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RESEARCH PAPER

Physiological characterization of two native yeasts in pure and mixed culture using fermentations of agave juice

Martha E. Nuñez-Guerrero^{1,2}, Elizabeth Salazar-Vázquez², Jesús B. Páez-Lerma², Raúl Rodríguez-Herrera¹, and Nicolás O. Soto-Cruz²

¹Universidad Autónoma de Coahuila, Faculty of Chemical Sciences, Laboratory of Molecular Biology. Blvd. V. Carranza e Ing. José Cardenas s/n, Saltillo Coahuila, México.

²Tecnológico Nacional de México/Instituto Tecnológico de Durango. Felipe Pescador 1830 Ote. 34080, Durango, Dgo., México.

Abstract

M.E. Nuñez-Guerrero, E. Salazar-Vázquez, J.B. Páez-Lerma, R. Rodríguez-Herrera, and N.O. Soto-Cruz. 2019. Physiological characterization of two native yeasts in pure and mixed culture using fermentations of agave juice. Cien. Inv. Agr. 46(1): 1-11. Yeast cells are subjected to diverse environmental conditions during the alcoholic fermentation of agave juice, causing different kinetic behaviors. Agave juice was used as culture medium to evaluate the kinetic behavior of *Saccharomyces cerevisiae* ITD-00185 and *Torulaspora delbrueckii* ITD-00014a, as pure and mixed cultures, under different inoculum sizes (1×10^5 , 1×10^6 , 1×10^7 and 1×10^8 cells mL⁻¹), and combined pH levels (3.5, 4.0 and 4.5) and temperatures (18 °C, 28 °C and 38 °C). *Saccharomyces cerevisiae* displayed high fermentation capacities at all inoculum concentrations assayed. However, *T. delbrueckii* required a high inoculum concentration ($\geq 1 \times 10^7$ cells mL⁻¹) to perform at fermentation levels similar to *S. cerevisiae*. Low temperatures (18 °C) slowed fermentation, while high temperatures (38 °C) adversely affected the development of the yeast strains, especially *T. delbrueckii*. The best temperature was 28 °C in all fermentations. The pH level had a strong effect on the performance of the coculture, since the fermentation kinetics suggested a synergistic effect at pH 4.5, while an antagonistic effect was postulated at pH 3.5. In all of the mixed culture cases, a positive effect at 28 °C, especially at pH 4.0 and 4.5, was demonstrated by greater levels of sugar consumption and ethanol production (~20%, $p < 0.05$) compared to fermentations of the *S. cerevisiae* monoculture. The coculture results allow us to postulate that a complex interaction exists between the two yeasts, which could be synergistic or antagonistic, as the environmental conditions change.

Key-words: Coculture, inoculum concentration, pH, Temperature.

Introduction

Several different alcoholic beverages are derived from agave plants such as pulque, tequila, bacanora and mezcal. However, only tequila, and more recently mezcal, have achieved interna-

tional recognition. Mezcal is a Mexican regional beverage with an origin denomination (Norma Oficial Mexicana: NOM-070.SCFI-1994). Tequila and mezcal are both protected under the North American Free Trade Agreement (NAFTA) and an "Agreement between the European Union and the United Mexican States on the mutual recognition and protection of designations for spirit drinks".

Tequila is obtained by distilling fermented sugar from *Agave tequilana*, whereas mezcal is obtained from *Agave duranguensis* juice as well as other *Agave* species (Soto-García *et al.*, 2009).

Fermentation is a critical stage for the quality and productivity of the mezcal production process. This stage is performed spontaneously during artisan processes, which can cause fermentation to occur over different lengths of time, the presence of undesirable products (e.g., methanol and furfural) and leave high amount of residual, nontransformed sugar. *Saccharomyces* and non-*Saccharomyces* yeasts are responsible for the alcoholic fermentation of agave juice (Escalante-Minakata *et al.*, 2008; Lappe-Oliveras *et al.*, 2008; Narváez-Zapata *et al.*, 2010; Páez-Lerma *et al.*, 2013). Yeast population changes that occur during the alcoholic fermentation of *A. duranguensis* juice for mezcal production were studied by Páez-Lerma *et al.* (2013). They found that the early stage of the fermentation process was conducted by highly diverse yeast populations, which contained species such as *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Candida diversa*, *Pichia fermentans* and *Hanseniaspora uvarum*; however, *S. cerevisiae* was the only yeast found at the end of the fermentation process. This finding is similar to the yeast behavior reported during grape fermentation for wine production (Esteve-Zarzoso *et al.*, 1998; Pretorius *et al.* 2003; Caridi, 2003).

