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Identification of acetic acid bacteria in traditionally produced vinegar and mother of vinegar by using different molecular techniques

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ABSTRACT

Culture-dependent and culture-independent methods were combined for the investigation of acetic acid bacteria (AAB) populations in traditionally produced vinegars and mother of vinegar samples obtained from apple and grape. The culture-independent denaturing gradient gel electrophoresis (DGGE) analysis, which targeted the V7–V8 regions of the 16S rRNA gene, showed that *Komagataeibacter hansenii* and *Komagataeibacter europaeus*/*Komagataeibacter xylinus* were the most dominant species in almost all of the samples analyzed directly. The culture-independent GTG_5-rep PCR fingerprinting was used in the preliminary characterization of AAB isolates and species-level identification was carried out by sequencing of the 16S rRNA gene, 16S–23S rDNA internally transcribed to the spacer (ITS) region and *tuf* gene. *Acetobacter okinawensis* was frequently isolated from samples obtained from apple while *K. europaeus* was identified as the dominant species, followed by *Acetobacter indonesiensis* in the samples originating from grape. In addition to common molecular techniques, real-time PCR intercalating dye assays, including DNA melting temperature (Tm) and high resolution melting analysis (HRM), were applied to acetic acid bacterial isolates for the first time. The target sequence of ITS region generated species-specific HRM profiles and Tm values allowed discrimination at species level.

1. Introduction

Vinegar is the product of a two-step fermentation process involving the alcoholic fermentation of sugars into ethanol by yeasts, and, subsequently, the oxidation of ethanol into acetic acid by acetic acid bacteria (AAB). Vinegar has been renowned as a food preservative since ancient times and is still used today in the food industry as a highly sought-after condiment and a multi-functional product utilized for pickling, preserving and flavor-balancing purposes (Sengun and Karapinar, 2004; Steinkraus, 2002). All raw materials containing sugar or fruits can be employed as starting substances for producing vinegar. Several different types of vinegars are produced around the world by using diverse manufacturing techniques and raw materials native to specific areas. In general, two well-defined methods, namely, slow surface culture fermentation and fast submerged fermentation, are used in vinegar manufacturing (Tesfaye et al., 2002). The slow method is also called traditional vinegar production and the AAB grow on the surface of the liquid during the fermentation process, which may take from several weeks up to a few months (Nanda et al., 2001). A non-toxic film composed of AAB and cellulose accumulates on the surface of the vinegar throughout the oxidation process. This film, called "mother of vinegar", is used in the back-slopping practice as an indigenous starter culture to trigger acetification in the production of traditional vinegar (Holzapfel, 2002; Hidalgo et al., 2010).

As a result of the two stage fermentation, several microbial species show competitive activity in the vinegar throughout the production process (Holzapfel, 2002). The microbial transformations give the characteristic taste and fragrance to vinegar and the microbial metabolites have beneficial health effects (Shimoji et al., 2002; Stasiak and Blażejak, 2009). Acetic acid bacteria, which carry out the second transformation, namely oxidative fermentation, are one of the main microbial populations in vinegar.

The three genera of acetic acid bacteria, *Acetobacter* (*A.*), *Gluconacetobacter* (*Ga.*) and *Komagataeibacter* (*K.*) are mainly responsible for acetic fermentation in vinegar. The species of *Acetobacter aceti*, *Acetobacter malorum*, *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Komagataeibacter europaeus*, *Komagataeibacter hansenii*, *Komagataeibacter intermedius*, *Komagataeibacter oboediens*, *Komagataeibacter xylinus*, *Komagataeibacter medellinensis* and *Komagataeibacter maltiaceti* were previously detected in the vinegar ecosystem (Sievers et al., 1992; Trcek et al., 1997; Sokollek et al., 1998; Boesch et al., 1998; Trcek et al., 2000; De Vero et al., 2006; Gullo et al., 2006; Yamada et al., 2012; Castro et al., 2013; Slapsak et al., 2013). However, the profiles of AAB are unstable and show particular diversity in accordance with the raw material characteristics and production process features (Gullo et al., 2009).

