Antonie van Leeuwenhoek (2006) 89: 181–189
DOI 10.1007/s10482-005-9022-1

Fermentation behaviour and volatile compound production by agave and grape must yeasts in high sugar *Agave tequilana* and grape must fermentations

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Received 4 July 2005; accepted in revised form 25 October 2005

Key words: Agave, Tequila, Volatile compounds, Wine, Yeasts

Abstract

Few studies have been performed on the characterization of yeasts involved in the production of agave distilled beverages and their individual fermentation properties. In this study, a comparison and evaluation of yeasts of different origins in the tequila and wine industries were carried out for technological traits. Fermentations were carried out in high (300 g l^{-1}) and low (30 g l^{-1}) sugar concentrations of *Agave tequilana* juice, in musts obtained from Fiano (white) and Aglianico (red) grapes and in YPD medium (with 270 g l^{-1} of glucose added) as a control. Grape yeasts exhibited a reduced performance in high-sugar agave fermentation, while both agave and grape yeasts showed similar fermentation behaviour in grape musts. Production levels of volatile compounds by grape and agave yeasts differed in both fermentations.

Introduction

Mexico produces a range of distilled beverages obtained from fermented musts of different species of agave plants. The best known is tequila, obtained from *Agave tequilana* Weber var. azul juice, but other beverages, such as mezcal, sotol, and raicilla, can be mentioned also. The tequila process is divided into four main phases: cooking, milling, fermentation and distilling. The fermentations are carried out at 8–14° Brix (60–120 g l^{-1} of agave sugars), 28–37 °C and with yeasts from bakeries, wines or agave must, with or without inoculation. The principal sugar of agave must is fructose, which is obtained by the hydrolysis of a type of inulin during cooking. The main minerals of the agave must are calcium, magnesium and phosphate (laboratory data). In tequila fermentation with high sugar concentrations, nitrogen limitation is a serious problem which could be solved by the addition of exogenous nitrogen (Arrizon and Gschaedler 2002). The fermentation capability of the yeast strain may be a factor also (Manginot et al. 1998).

In spite of the diversity of agave distilled beverages in Mexico, there is only one recent report on microbial populations in tequila fermentation (Lachance 1995) and little is known on the potential

of *Saccharomyces* and non-*Saccharomyces* species present in agave beverages (Fiore et al. 2005). Non-*Saccharomyces* yeasts are known to influence the sensory quality of wines (Romano et al. 2003), suggesting that this may be the case also in tequila production. In this study, fermentations were performed at high (300 g l^{-1}) and low (30 g l^{-1}) agave sugar concentrations and in grape must with *S. cerevisiae* and non-*Saccharomyces* strains isolated from grape and agave musts, in order to compare and evaluate their technological potential as fermentation yields, volatile compounds production and the capacity to ferment at high sugar concentration.

Materials and methods

Sixteen yeast strains belonging to two different collections were used. Collection 1 (from Basilicata University, Italy): three *Kloeckera apiculata* strains (20EI5, 20EII5, 7EI3), two *S. cerevisiae* strains (4LBI3, AGME7) from Aglianico-Vulture red grapes (Basilicata region), one *S. cerevisiae* strain (NDAM2) from Nero d'Avola red grapes (Sicily region) and one *S. cerevisiae* strain (FIMA3) from Fiano white grapes (Campania region). Collection 2 (from CIATEJ, Mexico): one *Candida krusei* strain (SO2) and one *S. cerevisiae* strain (SOM) from Sotol must (*Dacilirium*) in Chihuahua state, one *Candida magnoliae* strain (OFF1) and one *S. cerevisiae* strain (CHA) from Mezcal must (*Agave cupreata*) in Guerrero state, one *Kloeckera africana* strain (TE4) and three *S. cerevisiae* strains (GU4, MG, AR5) from Tequila must (*Agave tequilana*) and one *S. cerevisiae* strain (RG1) from Raicilla must (*A. maximiliana*) in Jalisco state.

Agave tequilana *and grape must fermentations*

Fermentations were carried out with agave and grape strains of *S. cerevisiae*, whereas sequential fermentations with non-*Saccharomyces* involved inoculation on the 4th day of *S. cerevisiae* strain FIMA3. All the fermentations were performed in duplicate.

