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Strain typing of acetic acid bacteria responsible for vinegar production by the submerged elaboration method

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ABSTRACT

Strain typing of 103 acetic acid bacteria isolates from vinegars elaborated by the submerged method from ciders, wines and spirit ethanol, was carried on in this study. Two different molecular methods were utilised: pulsed field gel electrophoresis (PFGE) of total DNA digests with a number of restriction enzymes, and enterobacterial repetitive intergenic consensus (ERIC) – PCR analysis. The comparative study of both methods showed that restriction fragment PFGE of *SpeI* digests of total DNA was a suitable method for strain typing and for determining which strains were present in vinegar fermentations. Results showed that strains of the species *Gluconacetobacter europaeus* were the most frequent leader strains of fermentations by the submerged method in the studied vinegars, and among them strain R1 was the predominant one. Results showed as well that mixed populations (at least two different strains) occurred in vinegars from cider and wine, whereas unique strains were found in spirit vinegars, which offered the most stressing conditions for bacterial growth.

1. Introduction

A variety of vinegars is produced in most Mediterranean countries and extensively used as a condiment and as an efficient acidifying agent for food preservation. Vinegar is produced by two well-defined methods: a slow surface process, in which acetic acid bacteria (AAB) are placed on the air–liquid interface in direct contact with atmospheric oxygen, and a fast submerged process, in which AAB are submerged in the acetifying liquid and a continuous strong aeration is applied to provide the necessary oxygen for acetic fermentation to take place. Generally, the surface process is employed for elaborating traditional vinegars and the submerged process is employed for the elaboration of most commercial vinegars of major consumption.

AAB are the microorganisms responsible for the transformation of ethanol into acetic acid, and it should be pointed out that not all the strains of a certain species have the same ability to oxidize ethanol into acetic acid (Gullo and Giudici, 2008). Therefore, discriminating among AAB strains of the same species is important to determine how many strains are involved in a fermentation process and which one is leading the transformation. DNA-based typing methods that have been successfully used for identification to strain level of AAB from a variety of origins are the following: enterobacterial repetitive intergenic consensus sequence amplification (ERIC-PCR) (González et al., 2004, 2005; Gullo and Giudici, 2008; Gullo et al., 2009; Nanda et al., 2001), random amplified polymorphic DNA (RAPD-PCR) analysis (Nanda et al., 2001; Prieto et al., 2007; Trcek et al., 1997), and pulsed field gel electrophoresis (PFGE) of genomic restriction fragments applied to a wide range of bacterial isolates (López et al., 2007). Microbiological studies reported in the last years have focused on characterisation of AAB from traditional balsamic vinegars (De Vero et al., 2006; Gullo et al., 2006, 2009; Gullo and Giudici, 2008; Ilabaca et al., 2008), rice vinegar (Haruta et al., 2006) and some other vegetable products such as grapes or cocoa beans (De Vuyst et al., 2008; Prieto et al., 2007), and very few reports can be found on strain characterization of AAB from vinegars produced by the submerged method (Callejón et al., 2008; Schüller et al., 2000; Trcek et al., 2000).

The objectives of the present study were searching for appropriate and efficient DNA-based molecular methods to type AAB strains, and to characterize up to strain level AAB isolates from vinegars elaborated by the submerged method from cider, wine and spirit ethanol.

2. Materials and methods

2.1. Vinegar sampling

Vinegar samples were aseptically taken from 30,000 l bioreactors (Frings Xuzhou Bio- and Chemical Technology Co., Ltd.) optimized

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for the submerged production of vinegar, of the company Vinagrerías Riojanas S. A., containing either wine, cider or spirit vinegars in full fermentation (fermentation rate = 0.17–0.25 acetic degrees/h). A total of 58 samples (34 wine vinegars, 20 cider vinegars and 4 spirit vinegars) were collected during the period from November 2007 to December 2008. Samples of 25 ml were collected in 50 ml sterile tubes and transported with continuous agitation, which favoured aerobic conditions. Samples were rapidly submitted (within 10 min) to microbiological analysis in the laboratory.

2.2. Culture media and growth conditions

25 ml samples of wine and cider vinegars were centrifuged at 2100×*g* (Megafuge Heraeus, Thermo Scientific, Wilmington, USA) for 10 min. Cell pellets of approximately 1 ml volume were collected, and 100 μl of each sample were cultivated for 5 days on GY agar plates [5% glucose (Panreac Química S.A., Barcelona, Spain), 1% yeast extract (Scharlau Chemie S A, Barcelona, Spain) and 1.5% agar (Becton–Dickinson, Madrid, Spain)] at 30 °C under aerobic conditions. 25 ml samples of spirit ethanol vinegars were centrifuged and cell pellets were cultivated in GY broth for 48 h at 30 °C with continuous and vigorous agitation in order to adapt AAB cells to growing in the culture medium. These samples were subsequently cultivated on GY agar plates following the same procedure as described for wine and cider vinegars. Colonies were submitted to gram staining and morphological analysis by optical microscopy. Three colonies were randomly taken from each vinegar sample. Isolates thus selected were considered to represent the numerically dominant strains present in the vinegar samples. AAB isolates were sub-cultured to purity on GY agar plates and were typed to species level by sequence analysis of the amplicon obtained by PCR of the 16S–23S intergenic region in a previous study (Fernández-Pérez et al., submitted for publication). A total of 103 pure isolates were recovered and stored in 20% sterile skim milk (Becton–Dickinson) at −20 °C.

