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Evaluation of autochthonous *Saccharomyces bayanus* strains under stress conditions for making ice ciders

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ABSTRACT

The product diversification policy being carried out by the Asturian cider industry includes the development of beverages similar to so-called ice ciders. During apple juice fermentations, yeast cells are affected by several stress conditions. In this study, 74 *Saccharomyces bayanus* strains isolated from Asturian cider, have been analyzed in synthetic media for their growth ability, sugar fermentation capacity and acetic acid production under the stress conditions that occurr during the production of ice cider. According to the data obtained 23 strains high proliferation capabilities, sugar fermentation capacity and low acetic acid production have been differentiated. Ten strains were chosen to produce ice ciders from apple juice (31.8 ºBrix) at 12 °C. The products obtained were characterized by not having developed the malolactic conversion and their low contents of acetic acid, methanol and ethyl acetate. Significant differences among ciders were detected for alcoholic degree, total acidity, and contents of glycerol, pyruvic, fumaric and shikimic acids due to the yeast strain used. A Multiple Correspondence Analysis showed four strains associated with sensory quality variables. Our results open up the possibility of using autochthonous *S. bayanus* strains as starters in the making of Asturian ice ciders.

1. Introduction

Ice cider is obtained by the fermentation of apple juice with has high sugar content. The Quebec legislation provides that the sugar content of juice must be higher than 30 ºBrix and the finished product must have a minimum residual sugar content of 130 g L^{-1}, and an alcoholic degree between 7 and 13%. There are two methods to obtain a high-sugar content juice. The most commonly used is called cryo-concentration in which the fruit is pressed and the resulting juice is frozen in containers. The second technique, known as cryo-extraction consists of freezing picked apples and pressing until the juice is squeezed from them (Kirkey & Braden, 2014). The juice is then inoculated with yeasts, and fermentation is carried out at 15–17 °C for some months; the alcoholic fermentation typically ends while there is a still considerable content of residual sugars present (Nurgel, Pickering, & Inglis, 2004).

During the production of ice cider, yeasts are subjected to large hyperosmotic stress due to the high solutes concentrations. In wines, this stress has been associated with cell shrinkage, reduced peak cell concentration, yeast biomass accumulation throughout fermentation, and the production of high levels of glycerol and acetic acid in the final product (Pigeau & Inglis, 2005). In addition, as fermentation progresses the ethanol concentration increases and the cells are exposed to an increasingly toxic level of ethanol the effects of which on the physiology of yeast include growth inhibition, reduced cell size and viability, and increased membrane permeability (Gibson, Lawrence, Leclaire, Powell, & Smart, 2007). As a consequence of these stress conditions fermentations are often sluggish, taking months to reach the desired ethanol level, also resulting in high levels of volatile acidity (Kontkanen, Inglis, Pickering, & Reynolds, 2004).

The development of special sweet ciders similar to those recognized as ice ciders is part of the present policy for diversification of the Asturian cider making sector. In this sense, the use of

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selected local yeast strains as starters for the production of this kind of cider should ensure the capacity to detect and respond to the above mentioned stress conditions without significant viability losses, giving ciders their typical sensory characteristics (Bauer & Pretorius, 2000; Querol, Barrio, Huerta, & Ramón, 1992).

Saccharomyces bayanus has been found among the yeasts present in fermenting cider habitats (Pando, Querol, & Suárez, 2010; Suárez, Pando, Fernández, Querol, & Madrera, 2007a). Several studies have reported that *S. bayanus* is better suited to grow at lower temperatures than *S. cerevisiae* (López-Malo, Querol, & Guillamón, 2013; Belloch, Orlic, Barrio, & Querol, 2008; Kishimoto & Goto, 1995). *S. bayanus* and *S. kudriavzevii* are considered the most psychrotrophic species of the *Saccharomyces* genus (Belloch et al., 2008; Salvadó et al., 2011). Moreover, this cryotolerant species produces higher amounts of glycerol and less acetic acid than *S. cerevisiae*, this ability being strain-dependent (Bellon, Yang, Day, Inglis, & Chambers, 2015; Castellari et al., 1994; Masneuf-Pomarède, Bely, Marullo, Lonvaud-Funel, & Dubourdieu, 2010). These characteristics make *S. bayanus* strains suitable for the making of ice ciders.

The present study proposes a selection procedure of *S. bayanus* strains for the production of Asturian naturally sweet ciders. For this purpose, a screening study of seventy four strains was done based on their growth ability, sugar fermentation and acetic acid production under stress conditions. Subsequently, ten yeast strains were chosen for the production of ice cider and characterized on the basis of the chemical composition and sensory quality of the resulting ciders.

2. Material and methods

2.1. Yeast strains and inoculum preparation

Seventy four *S. bayanus* strains belonging to the SERIDA culture collection (CCAS) were used in this study (Table 1). They have been isolated from Asturian cellars, identified by RFLP analysis of the 5.8S-ITS ribosomal region and characterized by mtDNA restriction analysis (Pando et al., 2010; Suárez, Pando, González, & Querol, 2007b).