Fermentation in Agave tequilana *must and YPD*

The *Agave tequilana* must was prepared by filtering concentrated *A. tequilana* juice (29° Brix) provided by a tequila factory, then the juice was concentrated at 55 °C in an evaporator with vacuum until it reached 300 g l^{-1} of agave sugar. The *A. tequilana* must for fermentation at high and low sugar concentrations was prepared as follows: (1) high sugar agave must (HSAF) was obtained by sterilizing (121 °C, 15 min) the concentrated agave juice (300 g l^{-1} of agave sugar) supplemented with 4 g l^{-1} of ammonium sulfate, (2) low sugar agave must (LSAF) was composed of concentrated *A. tequilana* juice diluted to 30 g l^{-1} and supplemented with 0.4 g l^{-1} of ammonium sulfate, then sterilized at 121 °C for 15 min. The laboratory medium YPD (2 % w/w peptone; 2 % w/w yeast extract), containing 270 g l^{-1} of glucose, was used as a control. For both agave-based musts and the YPD medium, fermentations were carried out at 35 °C for 72 h in 500-ml flasks containing 180 ml of must. The initial yeast population inoculated was 10×10^6 cells ml^{-1}.

Fermentations in grape must

Fermentations in grape must were performed in 130-ml Erlenmeyer flasks containing 100 ml of grape must sterilized at 100 °C for 20 min. Each sample was inoculated with 10^4 cells ml^{-1} from a 48 h pre-culture grown in the same must. The grape must surface was covered with a thin layer of sterilized paraffin oil in order to avoid air contact and the samples were incubated statically at 26 °C until CO_2 evolution ceased. This practice avoids the evaporation of liquid and mass losses and the weight loss recorded is a function of the CO_2 evolved. The determination of weight loss in the samples was used as a parameter to follow the fermentation process and was monitored every day in order to evaluate the quantity (in grams) of CO_2 evolved, which was used to express strain fermentation power at the end of the process.

All strains were tested in two different grape musts: Aglianico Vulture (red grape) and Fiano (white grape). Single-culture fermentations were

carried out with agave and grape strains of *S. cerevisiae*, whereas sequential fermentations with non-*Saccharomyces* species were completed by inoculation at day 4 with *S. cerevisiae* strain FIMA3.

At the end of all fermentations, samples were collected, refrigerated for 1 day at 2 °C, racked and stored at −20 °C until required for analysis.

Analytical determinations

Ethanol production and sugar and nitrogen consumption were determined by the methods of Boehringer and Jacob (1964), Miller (1959) and Chaney and Marbach (1962), respectively. By-products were determined by gas chromatographic analysis. For agave fermentation products, initial and final samples were distilled at 95 °C and volatile compounds and analyses were carried out in a Hewlett-Packard 5890 gas chromatograph with a flame ionisation detector (FID). One microlitre of distillate was injected into a HP-Innowax PEG column (60 m×320μm^2). The temperature program was 50 °C (6 min), followed by an increase of 10 °C min^{-1} to 160 °C and a final treatment at 20 °C min^{-1} to 220 °C (8 min). Injector and detector temperature was 250 °C. The carrier gas was nitrogen.

In the case of grape fermentation, 1 μl of experimental wine was injected into a glass column (180 cm × 2 mm) packed with 80/120 Carbopack B:5% Carbowax 20 M (Supelco). A Carlo Erba Fractovap series 2350 gas chromatograph equipped with a LT programmer model 220 and a FID linked to a Carlo Erba Mega series integrator and recorder were used. The column was run from 60 °C to 198 °C at the rate of 6 °C min^{-1}. The carrier gas was nitrogen at a flow rate of 20 ml min^{-1}. Each sample was preloaded with *n*-butanol at a concentration of 100 mg l^{-1}.

Statistical analysis

All data from the HASF and LASF samples were evaluated by Statgraphics (ver. 4). Data from grape must fermentation underwent statistical analysis (PCA) using the software 'Statistics for Windows', ver. 5.0, 97 edition (Statsoft).