Standardizing the fermentation process could be achieved by using a starter culture (inoculant), formulated with selected yeast strains, which could possibly achieve better utilization of the fermentable sugars (Nuñez-Guerrero *et al.*, 2016) while meeting the requirements of the Mexican Official Norm. Nuñez-Guerrero *et al.* (2016) demonstrated that a yeast mixture composed of 75% *S. cerevisiae* and 25% *T. delbrueckii* produced mezcal with a high yield and richness of volatile compounds, which resulted in better acceptability in sensory tests.

Therefore, the aim of this work was to evaluate the kinetic behavior of the abovementioned inoculant

(75% *S. cerevisiae* and 25% *T. delbrueckii*), as well as the individual yeasts, with reference to pH, temperature and inoculum size, during the alcoholic fermentation of agave juice.

Materials and Methods

Microorganisms

Saccharomyces cerevisiae ITD-00185 and *Torulaspora delbrueckii* ITD-00014a strains were obtained from the yeasts collection of the Microbial Biotechnology Laboratory at the Instituto Tecnológico de Durango. These yeast strains were selected in a previous work (Nuñez-Guerrero *et al.*, 2016) and were activated on Yeast-Peptone-Dextrose (YPD) agar (1% yeast extract, 2% dextrose, 2% casein peptone, 2% agar), incubated at 28 °C for 12 h and recultured in YPD broth (1% yeast extract, 2% dextrose, 2% casein peptone) at 28 °C for 12 h. The cells were stained with methylene blue and counted using a Neubauer chamber.

Agave juice

Agave duranguensis juice with an initial sugar concentration of $120 \pm 5 \text{ g L}^{-1}$ was kindly donated by *Productora Mexicana de Mezcal S. DE R.L.M.I.* Its total nitrogen was measured using the Micro-Kjeldahl method as described previously in De los Ríos-Deras *et al.*, (2015). The agave broth had a nitrogen concentration of 0.49 g N L^{-1} , and ammonium sulfate was added to reach 1.64 g N L^{-1} , which corresponds to a C/N ratio of 73 g C/g N. This C/N ratio has been demonstrated as the best C/N ratio for agave must fermentation during mezcal production (De los Ríos-Deras *et al.*, 2015).

Effect of inoculum concentrations

Fermentation assays were performed using pure cultures of each yeast strain. A mixture culture

(75% *S. cerevisiae* and 25% *T. delbrueckii*) was also used as inoculum as previously assayed in Nuñez-Guerrero *et al.*, (2016). The measurements of the fermentation kinetics that were used to evaluate the inoculum concentration effect were performed in glass tubes (20×150 mm), which were filled with 15 mL of agave juice and inoculated with either 1×10^5 , 1×10^6 , 1×10^7 and 1×10^8 cells mL⁻¹ of agave culture in triplicate.

Combined effect pH-temperature

A 3² factorial design was performed to evaluate the effects of pH (3.5, 4.0 and 4.5) and temperature (18 °C, 28 °C and 38 °C). *S. cerevisiae* ITD-00185, *T. delbrueckii* ITD-00014a and the mixed culture (75% *S. cerevisiae* and 25% *T. delbrueckii*) were each assayed. Fermentation kinetics were performed in triplicate using agave juice at an initial sugar concentration of 120 g L⁻¹ and a C/N ratio of 73 g C/g N as the substrate. Fermentations were started using an inoculum of 10⁷ cells mL⁻¹ and incubated at 28 °C for 72 h. Samples were analyzed every 8 h by high-performance liquid chromatography as previously described by De los Ríos-Deras *et al.*, (2015) to determine the glucose, fructose and ethanol concentrations.

Statistical analysis

The one-way ANOVA module of Statistica 7.0 (StatSoft Inc, USA) was used to check for significant differences among treatments using Fisher's test for least significant differences (LSD) among the means.

Results and Discussion

Nuñez-Guerrero *et al.* (2016) reported that a mixture of yeasts composed of 75% *S. cerevisiae* and 25% *T. delbrueckii* could potentially be used for the development of an inoculant for the production of mezcal and other related spirits. Nevertheless, it is

necessary to establish the effect of the inoculum size and to perform some physiological studies before using the inoculant. This is because yeast cells can face environmental conditions during the alcoholic fermentation stage. Hohman and Mayer (2003) noted that yeasts typically face diverse conditions that primarily affect their cellular structures and different macromolecules, especially lipids, proteins and nucleic acids. Two of the most significant factors that may affect the yeasts' performance are the pH and the temperature (Viegas *et al.*, 1989; Mauricio and Salmon, 1992; Alexandre *et al.*, 1994a,b; Pampulha and Loureiro-Dias, 1990).

