Screening of cider yeasts for sparkling cider production (Champenoise method)

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ABSTRACT

A total of 350 colonies isolated from a cider cellar in Asturias (Spain) were identified by rDNA ITS-RFLP restriction analysis. *Saccharomyces* spp. strains were characterized by mitochondrial DNA (mtDNA) restriction analysis. Fifty-four different *Saccharomyces* spp. strains were identified and tested to ascertain their capacity to carry out secondary fermentation of sparkling ciders. The screening of yeasts to determine their principal enological characteristics (tolerance to ethanol, production of volatile acidity and hydrogen sulphide) was accomplished by means of rapid, non-expensive assays (plate agar). As a result, 13 (24%) of the 54 initial *Saccharomyces* spp. yeast strains were eliminated. The technological properties assessed were flocculation capacity, ethanol and sulphite tolerance, and production of major volatiles. Ten *Saccharomyces cerevisiae* strains were characterized as true flocculants; all of these strains were able to grow in ethanolic medium and in the presence of 200 mg/l of sulphite. Applying cluster analysis to the production of amyl alcohols, isobutanol, propanol and 2-phenylethanol, the strains were classified in two natural groups. Two flocculent yeast strains referred to as 3′ and 50′, representative of the each statistical group, were selected together with two reference strains (*Saccharomyces bayanus* C6 and *S. cerevisiae* Levuline CHP) to elaborate four sparkling ciders by the Champenoise method. The analysis of variance ($p < 0.01$) among ciders revealed that glycerol, acetaldehyde, ethyl acetate, methanol, propanol, *i*-butanol and 2-phenylethanol were significantly influenced by the secondary yeast strain. The results of sensory analysis indicated that all the sparkling ciders were scored as good. No significant differences among sparkling ciders were found for odour attributes and taste intensity.

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1. Introduction

Sparkling ciders made by the Champenoise method, which is characterized by a secondary fermentation in bottle, is protected by the Spanish Designation of Origin "Sidra de Asturias". To perform the secondary fermentation, a base cider is mixed with a combination of yeast cultures, sucrose and bentonite. Fermentation and maturation on lees occur in the same bottles that will subsequently be purchased by the consumer. Autolysis of yeasts takes place during aging and several compounds that may substantially modify the organoleptic and foaming properties of the cider are released (Suárez et al., 2005; Picinelli et al., 2005; Rodríguez et al., 2007).

The use of isolated strains of *Saccharomyces cerevisiae* is an interesting strategy for maintaining the quality and reproducibility of fermented beverages. The use of selected local yeasts is believed to be much more effective, since these yeasts are presumed to be more competitive as a result of being better acclimated to environmental conditions. Moreover, the selection of suitable local yeasts assures the maintenance of the typical sensory properties of the fermented products produced in any given region (Degré, 1993; Melero, 1992; Querol et al., 1992a). In addition to the selection criteria generally applied to cider starter yeasts (Cabranes et al., 1997, 1998), the yeast strains chosen to carry out secondary fermentation must satisfy a series of additional characteristics. They must be able to ferment in reducing conditions (bottle), in the presence of ethanol, at low temperature and under high CO_2 pressure, possess a high flocculation capacity and not adhere to glass in order to facilitate their removal from cider when the cider-making process is completed (Bidan et al., 1986; Martínez-Rodríguez et al., 2001).

The present study proposes a selection procedure of *Saccharomyces* spp. strains for the production of sparkling ciders under industrial conditions (Champenoise method). Yeast strains isolated from a cider cellar in Asturias (Spain) were identified by rDNA ITS-RFLP restriction analysis. *Saccharomyces* spp. strains were then characterized by mitochondrial DNA (mtDNA) restriction analysis, 54 different *Saccharomyces* spp. strains were identified. The screening of yeasts was accomplished by rapid and non-expansive assays (plate agar) to determine their tolerance to alcohol, production of volatile acidity and hydrogen sulphide. Concurrently, a study of the flocculation capacity of

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pre-selected yeasts and their flocculation phenotypes was performed. Technological characteristics of secondary fermentation yeast strains were also tested. Finally, four sparkling ciders were made from the same base cider using two of the flocculent strains selected by applying the proposed methodology and two reference yeast strains.

2. Material and methods

2.1. Yeast strains

The indigenous yeast strains used in this study were isolated from a single cider cellar in "El Gaitero S.A." (Asturias) at different fermentation stages. A total of 350 yeast colonies were isolated and analyzed.

2.2. rDNA and mtDNA restriction analysis

Yeast colonies were identified by amplification and restriction of rRNA gene region (Esteve-Zarzoso et al., 1999) using the primers ITS1 and ITS4 described by White et al. (1990). Amplification, restriction and electrophoresis conditions as well as the comparison of the DNA fragments against reference strains and the database compiled by Esteve-Zarzoso et al. (1999) and Fernández-Espinar et al. (2000) have been described elsewhere (Suárez et al., 2007a).