Identification of AAB based only on morphological, biochemical, and physiological characteristics is not reliable and, therefore, is insufficient

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because of the poor reproducibility and discriminatory power of these phenotypic tests (Cleenwerck and De Vos, 2008; Papalexandratou et al., 2009). For this reason, nucleic acid-based molecular methods are now used to characterize and identify isolates of AAB from wine and vinegar ecosystems. These have included Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) (Gonzalez et al., 2004; Gullo et al., 2009; Nanda et al., 2001), Repetitive Extragenic Palindromic-PCR (REP-PCR) (González et al., 2004), $(GTG)_5$-rep-PCR fingerprinting (De Vuyst et al., 2008) and RAPD-PCR (Trcek et al., 1997; Bartowsky et al., 2003; Nanda et al., 2001). A reliable taxonomic identification is obtained when these techniques are combined with the sequencing of 16S rDNA genes (Gonzalez, 2005) and internal transcribed spacer sequences (ITS) of the 16S–23S rDNA genes (Gonzalez and Mas, 2011). Restriction fragment length polymorphism (RFLP) analysis of the ribosomal genes or their spacer regions has also been used for the identification of AAB present in food-related ecosystems (Gullo et al., 2006; Ruiz et al., 2000; Trcek and Teuber, 2002; Trcek, 2005; Vegas et al., 2010).

The combination of culture-independent methods with culture-dependent methods in a polyphasic system is recommended as an effective approach to overcome the difficulties regarding the isolation and cultivation of AAB strains (Cleenwerck and De Vos, 2008; Gullo et al., 2009; Sengun and Karapinar, 2011). In the last decade, several studies using the culture-independent DGGE and Temporal Temperature Gradient Gel Electrophoresis (TTGE) techniques were reported for the characterization of the microbial community in vinegar and the determination of population dynamics of AAB during fermentation (De Vero et al., 2006; De Vero and Giudici, 2008; Ilabaca et al., 2008). In addition, the real-time PCR technique has also been proposed for culture-independent detection of different genera, or species, of AAB (Andorra et al., 2008; Torija et al., 2010; Valera et al., 2013). Recently, the intercalating dye-based real-time PCR analysis involved in specific melting temperature (Tm) and high-resolution melting analysis were applied as a highly promising new approach for confirming the identification and grouping of the culturable strains belonging to different bacterial species (Kao et al., 2007; Juvonen et al., 2008; Kesmen et al., 2014), but not yet to AAB. These new approaches provide a rapid and reliable tool for the detection of small differences in the target DNA sequences of closely related species.

The identification of indigenous AAB has critical importance to improve the process control, overcome unpredictable fermentation problems and select the most suitable strains as the potential starter culture. Therefore, in this study, we aimed to detect and compare AAB populations in grape and apple vinegar and in mother of vinegar samples obtained from different regions of Turkey. Thus the culture-independent PCR-DGGE technique was combined with culture-dependent molecular techniques, including $(GTG)_5$-rep-PCR and sequence analysis of the 16S rRNA gene, 16S–23S rRNA internal transcribed sequences (ITS) region and *tuf* gene for identification and characterization of AAB isolated from analyzed samples. Furthermore, real-time PCR intercalating dye-based analysis was applied to AAB isolates to obtain species-level discrimination.

2. Materials and methods

2.1. Vinegar and mother of vinegar samples

Vinegar and mother of vinegar samples produced by the spontaneous fermentation method were obtained from three different local producers in Kayseri and Düzce, in Turkey. The samples, consisting of 2 grape vinegars (bg and eg) and their mothers (BG and EG), 2 apple vinegars (da and ha) and their mothers (DA and HA), were collected at the end of the acetic fermentation from wood barrels in which traditional surface fermentation is carried out. The acetic acid content of the vinegar samples were reported by producers as 3.96, 4.08, 4.28 and 4.37% for the samples ha, da, bg and eg respectively. After the sampling process, the vinegar and mother of vinegar samples were analyzed for AAB using cultural and molecular methods.