Initial inoculum concentration

Several fermentation kinetics were performed to determine the effects of the inoculum concentration (Fig. 1) to define the initial concentration of yeast cells that are required for the inoculant to promote efficient fermentation development. These experiments were performed for the mixed culture inoculant and for the separate yeast strains.

Figure 1A (1×10^5 cells mL⁻¹) shows that *S. cerevisiae* displayed high fermentation levels at this inoculum concentration, consuming almost all of the sugars and producing 65 g ethanol L⁻¹ by the end of the incubation time (72 h). A similar kinetic behavior was produced by the mixed yeast culture, reaching similar sugar consumption and ethanol production levels but in a slightly less time (66 h). Nevertheless, *T. delbrueckii* showed very a limited fermentation activity at this inoculum concentration, with only minor sugar consumption (~23 g L⁻¹) and limited ethanol production (~10 g L⁻¹). Similar findings are shown in Figure 1B (1×10^6 cells mL⁻¹). *S. cerevisiae* and the mixed culture both performed very well and completely consuming the carbohydrates completely after 50–60 h of incubation; however, *T. delbrueckii* again showed a very limited fermentation capacity. The mixed culture showed slightly better kinetic

behavior than the fermentation resulting from the inoculation of solely *S. cerevisiae*, which is a consistent finding for both inoculum concentrations (1×10^5 and 1×10^6 cells mL^{-1}). This finding suggests a mechanism of collaboration or synergism between *S. cerevisiae* ITD-00185 and *T. delbrueckii* ITD-00014a at the proportions used in this work, as previously reported by our research group (Nuñez-Guerrero *et al.*, 2016). In contrast, the poor performance of *T. delbrueckii* at these inoculum concentrations can be explained by chemical inhibition due to several compounds that are produced by the native microflora or low tolerance to osmotic stress, which have been suggested previously (Nishino *et al.*, 1985; Hohmann, 2002). Another cause may be the inhibition of non-*Saccharomyces* yeasts by the native microflora of the agave juice or by cell-to-cell contact with *S. cerevisiae*, which has also been suggested previously (Nissen and Arneborg,

2003). Nevertheless, Taillandier *et al.* (2014) discarded cell-to-cell contact as well as substrate competition as the mechanisms inhibiting *T. delbrueckii*, instead suggesting inhibition by an *S. cerevisiae*-produced metabolite as the likely cause of these interactions.

Figure 1C shows the small difference between the kinetic performances at this inoculum concentration (1×10^7 cells mL^{-1}) between the mixed culture and solely *S. cerevisiae*, with the mixed culture showing the best ethanol production. This figure also shows an improved kinetic behavior for *T. delbrueckii* in comparison to the previous inoculum sizes because it was able to consume almost all of the available sugars by the end of incubation period. Figure 1D shows virtually no differences between the kinetic performances of *S. cerevisiae* and *T. delbrueckii* inoculated separately or in a mixed culture at an inoculum concentration of 1×10^8 cells mL^{-1} .