2.3. Strain typing by ERIC (enterobacterial repetitive intergenic consensus) -PCR

A total of 90 AAB pure isolates were grown onto GY agar plates at 30 °C for 3 days under aerobic conditions for DNA extraction. The DNA extraction method that was carried out was as follows. Cells from this fresh culture were suspended in 200 μl of lysis buffer (50 mM Tris/HCl, pH 8; 10 mM β-mercaptoethanol). Samples were vigorously vortexed and reposed for 15 min at room temperature. They were incubated at 100 °C for 10 min, vortexed and frozen to −80 °C. Samples were unfrozen and submitted to precipitation (Sambrook et al., 1989). 200 μl of solution II (0.2 N NaOH; 1% SDS) was added over 100 μl of sample, mixed manually and kept on ice for 3 min. 150 μl of solution III (3 M potassium; 5 M acetate) was added to the mix and agitated for 10 s and kept on ice for 3–5 min. The sample was centrifuged at 14,800×*g* (Biofuge Heraeus, Thermo Scientific) for 5 min at 4 °C. The supernatant was recovered in a sterile microtube and DNA was precipitated with 2 vol of ethanol (96%) at 4 °C and resting on ice for 2 min. The sample was centrifuged at 12,000×*g* at 4 °C for 5 min and the supernatant was eliminated. The DNA was dissolved in 50 μl of TE buffer (10 mM Tris/Cl pH 8; 1 mM EDTA pH 8) and DNAses were inactivated by heating in a water bath at 85 °C for 15 min. DNA quantification was performed with the apparatus NanoDrop (Thermo Scientific) and adjusted between 80 and 150 ng/μl. The oligonucleotide primers used for the amplification of the ERIC-PCR were those described by Versalovic et al. (1991): ERIC1 (5′ATCGAAGCTCCTGGGGATTCAC3′) and ERIC2 (5′AAGTAAGTGACTGGGGTGAGCG3′) (synthesized by Sigma Aldrich, Madrid, Spain). PCR amplification was carried out in a final volume of 50 μl following the conditions described by González et al. (2004). The amplification products were resolved by electrophoresis in 1.5% (w/v) agarose gels, separated at 80 V for 1 h and 45 min, stained with ethidium bromide and photographed. ERIC-PCR patterns were classified as indistinguishable, closely related or unrelated when they were identical, differed in 1–3 bands, or differed in more than 3 bands respectively.

2.4. Strain typing by restriction fragment PFGE of total DNA digested with SpeI

AAB pure isolates were cultured for 24 h in GY broth with vigorous and continuous shaking to reach an optical density of 0.8–1.2 at 660 nm. Three ml samples of these fresh cultures were centrifuged and cells were washed once with 3 ml sterile saline solution. Cells were resuspended in 100 μl of storage buffer (10 mM Tris/HCl pH 8, 10 mM EDTA pH 8). The suspension was warmed at 50 °C and 100 μl of 1% pulse field certified agarose (D-5 Pronadisa Hispanlab S.A., Madrid, Spain) in TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8) at the same temperature was added. The suspension was allowed to solidify in molds and they were treated following the method described by López et al. (2007) for cell lysis and DNA isolation under immobilised conditions. Before restriction enzyme digestion, agarose blocks were cut (slices 1–2 mm) and balanced for 30 min at room temperature in 100 μl of the appropriate restriction enzyme buffer. The following restriction endonucleases were tested: *SfiI*, *XbaI*, *NotI*, *AluI*, *SmaI* and *SpeI*. Digestions with *SpeI* (New England Biolabs, Beverly, MA) were the most effective and DNAs were incubated with this enzyme for pattern comparison. *SpeI* digestions were performed overnight at 37 °C in a 100 μl total volume of the specific buffer with 5 U of restriction enzyme. Before loading, gel blocks were washed with 1 ml of TBE for 8 min at 52 °C. DNA fragments were separated in 1% (wt/vol) agarose (D-5 Pronadisa) in TBE buffer with a CHEF DR II system (Bio-Rad Laboratories, CA, USA). A total of 77 pure isolates were analysed by this PFGE method. Electrophoresis was performed at 14 °C at a constant voltage of 4.5 V/cm with a switch time ramped from 5 to 45 s over 24 h period. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed under UV light. Lambda ladder PFG marker (New England Biolabs) was used as molecular size standard. PFGE patterns were classified, like in the case of ERIC-PCR analyses, as indistinguishable, closely related or unrelated when they were identical, differed in 1–3 bands, or differed in more than 3 bands respectively, according to published criteria for bacterial strain typing (Tenover et al., 1995).

2.5. Reproducibility study

To determine the percentage of similarity necessary for strain discrimination, reproducibility studies were carried out according to López et al. (2008). The level of similarity obtained between repeats of the same isolate when included within the dendrogram for all strains, established the discriminatory threshold below which patterns were considered to be different.

2.6. Numerical analysis of gel images

The GelCompar 2.5 software (Applied Maths, Kortrijd, Belgium) was used for conversion, normalization, and further processing of images. Comparison of the obtained restriction fragment PFGE and ERIC-PCR patterns was performed with Dice coefficient and the Unweighted Pair Group Method using Arithmetic averages (UPGMA). The cophenetic correlation value was calculated for the dendrograms. This parameter is a measure of the reliability of the calculated distances in the dendrogram (Sokal and Rohlf, 1962).