Saccharomyces spp. strains were differentiated by mitochondrial DNA (mtDNA) restriction analysis. DNA was extracted and the mtDNA restriction patterns of the strains determined following the method by Querol et al. (1992b), using *Hinf I* (Boehringer Mannhein, Germany) as the restriction endonuclease.

2.3. Yeast reference standards

The strains *S. cerevisiae* L2056 and *Pichia fermentans* (ref: CECT 11773) were kindly supplied by Dr. Rodríguez-Vico (Department of Biochemistry and Molecular Biology, University of Almeria) to be used as control strains in the sulphite tolerance assays.

Two yeasts were used as reference standards in the making of sparkling ciders: a commercial wine yeast *S. cerevisiae* LEVULINE CHP (Oenofrance, France) and the indigenous cider yeast *Saccharomyces bayanus* C6 from our Research Centre collection (C6).

2.4. Pre-selection of yeast strains

2.4.1. High-ethanol growth
This capacity was observed after incubation of the strains for 48 h at 30 °C on "ESA" medium (0.5% yeast extract, 0.5% peptone, 2% glucose, 12% (v/v) ethanol, 0.015% sodium metabisulphite, 1.5% agar).

2.4.2. Acetic acid production
This characteristic was tested by incubating the strains for 72 h at 30 °C on a "Chalk Agar" medium (0.3% yeast extract, 1% glucose, 0.3% calcium carbonate, 1.5% agar) according to the method described by Lemaresquier et al. (1995). The capacity of yeasts to produce acetic acid is observed by the formation of a surrounding transparent halo. 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).

2.4.3. Hydrogen sulphide production
The commercial medium "BiGGY" agar (Becton Dickinson, France) was used to determine the ability of yeast strains to produce H_2S. Colonies turn black or brown after 24 h at 30 °C on this medium or remain white, depending on hydrogen sulphide production. The following scale was established following this procedure: white, 0 (no production); light brown, 1 (low production); dark brown, 2 (medium production); black, 3 (high production).

2.5. Technological characterization

2.5.1. Visual flocculation and adherence to glass
The yeast strains were inoculated in 10 ml of apple juice, sterilized with 0.1% (v/v) dimethyldicarbonate (Merck) and incubated at 25 °C for 72 h. After incubation, the tubes were stirred (10 s) and the flocculation capacity was observed visually. The strains were classified as flocculent yeasts (FY) when they formed cellular aggregation in static culture and formed clumps again after being dispersed by shaking, whereas they were classified as non-flocculent yeasts (NF) when the cells did not form clumps after dispersal by shaking.

2.5.2. Spectrophotometric determination
The cell cultures obtained as described above were centrifuged and the cells were resuspended in 5 ml of Helm's tampon (3 mM calcium chloride, 50 mM acetate–acetic buffer, pH = 4.5). The degree of flocculation of the different strains was determined in terms of the ratio between the optical density at 620 nm of the culture suspension and that obtained after 10 min at rest ($OD_{10} \times 100/OD_0$). The following flocculation scale was established: ratio >90%, 0 (no flocculent); ratio between 70% and 90%, 1 (low flocculent); ratio between 30% and 70%, 2 (medium flocculent); ratio <30%, 3 (high flocculent).

2.5.3. Flocculation inhibition by sugars
The flocculent strains were cultured at 30 °C in "GPY" medium for 72 h under shaking (orbital shaker). The cultures were centrifuged at 5000 g for 5 min at room temperature and washed once with distilled water. Cells were suspended to a final density of 10^6 cfu/ml in 5 ml of Helm's tampon supplemented with mannose or glucose 1 M. The cell suspension was maintained at room temperature for 20 min, visually observing flocculation capacity in the presence of these sugars.

2.5.4. Sulphite tolerance
The flocculent strains were incubated for 72 h at 28 °C under constant shaking in YPD medium (1% yeast extract, 2% peptone, 2% glucose) supplemented with 0.02% and 0.04% sodium metabisulphite. Tolerance to SO_2 was observed by measuring the decrease in density.

2.5.5. Fermenting power
This value was determined after incubation of the flocculent strains in a model cider (2.5% sucrose, 0.1% yeast extract, 8.1% (v/v) ethanol), adjusting pH to 3.48 with HCl and sterilizing the model cider (121 °C, 5 min). Flasks containing 50 ml of this medium were inoculated with 1 ml of YPD culture of each yeast strain (10^6 cfu/ml). Two replicates per strain were closed with a Müller valve and incubated at 12 °C. Sampling was performed after 12, 36 and 47 days to determine residual sugars and ethanol production.

