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ORIGINAL PAPER

Effect of *Agave tequilana* age, cultivation field location and yeast strain on tequila fermentation process

L. Pinal · E. Cornejo · M. Arellano · E. Herrera · L. Nuñez · J. Arrizon · A. Gschaedler

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Abstract The effect of yeast strain, the agave age and the cultivation field location of agave were evaluated using kinetic parameters and volatile compound production in the tequila fermentation process. Fermentations were carried out with Agave juice obtained from two cultivation fields (CF1 and CF2), as well as two ages (4 and 8 years) and two *Saccharomyces cerevisiae* yeast strains (GU3 and AR5) isolated from tequila fermentation must. Sugar consumption and ethanol production varied as a function of cultivation field and agave age. The production of ethyl acetate, 1-propanol, isobutanol and amyl alcohols were influenced in varying degrees by yeast strain, agave age and cultivation field. Methanol production was only affected by the agave age and 2-phenylethanol was influenced only by yeast strain. This work showed that the use of younger *Agave tequilana* for tequila fermentation resulted in differences in sugar consumption, ethanol and volatile compounds production at the end of fermentation, which could affect the sensory quality of the final product.

Keywords Tequila · *Agave tequilana* · Fermentation kinetics · Volatile compounds

L. Pinal · M. Arellano · E. Herrera · L. Nuñez · J. Arrizon · A. Gschaedler (✉)
Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Avenida Normalistas 800, Col. Colinas de la Normal, 44270 Guadalajara, Jalisco, Mexico
e-mail: agschaedler@ciatej.net.mx

E. Cornejo
Departamento de Ingeniería Química, Centro Universitario de Ciencias Exactas e Ingenierías, Universidad de Guadalajara, Guadalajara, Jalisco, Mexico

Introduction

Tequila is a characteristic alcoholic beverage of Mexico. Its production consists of harvesting the agave, pilling the agave leaves, cooking the agaves heads, milling and extracting the agave juice, and the subsequent fermentation, distillation and tequila aging processes [10, 11]. One specific characteristic of the tequila 100% agave production is that *Agave tequilana* Weber blue variety species is allowed as the only source of sugar [19]. Taxonomically, *Agave tequilana* Weber blue variety is classified as a member of the Rigidae group within the genus *Agave* of the Agavaceae family. The physiological plant maturity takes between 7 and 10 years depending on cultivation conditions, and after flowering it senesces and then dies. Normally, the agaves are harvested between 6 and 10 years or just before flowering when the accumulated sugars (fructans) have a maximum concentration. These agave fructans consist of a complex mixture of fructooligosaccharides containing principally β (2-1) linkages, but also β (2-6) and branch moieties [14, 15]. The fermentation stage [3, 4, 21] and distillation [22] influence the production of important tequila volatile compounds [7] such as the higher alcohols, the esters and the carbonyls. Other compounds such as furfural [10], Maillard compounds and vanillin [16] are produced during cooking and extracted from wood in the tequila aging process. Similar to other alcoholic beverages, the production of these compounds is directly related to the characteristics of the raw material for must elaboration [1, 24, 26]. During the period 1999–2003, there was a decrease in the *A. tequilana* plant production in the two principal Jalisco production regions (Amatitan Valley and Los Altos) due to climate changes and plant blight by molds and bacteria. All of these conditions have caused death to the

A. tequilana plant in the regions of tequila production. Furthermore, the raw material consumption for the production of this beverage has increased due to higher tequila demand around the world. Therefore, tequila producers were forced to use younger agave plants (4–6 years) in order to overcome the lack of mature agaves. The current study evaluates the effect of the agave age, cultivation field and yeast strain with respect to the kinetic parameters and volatile compounds production in tequila fermentation 100% agave. Presently there are no scientific reports regarding the correlation of those factors.

Materials and methods

Yeast strain

The *Saccharomyces cerevisiae* strains AR5 and GU3 were isolated from tequila factories and conserved in the laboratory collection of the Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C.