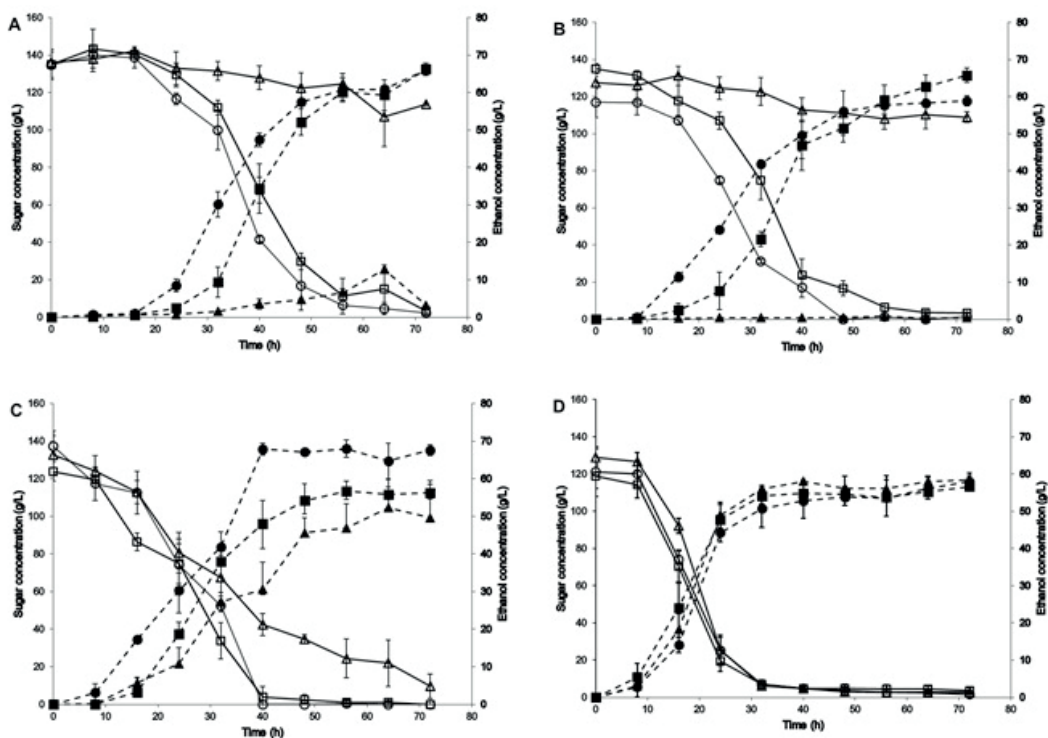


Figure 1. Fermentation kinetics in agave juice at different initial inoculum concentrations (cells mL^{-1}). A) 1×10^5 ; B) 1×10^6 ; C) 1×10^7 ; D) 1×10^8 . Sugar consumption by *S. cerevisiae* (\square), *T. delbrueckii* (\triangle) and mixed culture (\circ). Ethanol production by *S. cerevisiae* (\blacksquare), *T. delbrueckii* (\blacktriangle) and mixed culture (\bullet).

pH and temperature assays

Agave juice has a pH of approximately 4.0 and 28 °C is the average temperature in the agave producing regions during artisan mezcal production. The results obtained regarding the effects of pH and temperature using agave juice as the culture medium at an initial sugar concentration of 120 g L⁻¹ are shown in Figures 2-4. Low temperatures (18 °C) slowed fermentation because *S. cerevisiae* required 40 h to reach the stationary phase for the kinetic studies performed at pH 4.0 and 4.5, while the fermentation at pH 3.5 required 60 h to reach the stationary phase (Fig. 2A). The ethanol production at this temperature (Fig. 2B) was approximately 31 g L⁻¹ for every pH level tested. *S. cerevisiae* showed the best results at 28 °C

(Fig. 2C and 2D), where it consumed sugars and produced ethanol in superior quantities at all of the pH levels tested. In contrast, the fermentation kinetics at 38 °C (Fig. 2E and 2F) performed poorly, particularly at pH 3.5. The sugar consumption reached approximately 65 g L⁻¹ at pH 4.0 and 4.5 and only 45 g L⁻¹ at pH 3.5. Furthermore, the ethanol production at 38 °C was nearly 25 g L⁻¹ at pH 4.0 and 4.5 and 20 g L⁻¹ at pH 3.5. These data confirm that *S. cerevisiae* performed best at 28 °C (Table 1), particularly regarding its productivity, which was approximately 1 g of ethanol per L and per h. The fermentation results for *T. delbrueckii* are shown in Figure 3. This yeast species developed poorly under the tested growth conditions compared to *S. cerevisiae*. *Torulaspora delbrueckii* had a very slow fermentation at 18