2.5.6. Foam and volatiles production
The flocculent strains (two replicates per strain) were inoculated (10^6 cfu/ml) in test tubes with apple must sterilized

by adding 0.1% (v/v) dimethyldicarbonate (Merck) and incubated at 30 °C for 10 days. Foam height and foam persistence area were measured every day (Bagot et al., 1989). The chemical composition of the fermented apple juices was analyzed after 10 days.

2.6. Sparkling ciders

2.6.1. Production

Four sparkling ciders were produced from the same base cider using the "traditional or Champenoise method". Prior to secondary fermentation, the base cider was clarified and sterilized by cross-flow filtration, employing 0.22 μm ceramic membranes (Millipore Corporation). Two flocculent strains (ref: 3′ and 50′) chosen by applying the selection methodology and two control strains (dry wine strain Levuline CHP and a non-flocculent native cider strain C6) were used to inoculate the base cider. The base cider was supplemented with sucrose (18 g/l), ammonium sulphate (96 mg/l), thiamine (0.7 mg/l) and bentonite (3 g/hl) prior to inoculation with 2% (v/v) of each of the pure cultures. The flocculent strains were inoculated without bentonite. Secondary fermentation and aging were carried out at the cellar temperature (13–15 °C). Each sparkling cider was aged with the yeast strain for 1 month. For analytical determinations, three bottles were mixed and centrifuged at 5000 g (15 min, 10 °C).

2.6.2. Sensorial analysis

Sensory evaluation of the sparkling ciders was carried by a panel of eight people experienced in the sensory evaluation of cider, belonging to the technical staffs of the cider cellar and SERIDA. Ciders were randomly presented at 8 °C in flute-type glass. The ciders were slowly poured into the glasses, avoiding mixing the contents of the bottle during dispensing. Three aspects were evaluated: visual, odour and flavour. The attributes and methodology described elsewhere (Picinelli et al., 2005; Gallart et al., 2004) were used to evaluate the visual aspect. Foaming was assessed by means of a scale, defined as: 1 (poor), 2 (normal) and 3 (abundant). Odour and Taste intensities were assessed by means of a continuous seven-point scale, defined as follows: 1 (very low), 4 (moderate) and 7 (very high). Overall foam, overall sensation, odour and taste qualities were likewise evaluated by means of a seven-point scale defined as follows: 1 (deficient), 4 (correct) and 7 (excellent).

2.7. Analytical procedures

The viability of the yeasts was determined by plate counting onto Malt Extract Agar (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose, 2% agar) supplemented with 25 mg/l penicillin G potassium salt (Calbiochem, Nottingham, UK) and 100 mg/l streptomycin sulphate (Acofarma, Madrid, Spain) to inhibit bacterial growth. Plates were incubated for colony development at 28 °C for 2 days.

Enological parameters: density, pH, total and volatile acidities, alcoholic proof, free and total sulphur dioxide, free amino nitrogen and pressure in bottle, were determined following the methodology described in the Official Methods of Analysis of the UE (1998).

Sugars (sucrose, fructose, glucose) and glycerol were analyzed by high-performance liquid chromatography using an HPLC (Waters, Milford, MA, USA) apparatus, equipped with a 510 pump, a Waters 717-Plus automatic injector provided with temperature control and an IR 410 refraction index detector. The system was controlled by the Millenium v.3.1 software. Separation was carried out on a Sugar-Pak I cation-exchange column (Waters, Milford, MA, USA) according to the method optimised by Blanco et al. (1988). Standard solutions were prepared by dilution of the individual compounds in an ethanol/water mixture (5/95). Ethanol (HPLC quality) was purchased from Panreac (Barcelona, Spain) and ultra pure water was obtained from a Milli-Q system from Millipore (Milford, MA, USA).

Organic acids were determined by HPLC (Waters, Milford, MA, USA), using a diode array detector (DAD 996), at 206 nm. The column employed was a Spherisorb ODS-2 (250 mm × 0.46 mm i.d.; 3 μm particle size from Waters), the mobile phase was a 0.01 M potassium dihydrogen phosphate buffer solution of pH = 2.43. Elution was performed at 40 °C, in isocratic mode (flow rate 0.5 ml/min). The samples were microfiltered through a 0.45 μm-cellulose acetate filter and directly injected (10 μl) onto the chromatographic column. Standard solutions (malic acid, lactic acid, acetic acid) were prepared by dilution of the individual compounds in ultra pure water.