Results

In high-sugar agave fermentations (HASF), grape strains of *S. cerevisiae* exhibited a lower sugar consumption and a lower fermentation efficiency than agave strains (Figure 1), whereas in low agave sugar fermentations (LASF), the strains had similar recorded properties. Non-*Saccharomyces* grape strains did not ferment agave must in any conditions, whereas two of three non-*Saccharomyces* agave strains showed a lower fermentation activity than agave strains of *S. cerevisiae* both in LASF and HASF (data not shown).

In order to ascertain the existence of an inhibitory effect of high-sugar agave must on grape yeasts, fermentations were performed in YPD medium (270 g l^{-1} glucose). Grape and agave strains of *S. cerevisiae* exhibited similar levels both of sugar consumption and ethanol production and all non-*Saccharomyces* strains were able to consume sugar and to produce ethanol in YPD medium, although to a lesser extent than *S. cerevisiae* strains (data not shown).

In grape must fermentations, all *S. cerevisiae* strains exhibited good and similar yields (Figure 2a, b), although the fermentation rates were different. At the beginning, the fermentation was faster in Aglianico (Figure 1a) than in Fiano (Figure 2b) grape must. Conversely, non-*Saccharomyces* yeasts differed significantly as a function of their origin (Figure 2a, b). In particular, the three *K. apiculata* grape strains behaved similarly, exhibiting a general slow fermentation with a low carbon dioxide evolution until fermentation day 6. This result is in agreement with the widespread behaviour reported as typical of apiculate wine yeasts. Conversely, the apiculate strain from agave must, TE4 (*K. africana*), exhibited a good fermentation performance, particularly in Aglianico Vulture (Figure 2a). During the process, this strain had a high CO_2 evolution, which was at levels close to those of *S. cerevisiae* strains (about 6 g per 100 ml grape must). As regards the two agave strains of *Candida*, both exhibited a low CO_2 evolution corresponding to a sluggish fermentation, which is a typical behaviour of non-*Saccharomyces* yeasts. The agave strain SO2 (*C. krusei*) showed the worst fermentation performance in both the grape musts, highlighted by low CO_2 evolution (less than 2 g per 100 ml grape must) in the first 6 days of fermentation.