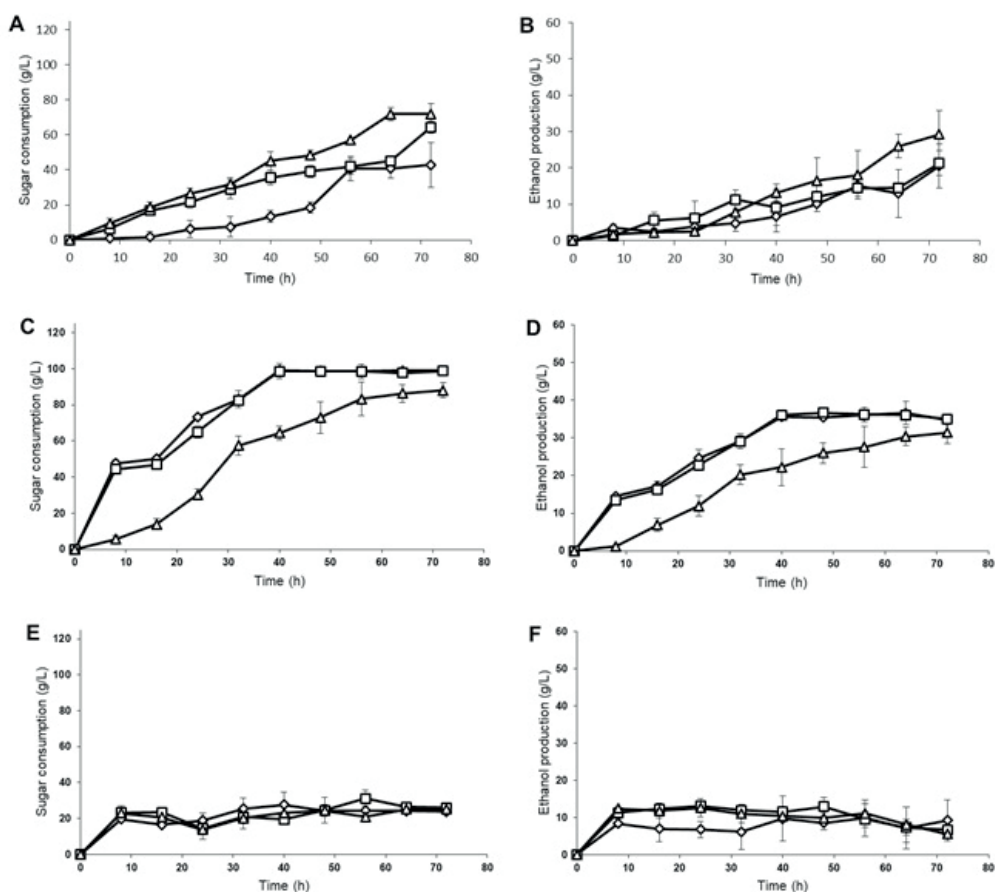


Figure 2. Fermentation kinetics of *Saccharomyces cerevisiae* ITD-00185 in agave juice at different temperatures and pH values. Sugar consumption (A, C, E) and ethanol production (B, D, F). Incubation temperature of 18 °C (A, B), 28 °C (C, D) and 38 °C (E, F). pH values of 3.5 (◇), 4.0 (□) and 4.5 (△).

°C, particularly at pH 3.5 (Fig. 3A and 3B). Its sugar consumption of approximately 70 g L⁻¹ was observed after 72 h incubation period at pH 4.0 and 4.5; however, its consumption was 43 g L⁻¹ at pH 3.5. *T. delbrueckii*'s ethanol production ranged from 21 (pH 3.5) to 29 g L⁻¹ (pH 4.5). Fermentation at 28 °C (Fig. 3C and 3D) was faster than at 18 °C in all cases. Comparison of the different fermentation levels at 28 °C showed that the *T. delbrueckii* cultures incubated at pH 3.5 and 4.0 showed similar behaviors; however, the slowest fermentation occurred at pH 4.5, in contrast to the behavior observed at 18 °C, where the slowest recorded fermentation occurred at pH 3.5. Importantly, at 28 °C, *T. delbrueckii* consumed

sugars and produced ethanol in amounts similar to *S. cerevisiae*; however, *T. delbrueckii* took 14 h longer to reach these levels. *T. delbrueckii* was practically unable to ferment sugars at 38 °C (Fig. 3E and 3F), independent of the pH level present. In all cases, its sugar consumption and ethanol production (approximately 20 g L⁻¹ and 10 g L⁻¹, respectively) were observed during the first 8 h. After this time, the fermentation process did not continue. Table 1 shows that, similar to *S. cerevisiae*, *T. delbrueckii* showed its best results at 28 °C.

The fermentation kinetics using the mixed culture are shown in Figure 4. It is important to note that