Major volatile compounds were analyzed in a Hewlett-Packard model 5890 series II (Agilent Technologies, Palo Alto, CA, USA) gas chromatograph equipped with a flame ionization detector (FID) and an FFAP semi-capillary column (30 m × 0.53 mm i.d.; phase thickness, 1.0 μm) supplied by Tecknokroma (Barcelona, Spain). Injection was carried out in splitless mode (1 min) employing helium as the carrier gas at 10 ml/min. The temperature gradient was as follows: 40 °C isotherm for 4 min, followed by a linear increase of 4 °C/min until 60 °C. This temperature was raised to 220 °C at a rate of 10 °C/min. Injector and detector temperatures were 240 and 275 °C, respectively. The microfiltered samples were directly injected into the chromatograph (1 μl). Standard solutions (acetaldehyde, ethyl acetate, methanol, 1-propanol, i-butanol, 1-butanol, amyl alcohols, ethyl lactate+ hexanol, 2-phenylethanol) were prepared by dilution of the individual compounds in an ethanol/water mixture (5/95).

Analysis of variance was performed to detect significant differences in the data (chemical variables and sensory attributes) depending on the yeast strain and cluster analysis (Ward's method) for finding natural groups among the samples, squared Euclidean distance was taken as a measure of proximity between strains. The similarities between mitochondrial DNA profiles for flocculent S. cerevisiae were determined by a Dice coefficient and the dendrogram was calculated with UPGMA method. The calculations were carried out by means of the SPSS (1994) software for Windows (version 11.5).

3. Results and discussion

3.1. Yeast identification and characterization molecular

Genetic profiles generated by rDNA ITS-PCR-RFLP analysis were used to identify the isolated strains by comparison to databases (Esteve-Zarzoso et al., 1999; Fernández-Espinar et al., 2000). Thirty percent of the isolated strains were non-Saccharomyces spp. yeasts, while the remaining 70% belonged to the Saccharomyces genus. This genus was represented by two species, S. cerevisiae and S. bayanus, which, respectively, constituted 51% and 19% of the total isolates. The presence of these species have been reported in other surveys of yeast flora in cider factories located in UK, France, Ireland and Spain (Beech and Davenport, 1970; Michel et al., 1988; Cabranes, 1994; Morrissey et al., 2004; Suárez et al., 2007a).

The 244 colonies identified as Saccharomyces genus were analyzed by mtDNA restriction profiles. Of the 179 colonies of S. cerevisiae yeast analyzed, 44 different restriction patterns were observed, which correspond to 44 different strains. As regards the S. bayanus yeast analyzed (65 colonies), 10 different strains were identified. These results showed that the degree of variability of Saccharomyces spp. strains, measured as the percentage of the different strains found among the colonies analyzed (22%), was

similar to that reported elsewhere in cider (Suárez et al., 2007b) and greater than the variability observed in studies on *Saccharomyces* spp. population during spontaneous fermentation of wines (Frezier and Dubourdieu, 1992; Querol et al., 1994; Gutiérrez et al., 1999; Torija et al., 2001; Schuller et al., 2005).

3.2. Selection of yeast strains

The methodology for selecting secondary fermentation yeast strains for making sparkling cider was divided into consecutive stages: analysis of the important enological characteristics, followed by determination of the flocculating ability of the yeast strains and characterization of flocculent strains.

Regarding enological characteristics, the screening of yeasts was performed by means of rapid, non-expensive tests (plate agar). The following characteristics were tested: high tolerance to alcohol, production of volatile acidity and hydrogen sulphide.

The methodology chosen to determine the ability to produce acetic acid and hydrogen sulphide allowed qualitative classification of the strains to be established according to the amounts produced. The *S. cerevisiae* strains developed on the "Chalk Agar" medium showed large differences in the size of the transparent halos. In our study, 13 of strains did not develop a halo, and were thus considered as non-acetic acid producers and only one strain developed a halo larger that 5 mm, hence being identified as a high acetic acid producer. Regarding *S. bayanus* yeasts, all the strains were classified as non or low producers. The ability of *S. bayanus* to produce less acetic acid than *S. cerevisiae* has been previously reported (Castellari et al., 1994; Guidici et al., 1995; Bertolini et al., 1996). The formation of hydrogen sulphide by yeasts is related to off odours in fermented beverages. Therefore, the ability of the indigenous yeasts to produce H_2S was also investigated. This production was assessed by pigmentation of the different strains on BiGGY medium. Fifty-four percent of the strains developed colonies which were white or light brown in colour. Only two *S. cerevisiae* strains developed black colonies, and were hence considered as high-H_2S producers. The formation of hydrogen sulphide during fermentation has been shown to be strain-dependent (Rankine, 1963; Guidici et al., 1993). On the other hand, all the *Saccharomyces* spp. strains analyzed presented similar abilities to grow in solid medium with 12% (v/v) ethanol.

The first criteria used for selecting yeast strains included the elimination of those strains considered high hydrogen sulphide producers together with those that show an ability for high or medium acetic production. Consequently, 13 colonies (24%) of the 54 initial strains were eliminated.