Agave must preparation

The musts for propagation and fermentation were obtained from *Agave tequilana* Weber blue variety juice of different ages (4 and 8 years) after cooking and from two cultivation fields (CF1 and CF2). The juices were filtered, diluted with distilled water to reach 8° Brix for inoculum media and 10° Brix for fermentation, and then sterilized (121°C, 15 min). In all cases, 1 g/l of ammonium sulfate (Sigma Aldrich Chemie, Steinheim, Germany) was added to the musts before sterilization.

Inoculum conditions

Saccharomyces cerevisiae strains (GU3 and AR5) were grown in 200 ml of must in 500 ml Erlenmeyer flasks with shaking (250 rpm) for 12 h at 30°C and harvested when the cell populations reached 180–250 $\times$ 10^6 cells/ml.

Fermentation conditions

Batch fermentations were prepared in 2 liter Erlenmeyer flasks containing 800 ml of sterilized must of *Agave tequilana*. The media was inoculated with an initial population of 20×10^6 cells/ml. Fermentations were carried out in duplicate without stirring at 35°C for 72 h. Culture samples were taken every 4 h to determine yeast population, sugar, ethanol and volatile compounds concentrations.

Analytical methods

Yeast population

The total concentration of yeast cells population was determined under light microscope using a Neubauer counting chamber.

Reducing sugars

The reducing sugar concentration in the medium was determined using 3,5-dinitrosalicylic acid reagent [18].

Ethanol

Each fermentation sample was distilled using a microdistiller with a vigreaux column. Five milliliters of must and 5 ml of distillated water were added to a bowl and boiled until 5 ml of distillate remained. The resultant ethanol concentration was measured using a dichromate reagent [9].

Volatile compounds

Analysis of volatile compounds of distillates was carried out in a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) with a flame ionization detector (FID) equipped with an HP-Innowax PEG column (60 m $\times$ 320 μm^2). The initial column temperature was 50°C for 6 min and was then ramped at 10°C per min to 160°C, followed by a 20°C per min ramp to 220°C. Injector and detector temperatures were maintained at 250°C. Sample size was 1 µl and the carrier gas was nitrogen.

Parameter calculation

Alcoholic efficiency was calculated from the ratio of the average ethanol produced at the end of fermentation and the theoretical ethanol production from the biochemical conversion of the sugar consumed. For maximum ethanol production, yeast population and sugar consumption rates (r_{p_max}, r_{x_max} and r_{s_max}, respectively), experimental data of ethanol, sugar and population concentrations were adjusted to a mathematical model using the Curve Expert 1.3 software (EBT Comm, Columbus, MS, USA). This model was further interpolated (100 points), and the maximum rates were obtained from the maximum slopes.

Statistical analysis

A combination of three treatments was applied in a multifactor experimental design; with the following variables: agave age (4 and 8 years), agave cultivation fields (CF1

and CF2) and yeast strains (GU3 and AR5). The variables were statistically evaluated for fermentation kinetics and the volatile compound content. ANOVA tests were carried out using the Statgraphics (StatPoint, Inc., Rockville, MD) software.

Results and discussion

Effect of the agave age, agave cultivation field and yeast strain on fermentation kinetics

The cell growth of both yeast strains was not affected by the agave age, cultivation field and yeast strain (Figs. 1a, b, 2a, b). This is observed also in the maximum growth rate values (Fig. 3a, b). It is well known that anaerobic conditions do not favor cell growth [31] as yeast cells try to maintain the redox balance in the absence of oxygen [23]. However, under aerobic conditions for inoculum propagation, the maximum growth rates for both yeast strains were two times higher than anaerobic fermentation (data not shown). The absence of oxygen therefore, could be the main factor affecting yeast cell growth during alcoholic fermentation. Contrary to cell growth and sugar consumption, ethanol production was influenced by the agave cultivation field location and yeast strain. The fermentations with CF1 agaves showed slower sugar consumption and ethanol production rates than fermentations with CF2 agaves (Fig. 3). The maximum ethanol production rates with CF1 agaves (Fig. 3a) were similar between AR5 and GU3 yeast strains for both 4- and 8-year-old agaves. However, maximum ethanol production rates of CF2 agaves (Fig. 3b) were considerably higher for the AR5 than for the GU3 yeast strain for the different agave ages. It has been found in wine fermentations that grape variety, the region and vineyard culture conditions influence the fermentation behavior [1, 24–26] due to variations in the chemical composition of the raw material [1, 29]. In tequila production, the only species allowed as principal raw material is the *Agave tequilana* Weber blue variety [19]. The agaves used in the present study came from the same region but from different cultivation fields. It has been observed in wine that differences in agricultural practices such as fertilization and soil mineral composition influence the fermentation behavior of grape must [20]. It may be possible, therefore, that the differences in fermentation between CF1 and CF2 agaves are a result of differences in