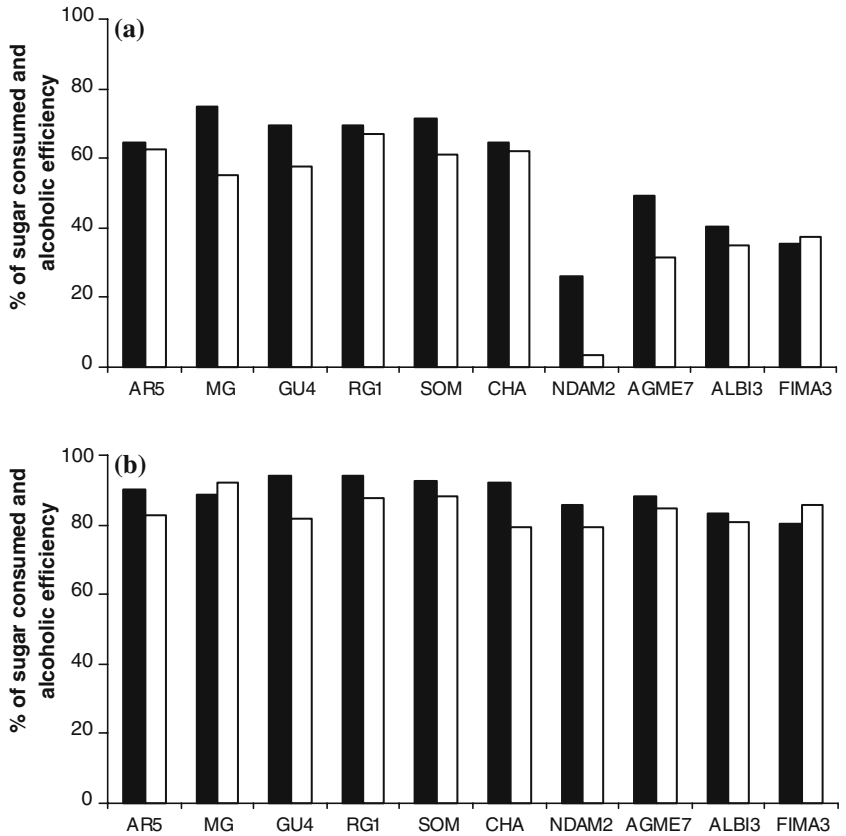

Figure 1. Percentage of sugar consumed (■) and alcoholic efficiency (□) in high *Agave tequilana* sugar (a) and YPD (b) fermentations, by *S. cerevisiae* strains from grape (NDAM2, AGME7, 4LBI3, FIMA3) and agave (AR5, MG, GU4, RG1, SOM, CHA) musts.

A correlation was found between the levels of volatiles produced and the fermentation behaviour in HASF, where agave strains of *S.cerevisiae* produced significantly more volatile compounds than grape strains (Figure 3). In particular, amyl alcohols were produced at the highest levels by agave strains; and methanol and 2-phenyl ethanol were found only in the samples fermented by agave strains (Figure 3).

By-products related to organoleptic quality were determined in the experimental wines.

Significant differences in the levels of by-products were found as a function of the yeast which performed the fermentation. In order to link differences in chemical compounds with yeast species or origin of the strains, the data were elaborated by principal component analysis (PCA). Figure 4 reports the diagrams obtained from this analysis, in particular Figure 4a shows the data related to the fermentation in Aglianico Vulture grape must, whereas Figure 4b reports the results obtained from the fermentation of the same strains in Fiano grape must. The statistical results from the two grape musts are reported in Table 1 as a function of the analytical variables considered. The PCA explained 65.8% and 45.5% of the total variance in Aglianico Vulture and Fiano, respectively, using the first and second components. In Aglianico, the variables which contributed mostly to the position of the experimental wines in the figure were ethyl acetate and isoamyl alcohol, which resulted in statistically significant parameters for component 1 (Table 1). In Fiano, the variability in the production of *n*-propanol, acetic acid, acetoin and amyl alcohols were the main variables and the variance was statistically significant for both components (Table 1). By comparative analysis of the two figures, differences appear which are established by the yeast species which performed the fermentation, or which are due to interaction

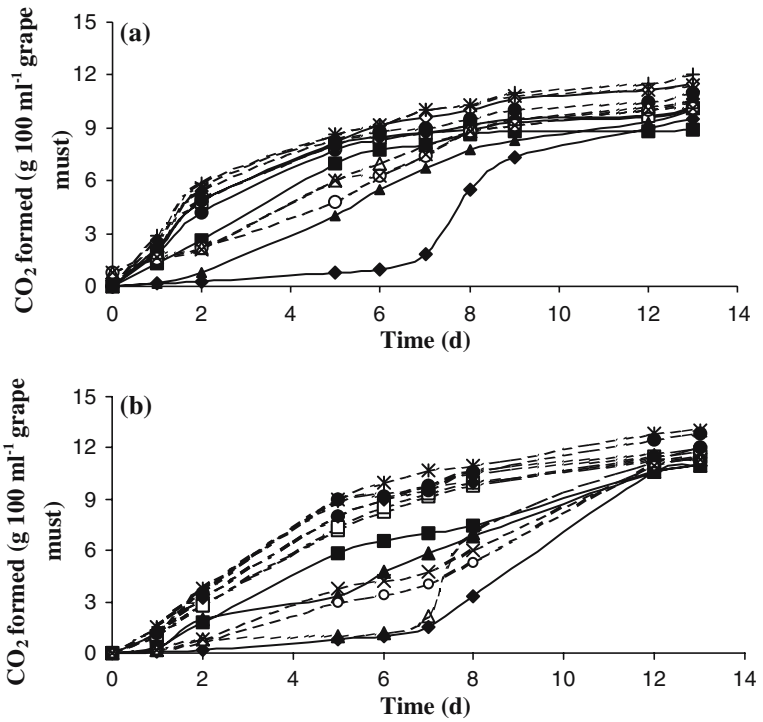

Figure 2. CO_2 production in Aglianico Vulture (a) and Fiano (b) grape fermentations by grape yeast strains: 7EI3 (-Δ-), 20EI5 (-O-), 20EII5 (-x-), NDAM2 (-+-), AGME7 (-*-), 4LBI3 (-●-), FIMA3 (-◆-) and by agave yeast strains: SO2 (◆), TE4 (■), OFF1 (▲), AR5 (x), MG (●), GU4 (+), RG1 (*), SOM (◇), CHA (□).