Flocculation may be defined as "the phenomenon wherein yeast cells adhere in clumps and sediment rapidly from the medium in which they are suspended" (Stewart and Russell, 1981). Flocculating is a very important property in itself in the brewing industry as well as in biotechnological applications due to the fact that it is often the primary method of yeast cell separation. This is especially so when considering the production of sparkling beverages that undergo fermentation in bottle, since flocculation of the yeasts facilitates the riddling and disgorging process (Bidan et al., 1986).

From this point of view, the flocculation property of yeast is a major factor when considering the selection of a yeast strain for sparkling cider made by the Champenoise method. Continuing with the selection procedure, the flocculation characteristics of 41 pre-selected yeasts were hence evaluated by two methods: visual observation and spectrophotometric determination according to the Helm's test. The visual method showed that flocculent yeasts group together phenotypes A and B described by Suzzi et al., 1984, whereas non-flocculent yeasts are similar to phenotype C. Visual

observation of the different cellular cultures allowed us to detect that 24% (10 colonies) of the total yeasts tested had the capacity to develop cellular aggregates and to flocculate. All of the strains possessing this capacity belong to *S. cerevisiae* species. The frequency of the flocculation character in the genus *Saccharomyces* has been reported by others (Stratford, 1989; Suzzi et al., 1997). However, it is worth noting that none of the *S. bayanus* strains had the capacity to flocculate, in contrast with the results reported by Suzzi et al., 1984.

When the Helm's test was used, the degree of flocculation observed among the *S. cerevisiae* strains varied between non-flocculent (68%) to medium flocculent (23%), whereas all the *S. bayanus* strains were classified as non-flocculent. The comparison between the two methods used to evaluate flocculation capacity allows us to state that all the strains (7 colonies) classified as medium flocculent by the Helm's test were characterized as flocculent yeasts by the visual method. Moreover, three strains visually classified as flocculent yeasts presented a low degree of flocculation according the spectrophotometric method. Therefore, both methods showed a number of differing results for any given strain. In spite of the importance and the interest of the flocculation phenomenon in yeasts, there is no established method for its determination (Speers and Ritcey, 1995). Even though the Helm's test is the basis of Analytical Methods of the American Society of Brewing Chemists (ASBC), it did not provide good results, since it could not detect some yeast visually classified as being flocculent. This fact has been previously reported elsewhere (Jin and Speers, 1998; Fajardo et al., 2003).

True flocculation capacity is considered as a reversible aggregation depending on calcium ions, since the removal of these ions from the medium by EDTA causes the aggregates to disappear. In our study, the flocs of all visual flocculent yeast strains were dispersed by 0.25 M EDTA. On the other hand, the intensity of flocculation may be affected by alcohol and carbohydrate contents (Soares et al., 2004; Claro et al., 2007). Three flocculation phenotypes have been described according to sugar specificity: Flo 1, flocculation of yeast was only inhibited by mannose (MS); the NewFlo phenotype, inhibited by glucose and mannose (GMS); and the mannose insensitive MI phenotype, following Stratford (1989) and Masy et al., 1992. The majority of brewery yeast strains belong to the NewFlo phenotype (Soares and Vroman, 2003). Eighty percent of the cider yeasts were mannose insensitive (MI), one strain was glucose–mannose sensitive and another mannose sensitive (Table 1). According to Masy et al. (1992), flocculation of yeasts classified as mannose insensitive could be produced by hydrophobic interactions or specific interactions not involving mannans (protein–protein, protein–lipid).

We selected the 10 *S. cerevisiae* yeast strains that visually exhibited the ability to flocculate for further technological characterization.

Table 1 summarizes the main enological and technological characteristics, together with the changes in flocculation intensity caused by sugars in the flocculent yeast strains. The resulting dendrogram from UPGMA clustering of the restriction analysis of mtDNA patterns of the flocculent yeast strains are shown in Fig. 1.

The sulphite tolerance of the 10 strains under testing and the control strains L2056 and CECT 11773 were similar for the two tested concentrations. All the strains were able to grow and metabolise sugars from YPD medium in the presence of 0.02% and 0.04% sodium methabisulphite (Table 1). The maximum admissible value in ciders for this compound, according to European Community legislation, is 200 mg/l.