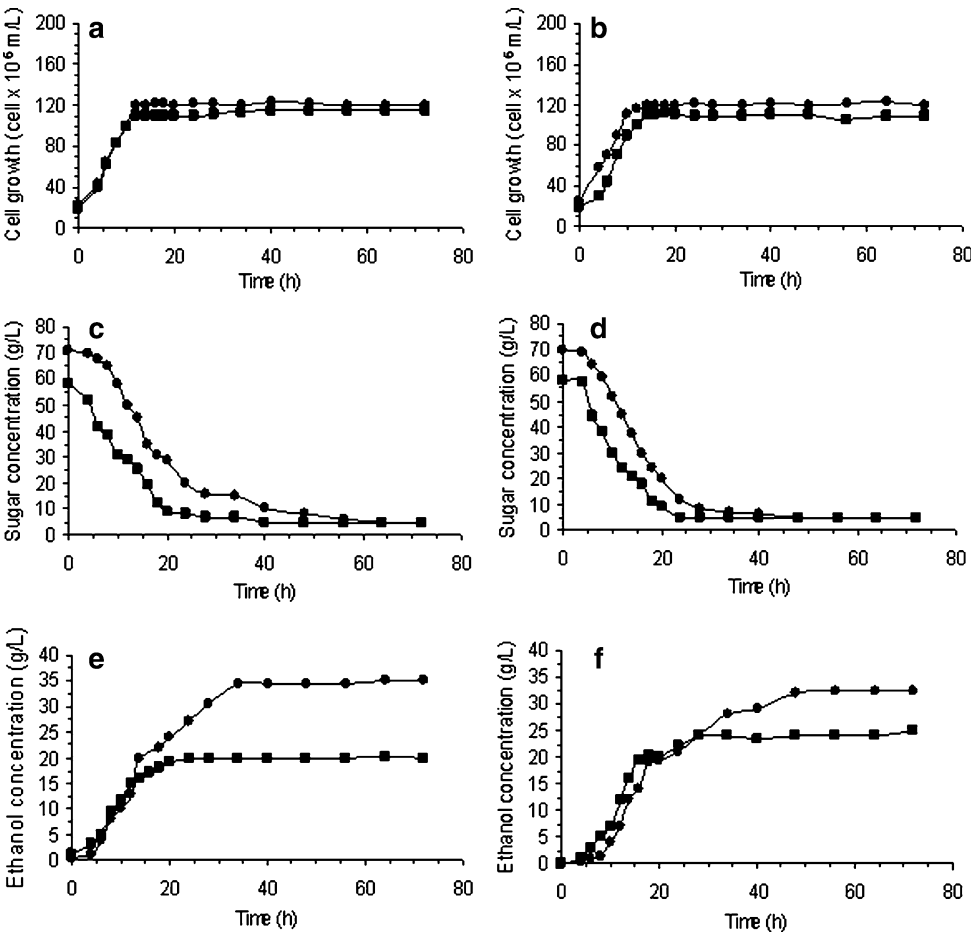

Fig. 1 Cell growth, sugar consumption and ethanol production for the *Saccharomyces cerevisiae* yeasts AR5 (**a**, **c**, **e**) and GU3 (**b**, **d**, **f**), respectively, in fermentations at 35°C using agaves of 4 (*filled square*) and 8 (*filled circle*) years from CF1

