylic alcohol concentration was 72.8 mg/L while propanol concentration was 14.0 mg/L. Even with the GRO3 strain, which produced the weakest higher ethanol concentration, this effect was observed in JA.

Regarding immobilization, Smogrovicová and Dömény (1999) observed that the higher-alcohol production increased when yeast was immobilized, especially concerning the production of 1-propanol. In our case, no differences in higher-alcohol concentrations were observed between free and immobilized fermentations. According to Virkajärvi and Pohjala (2000), the low concentration of higher alcohols produced by immobilized yeasts is due to the limitation of mass transfer within the alginate beads. Again, in our case, such a limitation could not be incriminated.

Investigating the strain effect, Sadoudi et al. (2012) found that the non-*Saccharomyces* produced a lower concentration of higher alcohols in Sauvignon Blanc juice for wine production than *S. cerevisiae* did. In our case, the non-*Saccharomyces* GRO3 (*P. kluyveri*), produced a lower concentration of higher alcohols than AR5 (*S. cerevisiae*) in both media studied, but the opposite was found with DU3 (*K. marxianus*), which produced higher concentrations than AR5 (*S. cerevisiae*). It is important to mention that Sadoudi et al. (2012) did not include *K. marxianus* in their study. In fermentations of agave juice, Diaz-Montaño, Délia, Estarrón-Espinosa, and Strehaiano (2008) reported that *S. cerevisiae* produced stronger concentrations of higher alcohols (amyl alcohol, n-propanol, isobutanol) in comparison with strains of the genus *Kloeckera*, which produced more ethyl acetate. In this work, the two non-*Saccharomyces* yeasts tested presented different behaviour; *K. marxianus* produced stronger concentrations of higher alcohols than *S. cerevisiae,* and *P. kluyveri* showed the lowest production. Thus, the case of non-*Saccharomyces* cannot be generalized. The production of higher alcohols depends directly on the yeast species, some of them producing more higher alcohols than *S. cerevisiae*, as observed with *K. marxianus* strain in this study.

3.6. Production of esters

The two non-*Saccharomyces* strains considered in this study showed a great production of esters (ethyl acetate and isoamyl acetate) in comparison with *S. cerevisiae*. The production was greater in M11 medium than that obtained in JA (Table 1). Significant statistical differences in the production of ethyl acetate were observed due to the strain and the medium (Table 3). Isoamyl acetate production was only detected in fermentations with strain GRO3, with higher production in M11 medium (Table 1).

The synthesis of esters has been studied in *S. cerevisiae*. They are formed enzymatically during fermentation and contribute to the fruity and floral sensory properties of the beverages. Flavour-active esters are the products of acetyltransferase activities catalysing the condensation reaction between acetyl-CoA and a higher alcohol or ethanol. The production of acetate esters during fermentation is dependent on the activity of at least three acetyltransferases in *S. cerevisiae*, and is correlated with the C:N ratio, unsaturated fatty acids, and free oxygen concentrations in the medium. It is significantly related to ATF1 and ATF2 gene expression (Saerens et al., 2008; Verstrepen et al., 2003). Non-*Saccharomyces* yeasts are generally considered to be greater ester producers (Ciani et al., 2010), which was confirmed in this study. In wine fermentation *Hanseniaspora* yeasts produce high concentrations of desirable volatile compounds such as ethyl acetate (fruity), phenyl ethyl acetate (floral), phenethylalcohol (rose) and acetoin (butter), which have an impact on the final aroma bouquet of the beverage (Moreira, Mendes, Hogg, & Vasconcelos, 2005; Romano et al., 2003). Other examples include studies performed with Pinot noir grape and Chardonnay juice, which were fermented with the yeasts *Pichia membranaefaciens* and *Kloeckera* in an attempt to increase the number of flavours in the wine.

High concentrations of ethyl acetate, isoamyl acetate, acetaldehyde and ethanol were observed (Mamede, Cardello, & Pastore, 2005). Nevertheless, no research has reported the regulatory mechanisms that allow this high production of esters.

The effect of cell immobilization on ester production was not significant according to the statistical analysis (Table 3) despite slight differences for AR5 and DU3 (Tables 1 and 2). In other studies using *S. cerevisiae* in beer and wine fermentation, cell immobilization was correlated with an increase in the production of esters (Mallouchos, Komaitis, Koutinas, & Kanellaki, 2003; Willaert & Nedovic, 2006). However such behaviour was not observed in this study, in which yeast strains and culture media were shown to be the dominant factors (Table 3).

In our study, non-*Saccharomyces* produced up to 130 times more ethyl acetate than *S. cerevisiae* under the same fermentation conditions, which opens up new perspectives for continuing the study of the non-*Saccharomyces* ester production metabolism.

3.7. PCA statistical analysis

Finally, a PCA analysis was carried out in order to observe the correlations between the different variables. Here 84.5% of variance was explained by 3 different components. An interesting finding in the PCA plot (Fig. 4) was that variables grouped the GRO3 strain at the left side and strains AR5 and DU3 on the opposite side. The GRO3 strain had a correlation with high yeast population, and ester

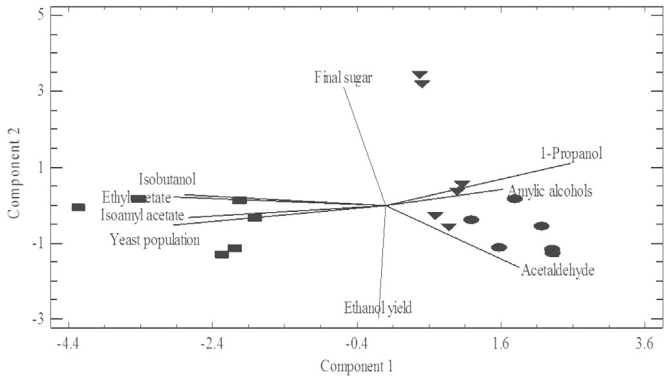

Fig. 4. Principal components plot of the volatile compounds produced and correlated with each yeast strain studied. ●AR5 (*Saccharomyces cerevisiae*), ▼DU3 (*Kluyveromyces marxianus*), ■GRO3 (*Pichia kluyveri*).

and isobutanol concentration. High residual sugar concentration was correlated with the DU3 strain in M11 medium. AR5 and DU3 strains were correlated with acetaldehyde, amylic alcohols and 1-propanol, meaning that these strains had similar profiles in JA. The PC1 explains incomplete fermentation, where less sugar consumption produces a smaller yeast population and high acetaldehyde concentration, but the ethanol yield is not affected.

4. Conclusions

The three strains showed different behaviours. Non-*Saccharomyces* proved to be greater ester producers in pure culture. The GRO3 strain not only produced the highest concentration of ethyl acetate, but also produced the highest concentration of isoamyl acetate and weaker higher-alcohol concentrations. The DU3 strain produced stronger ester concentrations in comparison with AR5, but also stronger higher-alcohol concentrations.

Generally speaking, the strategy of cell immobilization did not have obvious positive effects on the performance of the fermentation, regarding both sugar consumption and ester production.

Comparing the two media, the *S. cerevisiae* (AR5) strain was more robust, since it attained good results in both of the media studied, whereas non-*Saccharomyces* achieved better results in JA than in M11 medium. Thus, in studies with non-*Saccharomyces*, it is important to work with a medium as similar as possible to the natural medium in which the strain was isolated.

The main conclusion is that the non-*Saccharomyces* strains studied in this work were able to ferment the agave juice to produce tequila with an ethanol concentration similar to that of *S. cerevisiae* and with higher concentrations of esters.

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