The cells, stored at −80 °C, were revived by streaking onto GPY agar (20 g L^{-1} glucose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 20 g L^{-1} agar) and incubated at 30 °C for 48 h. One colony was used to inoculate 6 mL of GPY broth and incubated overnight at 30 °C on a shaker at 300 rpm. Next, the cultures were adjusted to an OD_{660} of 0.5 absorbance in a nanodrop 1000 (Thermo Scientific) by dilution with fresh GPY broth.

2.2. Yeast selection under stressful conditions

2.2.1. Synthetic media

Two synthetic media, MSH (102.37 g L^{-1} sucrose, 63.54 g L^{-1} glucose, 186.04 g L^{-1} fructose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract) and SSH (30 g L^{-1} glucose, 105 g L^{-1} fructose, 5 g L^{-1} peptone, 5 g L^{-1} yeast extract, 100 mlL^{-1} ethanol) were formulated to respectively represent a 32 ºBrix-apple juice and a cider with 9 ºBrix and 10% v/v ethanol. Both media were adjusted at pH 6.2 and solidified with agar (20 g L^{-1}).

Chalk Agar (CA) medium described by Lemaresquier et al. (1995) was modified to contain a concentration of sugars of 32 ºBrix as follows: 102.37 g L^{-1} sucrose, 63.54 g L^{-1} glucose, 186.04 g L^{-1} fructose, 3 g L^{-1} yeast extract, 3 g L^{-1} calcium carbonate, 15 g L^{-1} agar, (pH = 7.2).

Table 1
List of yeast strains used in this study.

Strain (CCAS number)	Cellar	City	Source
SB1, SB2, SB7	A	Villaviciosa	Fermenting must
C6, SB5	B	Siero	Fermenting must
SB3, SB4	C	Gijón	Fermenting must
SB8 to SB28	D	Villaviciosa	Fermenting must
SB29 to SB45, SB47 to SB49, SB51 to SB54, SB58 to SB66, SB70, SB73	E	Villaviciosa	Fermenting must
SB46, SB50, SB67, SB68, SB71, SB72	E	Villaviciosa	Apple juice
SB55 to SB57, SB69, SB74	E	Villaviciosa	Cider

CCAS: SERIDA culture collection.

2.2.2. Growth abilities

The inocula were 10-fold sequentially diluted in Ringer solution and 5 μL aliquots were spotted in a row onto MSH agar and SSH agar. A positive growth control, in which cells were not exposed to stress conditions was carried out on GPY agar at 30 °C. Growth was measured by comparing the number of colonies that appeared in different dilutions.

2.2.3. Sugar fermentation

The strains (200 μL) were inoculated in 10 mL of MSH and SSH broth with Durham tubes. Fermentation was observed by the presence of a gas bubble trapped inside the Durham tubes. Yeasts were classified according to the following scale: no bubble present, 0; presence of a small bubble, 1; bubble filled ¼ of Durham tube, 2; bubble filled ½ of the Durham tube, 3; bubble filled ¾ of the Durham tube, 4; bubble filled the Durham tube, 5.

2.2.4. Acetic acid production

This characteristic was tested by incubating 5 μL inoculum on modified-CA. The capacity of yeast to produce acetic acid was observed by the formation of a surrounding transparent halo (Suárez, Pando, Lastra, & Mangas, 2008). The yeasts were classified according to the following scale: halo <1 mm, 0 (no production); halo between 1 and 3 mm, 1 (low production); halo between 3 and 5 mm, 2 (medium production); halo >5 mm, 3 (high production).

All tests were carried out in duplicate at 12 °C over a period of 12 days and data were collected every three days.

2.2.5. Data analysis

Two cluster analyses were performed by using the V-PARVUS 2007 statistical package (Forina, Lanteri, Armanino, Casolino, & Casale, 2007). The first analysis was performed to evaluate the growth capacity of the 74 *S. bayanus* strains, by taking into account the highest dilutions at which colonies developed on MSH and SSH media at 6 and 9 days. A 74 × 74 similarity matrix, consisting of Ward's distances in which each observation was represented by a 4-dimensional vector, was used for hierarchical cluster analysis based on Ward's method (Murtagh & Legendre, 2014). The second analysis was performed to evaluate the ability of 48 pre-selected yeast strains to ferment sugars and to produce acetic acid, by taking into account the data obtained from the MSH and SSH media at 6, 9 and 12 days for sugar fermentation, and at 3, 6, 9 and 12 days for acetic acid production. A 48 × 48 similarity matrix, consisting of the Euclidean distances in which each observation was represented by a 10-dimensional vector, was used for the hierarchical cluster analysis based on the average linkage method.

2.3. Evaluation of yeasts for ice cider production

2.3.1. Fermentations

A natural apple juice (31.8 ºBrix) was obtained by pressing frozen whole apples (−8 °C) in the "El Gaitero" cellar (Villaviciosa, Asturias, Spain). The raw material consisted of a mixture of apple varieties belonging to the Protected Designation of Origin "Cider from Asturias".