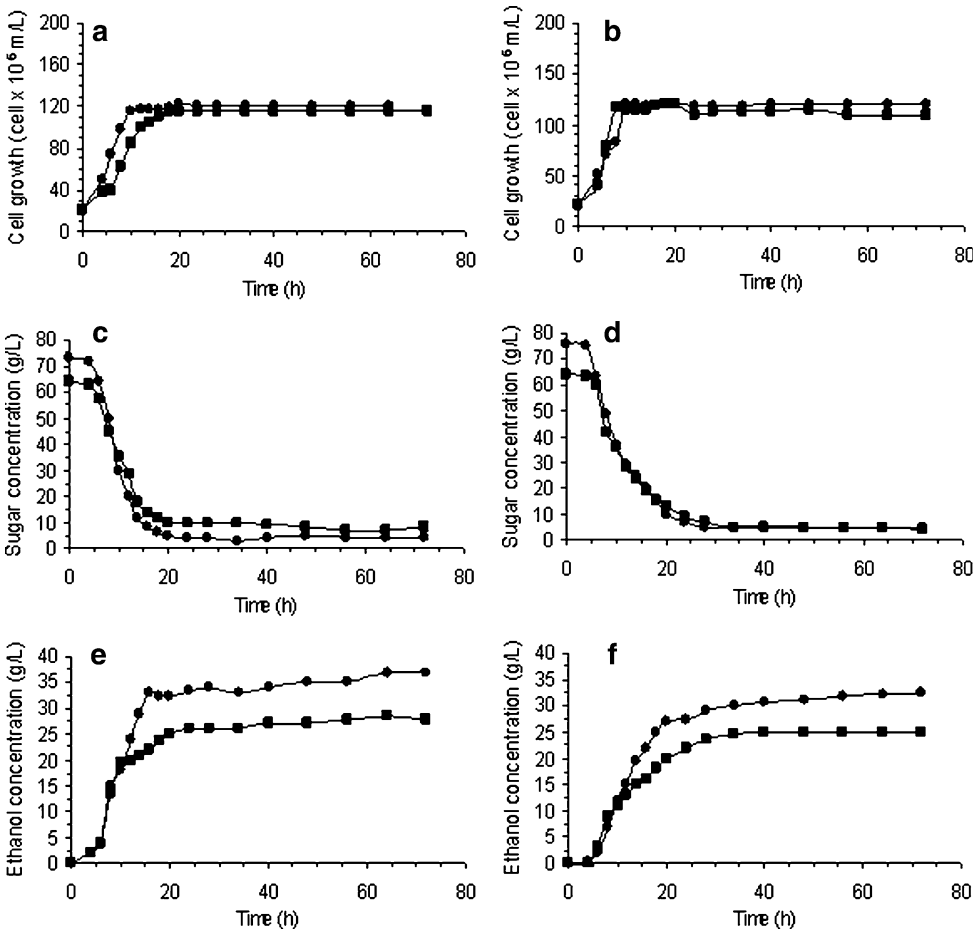

Fig. 2 Cell growth, sugar consumption and ethanol production for the *Saccharomyces cerevisiae* yeasts AR5 (**a**, **c**, **e**) and GU3 (**b**, **d**, **f**), respectively, in fermentations at 35°C using agaves of 4 (*filled square*) and 8 (*filled circle*) years from CF2

agricultural practices. Although the agave age did not have an influence over either the maximum sugar consumption or ethanol production rates, it affected the level amount of ethanol produced. Four-year-old agaves showed lower ethanol production than 8-year-old agaves independent of cultivation location (Figs. 1 e, f, 2, e, f). The difference in the amount of ethanol produced was also dependent upon the yeast strain. It can also be observed that musts obtained from agaves of different ages adjusted to 10 Brix degrees had different sugar concentrations (61.5 and 72.5 g/l for 4- and 8-year-old agaves, respectively), caused by differences in soluble solids due to pectin content (data not shown). According to the experience of agave producers, the difference can be due to the fertilization system and soil composition of each location, and these factors, probably influenced ethanol production. It has been found that nutrient limitations inhibit ethanol production in tequila [2, 7, 27] and other fermentations [6, 8, 17, 30, 32].