In order to estimate the ability of the flocculent strains to carry out secondary fermentation in bottle, their fermentation power in

Table 1
Characteristics of visual flocculent cider yeast strains

Strain	Helm test flocculation	Flocculation inhibition by sugar	Acetic acid production	Hydrogen sulphide production	Sulphite tolerance	Fermentation power (1)	Maximum height foam (mm)
3′	Low	MI	Low	No	+	1.4	8
19′	Middle	MI	Low	Middle	+	1.6	6
20′	Middle	MI	Low	Middle	+	1.5	11
25′	Middle	MI	Low	Middle	+	1.6	13
27′	Middle	GMS	Low	Middle	+	1.5	6
29′	Middle	MI	Low	Middle	+	1.5	9
32′	Low	MI	Low	Middle	+	1.4	10
34′	Middle	MI	Low	Middle	+	1.4	9
40′	Middle	MI	Low	Middle	+	1.5	9
50′	Low	MS	No	Middle	+	1.6	9

MI, mannose insensitive; GMS, glucose and mannose sensitive; MS, mannose sensitive; +, tolerance 200 mg/l SO_2; (1), ethanol production (%, v/v).
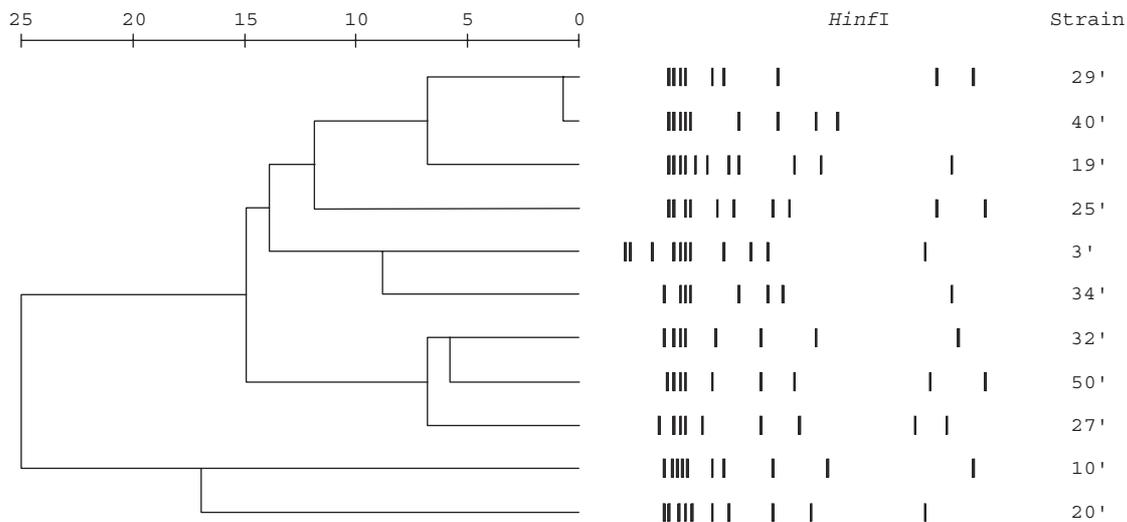
Fig. 1. Dendrogram resulting from UPGMA comparison of patterns obtained for flocculent *Saccharomyces cerevisiae* strains by mtDNA restriction with *Hinf*I endonuclease; mtDNA RFLP schematized patterns are also shown.

the model cider base was analyzed. After 36 days of incubation, only two fermentations inoculated with the strains referred to as 3′ and 34′ showed a concentration of residual fructose higher than 3 and 1 g/l, respectively. After 47 days, the 10 yeast strains completely metabolised the sugar to yield an increase of at least 1.4% ethanol (Table 1).

Regarding the perception of foam height, the highest values for foam height ranged between 6 and 13 mm and were measurable from the 3 to 5 days after inoculation (Table 1). During this time, consistent foam was developed on the surface of the test tubes. After 10 days, the foam height was lower than 1 mm for all of the assessed yeast strains, except for the strains referred to as 25′ and 32′, which maintained a height of 5 mm. Likewise, after 10 days a thin foam collar was observed around the cider surface in all the test tubes except in the aforementioned strains, which formed stable and persistent foam.

In parallel, the micro-fermentations of apple juice inoculated with different flocculent strains were evaluated by determination of residual sugar, acetic production and profiles of major volatiles. The resulting ciders presented residual sugars at concentrations lower than 3 g/l. Their alcoholic proof ranged from 5.2% to 5.9% (v/v) and the production of acetic acid was always lower than 0.4 g/l. Among the volatiles analyzed, the amount of ethyl acetate was always lower than 6 mg/l and the highest concentrations of acetaldehyde (>50 mg/l) were exhibited by the strains referred to as 20′ and 27′. The concentrations of higher alcohols varied considerably for all yeast strains, from 118 mg/l in apple juices

fermented with strain 50′ to 273 mg/l in micro-fermentations with strain 19′. Among higher alcohols, the compounds produced in the highest relative amounts were the amyl alcohols (>40%), and the lowest, 2-phenylethanol (<8%). All the tested flocculent strains fermented apple juice and produced an adequate volatile profile (Cabranes et al., 1997; Suárez et al., 2005), the low volatile acidity production by the selected yeast strains was also confirmed.