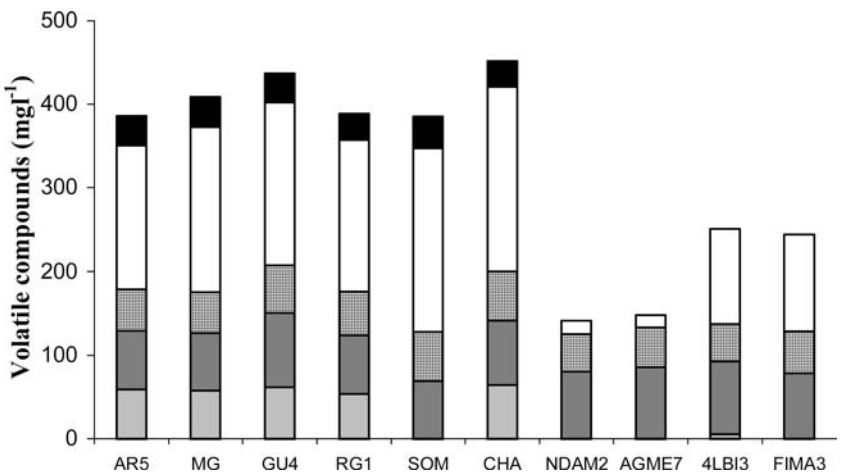

Figure 3. Volatile compounds in distillated samples from high sugar *A. tequilana* fermentation (HASF): 2-phenylethanol (■), amyl alcohols (□), isobutanol (▦), 1-propanol (▩), methanol (▩) by grape (NDAM2, AGME7, 4LBI3, FIMA3) and agave must (TE4, OFF1, AR5, MG, GU4, RG1, SOM, CHA) yeasts.

between the yeast and grape must composition. The different distribution of the experimental wines indicates a high yeast species/strain variability in the production level of secondary compounds. In particular, in Aglianico fermentation, agave and grape yeasts were located near,