The effect from agave age, cultivation field and yeast strain on volatile compounds production

The influence of yeast strain, agave age and cultivation field on volatile compounds differs depending on the compound analyzed. The data of volatile compounds presented in Table 1 represents the concentration at the end of the fermentation and its statistical analysis. There is a complex relationship between agave age, cultivation field, yeast strains and volatile compounds production. Each compound will be discussed in the following sections.

Ethyl acetate production was principally influenced by the yeast strain. As can be seen in Table 1, the GU3 yeast strain produced more ethyl acetate than the AR5 in fermentations with both agave cultivation fields (CF1 and CF2). In fermentations with CF1 agaves the age did not influence ethyl acetate production for either yeast strains (Table 1), whereas in fermentations with CF2 agaves, it was not possible to observe a direct influence of the agave age on the ethyl acetate production (Table 1). Ethyl acetate is produced by the action of alcohol-acetyl-transferase, which combines an ethanol molecule with an acyl group from acetyl-CoA [12, 13]; thus the differences observed were possibly a function of yeast metabolism variations between the strains.

Methanol concentration depended on agave age and cultivation field. In fermentations with CF1 agaves, the concentration of methanol was higher in 4-year-old agaves than in 8-year-old ones. As can be seen, the effect of agave

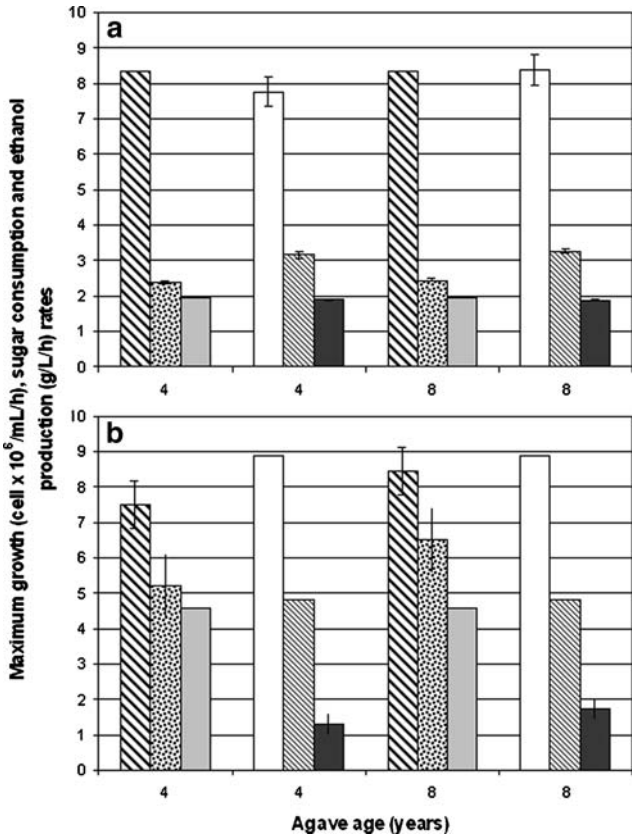

Fig. 3 Maximum growth (*stripped bars* and *open bars*), sugar consumption (*dotted bars* and *dot-stripped bars*) and ethanol production rates (*light-shaded bars* and *dark-shaded bars*) for the *S. cerevisiae* AR5 and GU3 yeasts, respectively, in CF1 (**a**) and CF2 (**b**)

age on methanol production was stronger in CF1 agaves than in CF2 agaves. It has been shown in different reports that methanol in wine and tequila is produced principally from the methoxyl groups of pectins present in the vegetal material of the must [28]. Therefore it may be possible that with older agaves, less methoxyl groups of pectins are available for methanol production.