2.2. Isolation of acetic acid bacteria (AAB)

10 ml of each of the 5 vinegar samples were diluted with 90 ml of Maximum Recovery Diluent (Merck, GmbH, Darmstadt, Germany), and homogenized for 2 min with a shaker (IKA, Germany). Serial decimal dilutions were prepared with the same diluent and subjected to the agar plate method for the isolation of AAB on GYC (5% D-glucose, 1% ethanol, 1% yeast extract, 1% $CaCO_3$, 0.05% bromocresol purple, 2% agar) and MYP (1% mannitol, 1% yeast extract, 0.3% peptone, 2% agar) agar plates. To inhibit yeast growth, 100 ppm cycloheximide (Sigma) was added to both agars. All plates were incubated at 30 °C for 5 days. For each sample, 10–15 catalase-positive, oxidase-negative, and Gram-negative colonies, showing different morphological characteristics were purified by streak-plate technique and subjected to further characterization. All isolates selected from samples were stored at −80 °C.

2.3. DNA extraction from pure cultures, vinegar and mothers of vinegar samples

Bacterial cells harvested from GYC and MYP agar were washed in 1 ml TE buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA) and resuspended in a 300 μl lysis buffer (100 mM Tris–HCl, pH 8.0, 10 mM EDTA, 2% SDS, 1% PVP, 0.15% NaCl). After homogenization, the bacterial DNA was extracted with the method described by Gullo et al. (2006). The total DNA, which was extracted directly from each vinegar and mother of vinegar sample, was used for PCR-DGGE analysis. The mother of vinegar samples were powdered under liquid nitrogen and vinegar samples were pelleted by centrifugation at 10 000 ×*g* for 10 min before performing DNA extraction as described by De Vero et al., 2006. The quantity and purity of the extracted DNA from the pure cultures and the samples were measured by using a microvolume UV/Vis spectrophotometer (UVS-99, ACTGene, USA) at 260 nm and standardized at the final concentration of 100 ng/μl.

2.4. PCR-DGGE analysis of vinegar and mother of vinegar samples

The V7–V8 region of the 16S rDNA was amplified by using DNA isolated from each vinegar and mother of vinegar sample. The primers WBAC1 (5′-GTC GTC AGC TCG TGT CGT GAG A-3′) and WBAC2 (5′-CCC GGG AAC GTA TTC ACC GCG-3′) were used to amplify an approximately 328 bp fragment of the target region (Lopez et al., 2003). A GC clamp (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C-3′) was attached to the WBAC1 primer, according to Lopez et al. (2003). All of the PCR amplifications were performed in a final volume of 50 μl, containing 25 μl of commercial PCR master mix (Dream Taq, Fermentas, USA), 40 pmol of forward primer with a GC clamp, 20 pmol of reverse primer and 100 ng sample DNA. The thermal cycler (TC-5000 gradient thermal cycler, Techne, UK) conditions were programmed in accordance with De Vero et al. (2006). The amplification products were checked by electrophoresis in 2% (w/v) agarose gel containing ethidium bromide and visualized under UV light. The sequence specific separation of the PCR products was performed on the Dcode TM Universal Mutation Detection System (BioRad, Hercules, USA) by using 1 mm polyacrylamide gel (8% [wt/vol] acrylamide–bisacrylamide 37.5:1), containing 30% to 60% urea–formamide denaturing gradient (100% corresponds to 7 m urea and 40% [w/v] formamide). Electrophoresis was performed at 60 °C in TAE buffer 1× (40 mM Tris base, 20 mM acetic acid glacial, 1 mM EDTA 0.5 M, pH 8.0 and dH2O) with a constant voltage of 150 V at 60 °C for about 4 h. After electrophoresis, the DGGE gels were viewed under UV transillumination (Gel Doc XR, BioRad) after being stained with ethidium bromide solution (50 μg/ml) for 20 min.