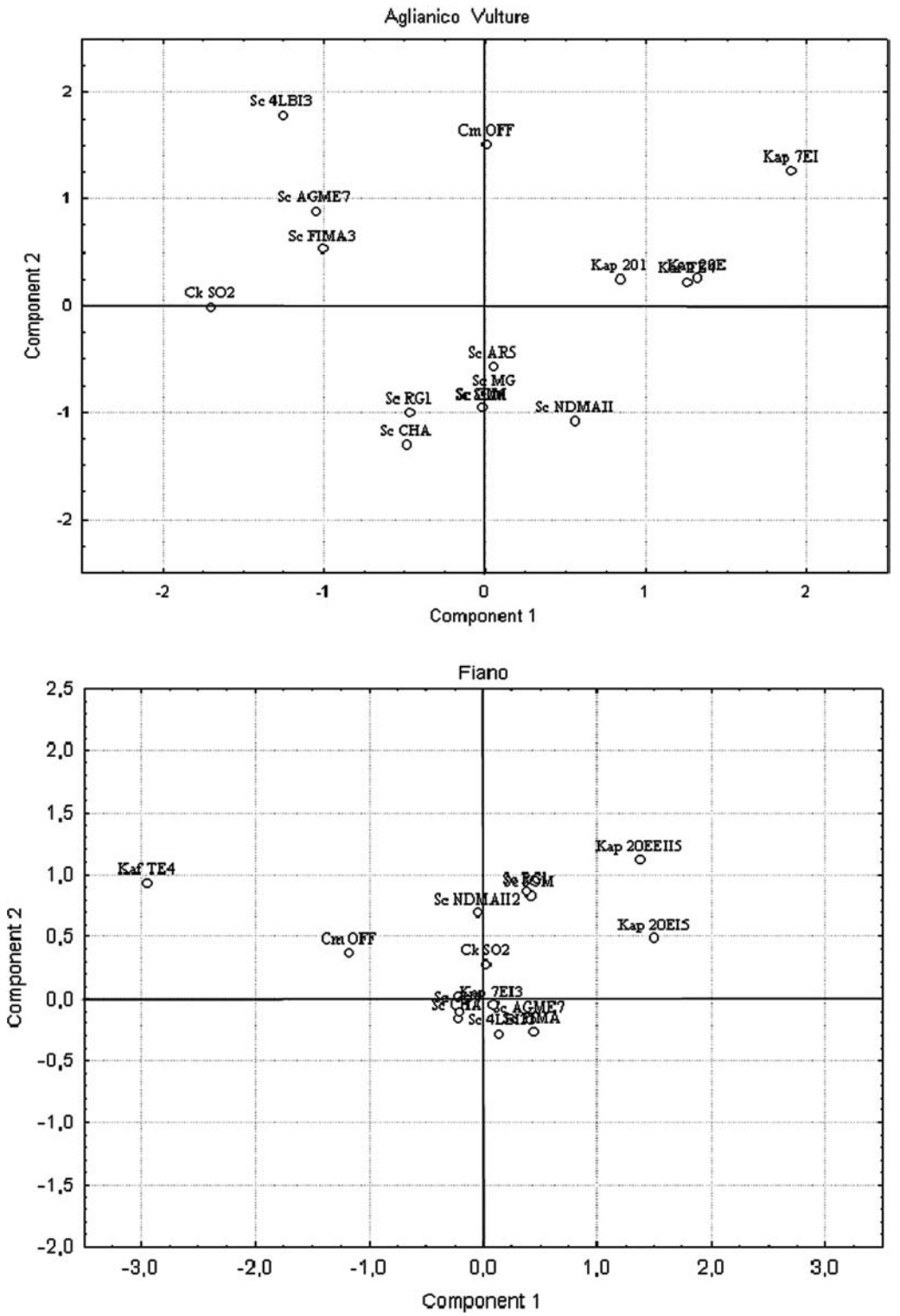

Figure 4. Discrimination of the experimental wines obtained with agave and grape yeasts (*S. cerevisiae* and non-*Saccharomyces*) by PCA based on secondary compounds produced in fermentation of the two grape musts.

Table 1. Loadings for the two principal components in the two grape must fermentations.

Variables	Fiano		Aglianico	
	Component 1	Component 2	Component 1	Component 2
Acetaldehyde	−0.31	−0.45	0.63	0.49
Ethyl acetate	0.01	0.28	0.79*	0.43
n-Propanol	−0.82*	−0.06	0.27	0.44
Isobutanol	0.44	−0.34	−0.40	0.64
Acetoin	−0.42	−0.80*	0.55	0.24
Acetic acid	0.85*	−0.44	−0.04	0.54
D-amyl alcohol	−0.44	−0.72*	−0.66	0.57
Isoamyl alcohol	0.83*	−0.39	−0.87*	0.28
Explained variance (%)	26.68	19.90	45.64	20.20
Total variance (%)	45.52		65.84	

* Parameters with significant variability.

generally as a function of their yeast species, but also of their origin. In fact, as regards *S. cerevisiae*, three grape strains are located together in the left superior quadrant, two agave strains together in the left inferior quadrant and another two agave strains on the line between the two inferior quadrants. Conversely, in Fiano fermentation, a more uniform distribution was determined, with the majority of the strains close to the centre of the figure and in the superior quadrants. Only four non-*Saccharomyces* yeasts, two grape and two agave strains, were located far from the others: two *K. apiculata* grape strains (20EI5, 20EII5), *K. africana* agave strain TE4 and *C. magnoliae* agave strain OFF1. The statistical analysis emphasised that the variables influencing the strain position in space are different, demonstrating that a strict correlation exists between yeast behaviour and the fermention substrate. The organoleptic quality of the fermented products is the result of this interaction.