The amyl alcohols and 1-methyl-propanol production were affected by the agave age and yeast strain. The production of amyl alcohols production increased more than 30% as the agave age augmented, independently to the yeast strain (Table 1). The production of 1-methyl-propanol increased between 25% and 54% with older agave (Table 1). It can also be seen that the GU3 yeast strain produced in general more amyl alcohols and 1-methyl-propanol than the AR5 yeast strain independent of agave age and cultivation field (Table 1). Similar to 1-methyl-propanol and amyl alcohols production, the 1-propanol concentration increased with older agaves. It can, thus, be observed that the differences in 1-propanol production related with agave age were more pronounced in CF2 agaves than in CF1 agaves. The yeast strain had less effect on 1-propanol than the amyl alcohols and 1-methyl-propanol productions. It is well known that higher alcohols can be produced by the catabolism of amino acids, known as the Ehrlich pathway, or by anabolism of amino acids under nitrogen limited conditions where more α-ceto acids are decarboxylated and transformed to aldehydes and to higher alcohols [5, 31]. In tequila fermentations, low levels of nitrogen are often present in the must due to the degradation of nitrogen by Maillard reactions during cooking [16]. It has also been observed that in the tequila fermentation process, the production of higher alcohols increases as the C/N ratio increases [4, 21]. Thus higher alcohols in tequila fermentation may be produced by anabolism of amino acids. The C/N ratio was higher in the must of 8-year-old agaves than in that of 4-year-old agaves, due to the fact that despite equal nitrogen supplementation in all fermentations the initial sugar concentration was lower in 4-year-old agaves than in 8-year-old agaves. This may have caused the increase in 1-propanol, isobutanol and amyl alcohols

Table 1 Final volatile compounds concentration (mg/l) produced in tequila fermentation using different agave ages and cultivation fields by two *S. cerevisiae* yeast strains

Volatile compound (mg/l)	CF1				CF2			
	GU3 yeast strain Agave age (years)		AR5 yeast strain Agave age (years)		GU3 yeast strain Agave age (years)		AR5 yeast strain Agave age (years)	
	4	8	4	8	4	8	4	8
Ethyl acetate	39^{a}	39^{a}	0^{b}	5^{b}	34^{a}	16^{a}	5^{b}	23^{b}
Methanol	145^{a}	40^{b}	150^{a}	31^{b}	110^{a}	58^{b}	88^{a}	60^{b}
1-propanol	18^{a}	25^{b}	14^{a}	19^{b}	22^{a}	28^{b}	14^{a}	36^{b}
1-methyl-propanol	28^{ac}	60^{ad}	12^{bc}	24^{bd}	36^{ac}	48^{ad}	14^{bc}	23^{bd}
Amyl alcohols	52^{a}	92^{b}	28^{a}	91^{b}	60^{a}	85^{b}	45^{a}	64^{b}
2-phenylethanol	8^{a}	8^{a}	22^{a}	31^{a}	8^{a}	8^{a}	18^{a}	7^{a}

[a–d] Significantly different in the production of each volatile compound

production at the end of the fermentation with older agaves.

The yeast strain and agave age did not showed statistical differences in the 2-phenyl-ethanol production. In spite of this result, the strain AR5 produced more 2-phenyl-ethanol than GU3 in 75% of the experiments. For the GU3, the production was the same for CF1 and CF2 agaves (Table 1). For the AR5 yeast strain, as agave age increased, the production of 2-phenyl- ethanol increased from 22 to 31 mg/l in fermentations with CF1 agaves and decreased from 18 to 7 mg/l in fermentations with CF2 agaves (Table 1). Contrary to the production patterns of 1-propanol, isobutanol and amyl alcohols, the production of 2-phenyl-ethanol was influenced only by yeast strain. It has been reported that in *Agave tequilana* juice fermentation, the production of 2-phenyl-ethanol was the principal difference in volatile compounds between yeast strains isolated from wine and agave must [3].

Conclusion

Of the variables investigated in this study the agave age proved to be an important factor affecting tequila fermentation. The production of ethanol and volatile compounds was influenced in different patterns by agave age, agave cultivation field and yeast strain. Studies focused on the influence of agricultural practices on agave production must be performed in the future, in order to clarify the roll of factors such us fertilization, ground type and environmental conditions of cultivation of the raw material on tequila fermentation.

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