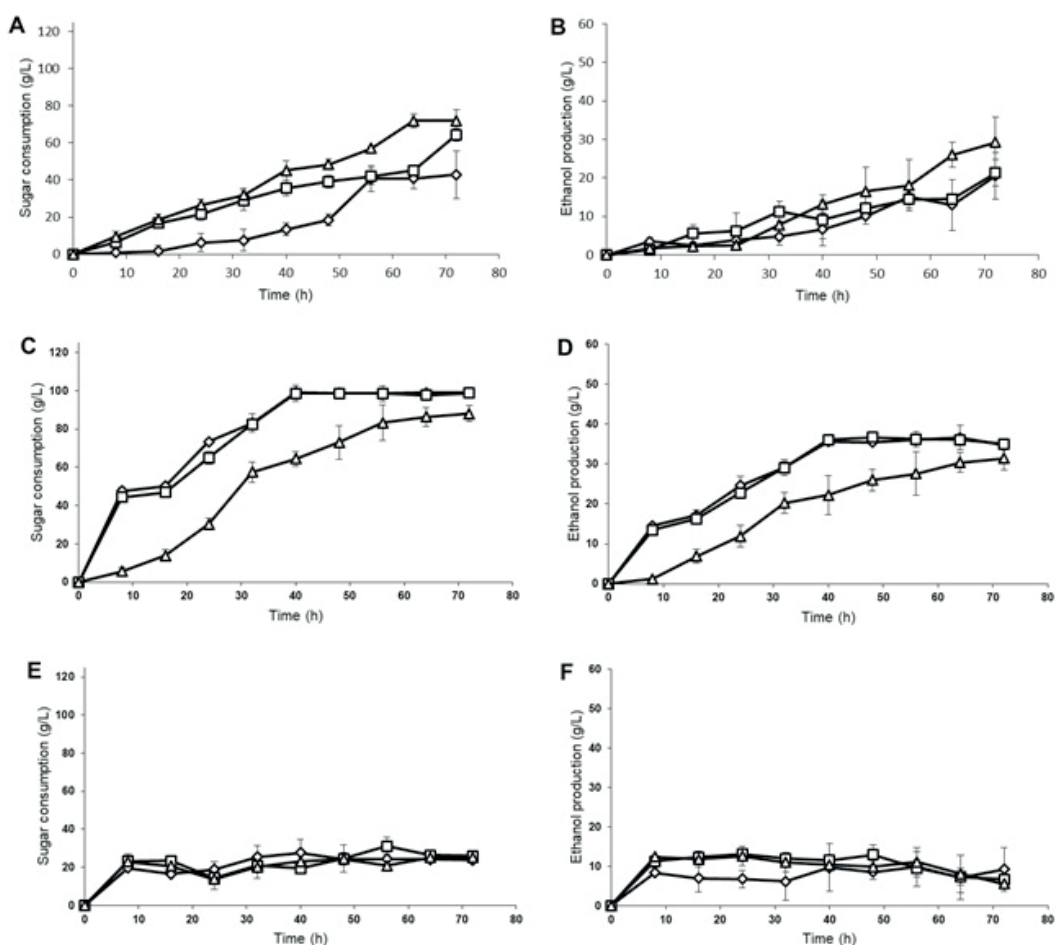


Figure 3. Fermentation kinetics of *Torulaspora delbrueckii* ITD-00014a in *Agave duranguensis* juice at different temperatures and pH values. Sugar consumption (A, C, E) and ethanol production (B, D, F). Incubation temperature of 18 °C (A, B), 28 °C (C, D) and 38 °C (E, F). pH values of 3.5 (◇), 4.0 (□) and 4.5 (△).

Table 1. Temperature and pH effect on the yield and productivity of ethanol using different inocula.

Temperature	Temperature	pH	$Y_{p/S}$ (g EtOH [g Sugar] ⁻¹)	Productivity (g EtOH L ⁻¹ h ⁻¹)
Saccharomyces cerevisiae	18°C	3.5	0.419±0.028	0.579±0.060
		4.0	0.330±0.009	0.701±0.076
		4.5	0.362±0.011	0.825±0.070
	28°C	3.5	0.387±0.010	0.958±0.072
		4.0	0.407±0.012	0.984±0.083
		4.5	0.404±0.016	1.005±0.040
	38°C	3.5	0.450±0.034	0.517±0.068
		4.0	0.390±0.009	0.651±0.080
		4.5	0.357±0.002	0.627±0.095
Torulaspora delbrueckii	18°C	3.5	0.429±0.104	0.167±0.042
		4.0	0.327±0.015	0.227±0.028
		4.5	0.357±0.038	0.329±0.043
	28°C	3.5	0.362±0.009	0.892±0.109
		4.0	0.366±0.009	0.903±0.120
		4.5	0.349±0.012	0.555±0.043
	38°C	3.5	0.360±0.047	0.243±0.033
		4.0	0.342±0.123	0.289±0.078
		4.5	0.371±0.131	0.259±0.061
Mixed culture (75% <i>S. cerevisiae</i> , 25% <i>T. delbrueckii</i>)	18°C	3.5	0.375±0.048	0.257±0.175
		4.0	0.364±0.030	0.805±0.119
		4.5	0.362±0.005	1.065±0.110
	28°C	3.5	0.465±0.008	1.249±0.154
		4.0	0.447±0.005	1.297±0.152
		4.5	0.433±0.009	1.320±0.152
	38°C	3.5	0.444±0.027	0.629±0.092
		4.0	0.481±0.041	0.542±0.078
		4.5	0.353±0.013	0.689±0.081

the combined concentration in the mixed culture was 1×10^7 cells mL⁻¹, which was equal to the fermentations performed in monoculture. Figures 4A and 4B show that, at 18 °C, the pH had a strong effect on the performance of the coculture of *S. cerevisiae* and *T. delbrueckii*. At pH 4.5, the fermentation profile (sugar consumption and ethanol production) was better than the profile observed for the *S. cerevisiae* monoculture, which suggests a synergistic effect between the two yeast species. Nevertheless, the fermentation profile at pH 3.5 was seriously affected, which may be attributed to an antagonistic effect. In all of the mixed culture cases tested, the most significant positive effect recorded was at 28 °C, especially at pH 4.0 and 4.5. This finding was demonstrated by higher