Fusel alcohols are quantitatively the largest group of flavour compounds in alcoholic beverages, their concentrations depending on the yeast and fermentation conditions. Therefore, in order to determine relationships between the strains, cluster analysis was performed taking the concentrations of amyl alcohols, isobutanol, propanol and 2-phenylethanol as variables. The results of the dendrogram show two natural groups at the 40% similarity level (Fig. 2). Group I included all of the apple juice fermentations with the minor values of higher alcohols (<200 mg/l), whereas Group II contained the experimental apple fermentations with the higher values of isobutanol (>60 mg/l).

3.3. Sparkling ciders

Once all the results were assessed, we selected two flocculent yeast strains (3′ and 50′) as being representative of the aforementioned natural groups of yeasts to make sparkling ciders under industrial conditions following the Champenoise method. Another
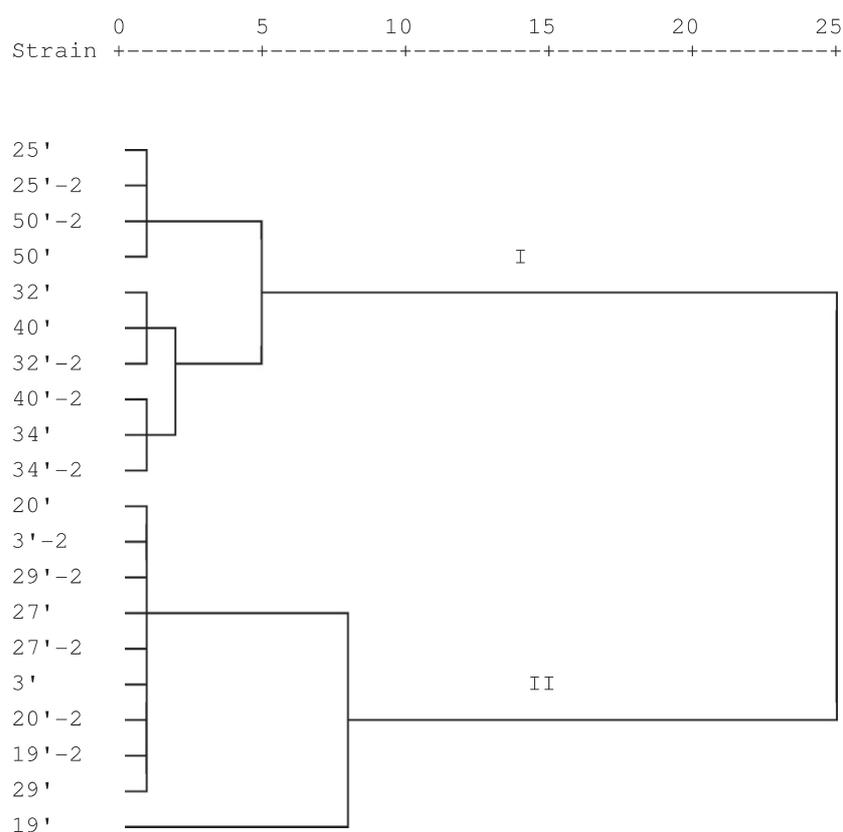
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            0         5        10        15        20        25
   Strain +---------+---------+---------+---------+---------+

   25' ─┐
   25'-2 ─┤
   50'-2 ─┤
   50' ─┘
   32' ─┐
   40' ─┤
   32'-2 ─┤
   40'-2 ─┐
   34' ─┤
   34'-2 ─┘

   20' ─┐
   3'-2 ─┤
   29'-2 ─┤
   27' ─┤
   27'-2 ─┤
   3' ─┤
   20'-2 ─┤
   19'-2 ─┤
   29' ─┤
   19' ─┘
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Fig. 2. Natural groups of floculent yeasts according to their fusel alcohol concentrations.