Discussion

Considering that grape yeasts exhibit a reduced fermentation performance at a high sugar concentration in agave musts, it appears evident that the starter culture has to be chosen as a function of the raw material. In our case, the relevant growth differences found between strains as a function of their isolation origin could be the result of yeast adaptation to different fermentation conditions. In the tequila process, the must obtained after cooking of the agave contains furfural (Cedeño 1995), Maillard compounds and vanillin (Mancilla-Margalli and López 2002). In contrast, in winemaking the grapes are harvested, crushed and used directly for fermentation. As furfural (Palmqvist et al. 1999) and vanillin (Fitzgerald et al. 2004) are toxic to yeasts, the concentrated agave juice probably contains these compounds at levels that affect the growth and activity of grape yeasts. Taking into account that *Saccharomyces* wine yeasts have a different preference for glucose and fructose, depending on environmental conditions (Berthels et al. 2004), and that *Agave tequilana* must contains principally fructose (Cedeño 1995), it is possible that *S. cerevisiae* agave strains possess a better capacity for fructose and glucose assimilation than grape strains.

In YPD, Aglianico and Fiano grape fermentations, agave strains of non-*Saccharomyces* species exhibited a higher efficiency than grape strains in growth, sugar consumption and ethanol production. As mezcal, sotol, raicilla and some tequila processes are produced by natural fermentations, in the presence of toxic compounds at temperatures of 20–37 °C or more, environmental pressure has caused a selection for tolerant yeasts. In particular, non-*Saccharomyces* agave strains possess a high tolerance to ethanol and sulphite (Fiore et al. 2005).

A significant, sometimes also considerable, yeast strain variability was observed in products, such as higher alcohols (Steger and Lambrechts 2000; Romano et al. 2003), resulting also in a potential considerable effect on the final organoleptic quality of the products. Our results confirm these findings. In the case of volatile production in

HASF, the levels formed by grape yeasts are not only the result of strain characteristics, but rather the result of the inhibition of fermentation; and the production of 2-phenyl-ethanol differentiates clearly agave and grape strains. The differences in 2-phenyl-ethanol formed could be related to the dependence of higher alcohols production to nitrogen demand (Torrea et al. 2003) and to the differences in nitrogen requirements between *Saccharomyces* strains (Manginot et al. 1998).

Methanol was recovered only in samples fermented with agave strains of *Saccharomyces*. There is evidence that *Saccharomyces* strains have enzymes with methyl-esterase activity, such as phosphatase-carboxyl-methyl-esterase (PM1-1), which has a regulatory function and performs methylation and demethylation reactions with different molecules (Lee et al. 1996; Wei et al. 2001); and this enzyme is well conserved in all eukaryote cells (Ogris et al. 1999). Therefore, it might be possible that agave strains possess a methyl esterase enzyme which, at the conditions tested, becomes active and can determine the formation of methanol.

Among non-*Saccharomyces* yeasts, one agave strain of *K. africana* exhibited the best fermentation in both grape fermentations. This behaviour differs considerably from other apiculate yeasts, which dominate the early phase of grape must transformation and then are replaced by the strongly fermentative yeast *S. cerevisiae* (Fleet and Heard 1993).

Even if only one *K. apiculata* agave strain has been used, our results confirm previous information (Lachance 1995), reporting that traditional agave fermentations represent a natural source of non-*Saccharomyces* yeasts which possess tolerance to environmental conditions. In our work, the agave yeasts studied were characterized by tolerance to high concentrations of ethanol and sugar.

Finally, it must be underlined that the yeast species/strain in inoculated fermentation exerts a prominent role on the final organoleptic quality, by transforming aromatic precursors of the must and by producing *ex novo* aromatic substances.

Conclusions

In conclusion, our results demonstrate that HASF generates more stress conditions for yeast performance than grape fermentations. Moreover, the different yeast behaviour as a function of sugar concentration and the significant diversity found at strain level for the parameters considered underline the advantage of using specific yeast strains for tequila and other Mexican agave beverages, suggesting research should be addressed towards a selection program of yeasts possessing suitable and desirable characteristics. In particular, the selection of specific strains for specific fermentations has to be performed as a function of the characteristics of the raw material, in order to take the major advantage from the combination of fermentation substrate/yeast strain.

Acknowledgements

This research was supported by the Consejo Nacional de la Ciencia y Tecnología de Mexico (CONACYT, project 32719-B). We also want to thank Arturo Rosas for technical support.

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