levels of sugar consumption and ethanol production (~20%, $p < 0.05$) compared to the production levels of the *S. cerevisiae* monoculture. Similarly, Lu *et al.* (2017) reported that the pH had a more significant effect on ethanol production than did temperature during alcoholic fermentation for wine production. Figures 4E and 4F show that 38 °C is an adverse temperature for the development of the tested yeasts. Table 1 shows that in the mixed cultures, a very positive effect was observed at 18 °C and pH 4.5 because the productivity increased by approximately 30% ($p < 0.05$) compared with the fermentation of the *S. cerevisiae* monoculture under the same conditions. A positive effect was observed at pH 4.0, showing a 15% ($p < 0.05$) increase in productivity, whereas the effect at pH

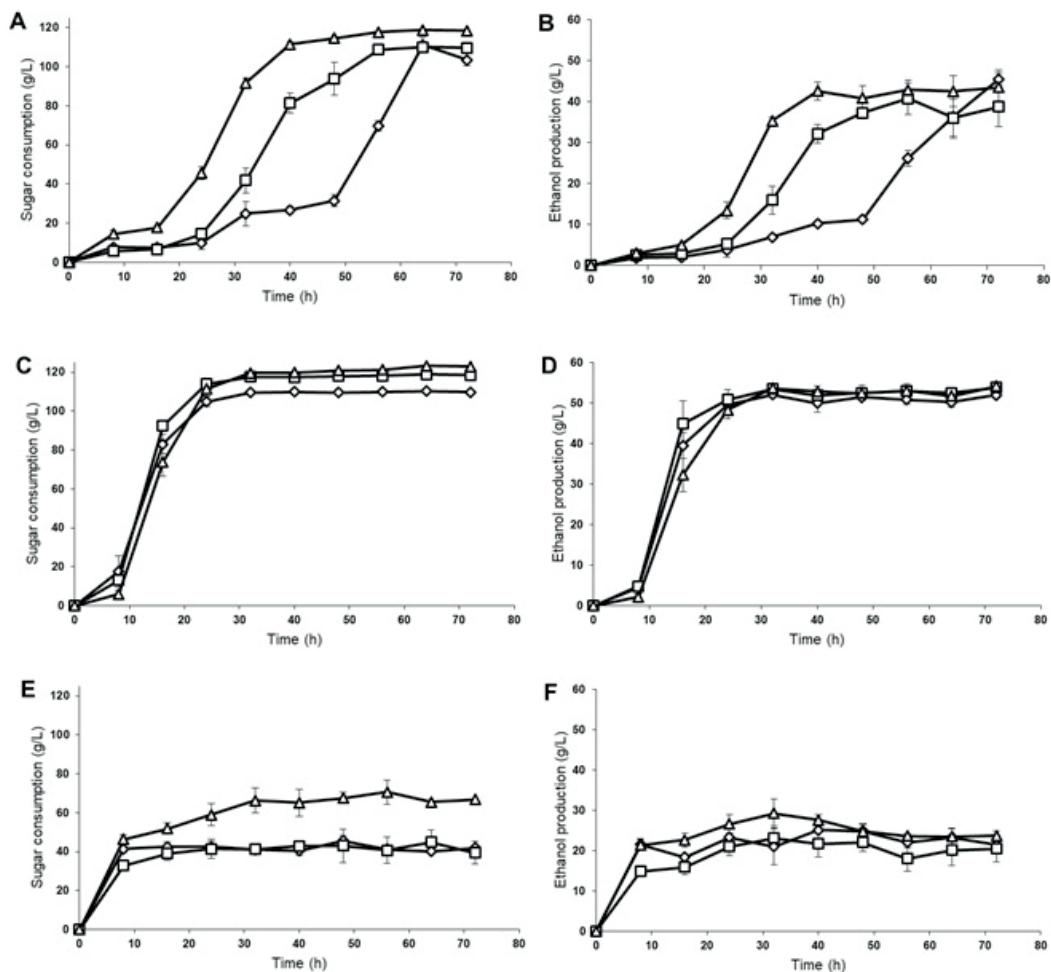


Figure 4. Fermentation kinetics of mixed cultures of *Saccharomyces cerevisiae* ITD-00185 and *Torulaspora delbrueckii* ITD-00014a in *Agave duranguensis* juice at different temperatures and pH values. Sugar consumption (A, C, E) and ethanol production (B, D, F). Incubation temperature of 18 °C (A, B), 28 °C (C, D) and 38 °C (E, F). pH values of 3.5 (◇), 4.0 (□) and 4.5 (△).