Table 2
Analytical characteristics of the base cider and sparkling ciders

	Base cider	Yeast strains			
		C6	Levuline	3′	50′
Alcoholic proof (%, v/v)	6.3 ± 0.01	7.5 ± 0.00	7.4 ± 0.00	7.4 ± 0.01	7.4 ± 0.00
Acetic acid (g/l)	0.8 ± 0.02	0.7 ± 0.02	0.8 ± 0.01	0.8 ± 0.03	0.7 ± 0.01
Glycerol (g/l)	4.7 ± 0.01	5.1 ± 0.01	5.2 ± 0.00	5.2 ± 0.00	5.1 ± 0.01
Acetaldehyde (mg/l)	8 ± 0.35	51 ± 1.13	36 ± 0.39	25 ± 1.19	33 ± 0.83
Ethyl acetate (mg/l)	53 ± 0.63	61 ± 0.62	66 ± 0.50	57 ± 1.59	62 ± 1.61
Methanol (mg/l)	65 ± 2.21	72 ± 0.92	76 ± 2.19	69 ± 1.81	70 ± 3.15
1-Propanol (mg/l)	13 ± 0.30	18 ± 0.06	23 ± 0.46	19 ± 0.10	21 ± 0.56
i-Butanol (mg/l)	39 ± 1.24	46 ± 0.71	51 ± 0.77	49 ± 0.18	48 ± 0.85
1-Butanol (mg/l)	5 ± 0.17	6 ± 0.05	7 ± 0.06	6 ± 0.03	6 ± 0.16
Amyl alcohols (mg/l)	200 ± 5.04	246 ± 2.40	263 ± 2.40	239 ± 2.92	245 ± 5.99
Ethyl lactate+hexanol (mg/l)	213 ± 3.51	244 ± 8.78	253 ± 2.97	235 ± 2.87	239 ± 4.78
2-Phenylethanol (mg/l)	26 ± 0.84	46 ± 1.60	38 ± 0.48	36 ± 0.48	35 ± 0.76

Each value in the table represents the mean value ± S.D. from three analyses.

two yeast strains, namely Levuline CHP (*S. cerevisiae*) and C6 (*S. bayanus*), were used as reference standards.

The analytical composition of the base cider and the corresponding sparkling ciders is shown in Table 2. Secondary fermentation in bottle was completed after 14 days by all of the yeast strains (residual fructose < 1 g/l), the alcohol proof ranged from 7.4 to 7.5% (v/v), no differences were detected among sparkling ciders and base cider, neither in terms of total and volatile acidities or pH. After the second fermentation process had finished, a sample was taken for microbiological determinations. Five colonies were randomly taken for mt-DNA analysis, resulting identical profiles than those found for the inoculated yeasts. At the end of fermentation, the number of viable yeasts was higher in the ciders made with C6 and Levuline (10^6 cfu/ml) as regards the sparkling ciders produced with the selected flocculent yeasts (10^4 cfu/ml). The sparkling ciders made with the commercial dry wine yeast Levuline contained the highest concentration of amyl alcohols and *i*-butanol, whereas the native cider yeast C6 exhibited the highest production of 2-phenylethanol. This result confirms the ability of the *S. bayanus* species to produce elevated amounts of this aromatic alcohol (Masneuf et al., 1998; Querol et al., 2003). The volatile composition of the sparkling ciders produced was similar to those reported by our research group in a previous paper (Rodríguez et al., 2007). The results of the analysis of variance ($p < 0.01$) revealed that the concentration of the analyzed compounds was significantly influenced by yeast strain, with the exception of 1-butanol, amyl alcohols, ethyl lactate+ hexanol and acetic acid.

Table 3
Mean values found the tasters in sensory attributes of sparkling ciders

Yeast	Visual		Odour		Taste		Overall sensation
	Foaming	Overall foam quality	Intensity	Quality	Intensity	Quality	
C6	3b	6.1b	4.9a	5.3a	5.6a	4.9a	5.0ab
Levuline	2.6a	6.1b	5.1a	5.5a	5.4a	5.4ab	5.8b
3′	3b	3.9a	5.5a	4.9a	4.9a	4.9a	4.6a
50′	3b	4.5a	5.0a	4.8a	5.0a	5.8b	5.0ab

Mean values with the same letter in a column indicate no significant differences between them (Duncan's test).
Foaming scale: 1–3; other attributes scale: 1–7.

The sensory attributes of sparkling ciders are shown in Table 3. In general terms, all the samples were scored as good. The yeasts selected for secondary fermentation established significant differences among the corresponding ciders for visual (overall foam quality) and flavour (taste quality and overall sensation). No significant differences were found for odour attributes or taste intensity. The cider made with the commercial yeast Levuline scored the best for overall sensation, whereas the sparkling cider made with the flocculent yeast strain referred to as 50′ obtained the best score for taste quality.

4. Conclusions

The present paper proposes a methodology for the selection of yeast strains for secondary fermentation of sparkling ciders. A total of 350 colonies isolated from a cider cellar were identified by rDNA-ITS-RFLP restriction and 54 different patterns of *Saccharomyces* spp. strains yeasts were identified by using mitochondrial DNA restriction analysis. The methodology was divided into consecutive stages, analysis of enological characteristics by means of rapid and non-expansive tests (plate agar) followed by determination of the technological properties. As result, 10 *S. cerevisiae* strains were found as true flocculants and were able to grow in ethanolic medium and in the presence of 200 mg/l of sulphite. Four sparkling ciders were produced using the "Champenoise method" with two flocculent strains chosen by applying the selection methodology together with two control strains. No significant differences among sparkling ciders were found for alcohol proof, total and volatile acidities. The results of the sensory analysis indicated that all of the sparkling ciders were of good quality.

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