3.5 was strongly negative, with a productivity decrease of 56% ($p < 0.05$). The positive effect of the coculture was very evident at 28 °C because the productivity increased an average of 31% ($p < 0.05$) in all three cases; whereas at 38 °C, pH 3.5 and 4.5 were not substantially affected in comparison with the *S. cerevisiae* monoculture. A negative effect on the productivity of the coculture was observed at 38 °C and pH 4.0 (-17%, $p < 0.05$).

Ciani *et al.* (2016) noted the complexity of the interaction between non-*Saccharomyces* and *Saccharomyces* yeast strains, which was influenced

by many abiotic (temperature, pH, nutrients) and biotic (cell-to-cell contact, proteinaceous antimicrobial compounds) factors. They also noted that the physiological and biochemical basis for these interactions were still unclear, though they suggested a multifactorial approach using omics methodologies to elucidate the yeast species interactions. Our research group previously postulated a synergistic interaction between *S. cerevisiae* ITD-00185 and *T. delbrueckii* ITD-00014a (Nuñez-Guerrero *et al.*, 2016). Nevertheless, the results presented in this work suggest that a more complex interaction occurs between the two yeast species because both positive (syn-

ergistic) and negative (antagonistic) interactions were recorded depending on the environmental conditions.

The main conclusions are the following. The inoculum concentration is an important parameter for the fermentation performance of the tested yeast species. *Saccharomyces cerevisiae* ITD-00185 showed high performance levels at all of the inoculum concentrations assayed; however, *T. delbrueckii* ITD-00014a required a high inoculum concentration ($\geq 1 \times 10^7$ cells mL⁻¹) to perform at fermentation levels similar to *S. cerevisiae*. Low temperatures (18 °C) slowed

fermentation; however, the highest temperature (38 °C) was adverse for the development of these yeast species, especially for *T. delbrueckii*. The best temperature for the yeast strains was 28 °C at which they underwent vigorous fermentation. Finally, we conclude that the coculture of the two yeast species produced results that are not simply the sum of each separately cultured yeast species' performance. Therefore, we postulate that a complex interaction exists between the two yeast species and that this interaction may be synergistic or antagonistic as the environmental conditions change. These interactions will be further elucidated in future experiments.

Resumen

M.E. Nuñez-Guerrero, E. Salazar-Vázquez, J.B. Páez-Lerma, R. Rodríguez-Herrera, y N.O. Soto-Cruz. 2019. Caracterización fisiológica de dos levaduras nativas en cultivo puro y mixto usando fermentaciones de jugo de agave. Cien. Inv. Agr. 46(1): 1-11. Las levaduras se someten a diversas condiciones ambientales durante la fermentación alcohólica de jugo de agave, causando diferentes comportamientos cinéticos. Se utilizó jugo de agave como medio de cultivo para evaluar el comportamiento cinético de *Saccharomyces cerevisiae* ITD-00185 y *Torulaspora delbrueckii* ITD-00014a, en cultivos puros y mezclados, en diferentes condiciones de tamaño de inóculo (1×10^5 , 1×10^6 , 1×10^7 y 1×10^8 células mL⁻¹) y combinaciones de pH (3.5, 4.0 y 4.5) y temperatura (18 °C, 28 °C y 38 °C). *Saccharomyces cerevisiae* mostró alta capacidad fermentativa en todas las concentraciones de inóculo. *Torulaspora delbrueckii* requirió alta concentración de inóculo ($\geq 1 \times 10^7$ células mL⁻¹) para fermentar como *S. cerevisiae*. A 18 °C se retardó la fermentación, mientras que a 38 °C se afectó negativamente al desarrollo de las levaduras, especialmente *T. delbrueckii*. La fermentación fue vigorosa para ambas levaduras a 28 °C. El pH tuvo un fuerte efecto sobre el rendimiento del co-cultivo, sugiriendo un efecto sinérgico a pH 4.5 y efecto antagónico a pH 3.5. Para cultivo mixto se observó un efecto positivo a 28 °C, especialmente a pH 4.0 y 4.5, demostrando mejor consumo de azúcar y producción de etanol (~ 20%, $p < 0,05$) comparado con el monocultivo de *S. cerevisiae*. Los resultados globales permiten postular una interacción compleja entre las dos levaduras, que puede ser sinérgica o antagónica, según las condiciones ambientales.

Palabras clave: Co-cultivo, concentración de inóculo, pH, temperatura.

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