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ORIGINAL ARTICLE

Acetobacter strains isolated during the acetification of blueberry (*Vaccinium corymbosum* L.) wine

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Significance and Impact of the Study: In this study, the Acetic Acid Bacteria population involved in blueberry vinegar production using Schützenbach method is first reported. Inoculation of an *Acetobacter cerevisiae* strain speeded up and conducted the process compared to spontaneous process. However, two genotypes of *Acetobacter pasteurianus* were also isolated in both processes (inoculated and spontaneous). These *Acet. pasteurianus* isolates can be exploited for studies of inoculation in vinegar production from blueberry or other raw material using Schützenbach method or other vinegar production methods.

Keywords
Acetobacter, highbush blueberries, Schützenbach method, starter culture, vinegar.

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Abstract

Highbush blueberries (*Vaccinium corymbosum* L.) are known to have positive health benefits. The production of blueberry vinegar is one method to preserve this seasonal fruit and allow extended consumption. In this study, blueberry wine acetification was performed with naturally occurring micro-organisms and with an inoculated *Acetobacter cerevisiae* strain. Acetifications were carried out in triplicate using the Schützenbach method. The successful spontaneous processes took up to 66% more time than the processes involving inoculation. The isolation of acetic acid bacteria (AAB) and the analysis of these AAB using molecular methods allowed the identification of the main genotypes responsible of the blueberry acetification. Although the *Acet. cerevisiae* strain was the predominant strain isolated from the inoculated process samples, *Acetobacter pasteurianus* was isolated from samples for both processes and was the only species present in the spontaneous acetification samples. To the best of our knowledge, this is the first report describing the identification and variability of AAB isolated during blueberry acetification. The isolated *Acet. pasteurianus* strains could be used for large-scale blueberry vinegar production or as a starter culture in studies of other vinegar production methods.

Introduction

Acetic acid bacteria (AAB) are important micro-organisms in the food and biotechnological industries because of their ability to oxidize many types of sugar and alcohols (De Ley *et al.* 1984). The production of vinegar is one of the most important industrial processes in which these bacteria are involved.

Vinegars derived from different raw materials, such as grapes, cereals, onions, persimmons, berries and whey, have been produced and studied (Solieri and Giudici 2009). In some of these studies, molecular techniques have been used to identify the AAB species and genotypes present in these niches. For instance, *Acetobacter malorum* was reported as the responsible from strawberry vinegar production by traditional method (Hidalgo *et al.* 2013) and *Acetobacter pasteurianus* was the dominant species identified in cereal vinegar elaborated by solid-state fermentation (Wu *et al.* 2010). In the single study carried out on AAB from blueberries, several different AAB genera were identified using biochemical tests (*Acetobacter*, *Gluconobacter*, *Asaia*, *Gluconacetobacter*

and *Swaminathania*) from different varieties of blueberry (Gerard *et al.* 2010).

Highbush blueberries (*Vaccinium corymbosum* L.) are a rich source of dietary antioxidants (Gu *et al.* 2004; Seeram 2006; Borges *et al.* 2010) that have multiple beneficial biological effects. This fruit is native to North America, and the largest blueberry industries in the world are in the United States and Chile. Currently, Chile has a dominant position in the Southern Hemisphere, and blueberry cultivation is an important economic activity in this country (Brazelton 2011).

As with any activity related to the production of fresh fruit, there are substandard fruit, seasonal surpluses and fruit waste generated during the cultivation, and these materials could be used to produce fruit vinegar. In the case of blueberries, the production of vinegar could be a good option to preserve the healthy properties of this fruit.

There are two well-defined methods used to produce vinegar: traditional or surface processes and submerged methods. The primary differences between these two methods are related to the time needed to complete the acetification process and to the quality of the final product. In the traditional method, the time required to obtain the expected level of acetic acid is longer than in the submerged method due to the strong aeration mechanism used in the latter method to meet the oxygen demand of the AAB. In traditional method, AAB grows on the air–liquid interface obtaining oxygen from atmospheric air, which slows down the acetification but allows gaining metabolic by-products from AAB that increase the sensorial complexity of the vinegar. Therefore, as consequence of the production system, the elaboration time is lengthier but the quality of the final product is significantly higher in the traditional method.

Alternative systems for the production of vinegar have been designed to achieve faster rates of production than those for traditional methods while preserving the quality of the final product to the greatest extent possible. These alternative methods, among which is the Schützenbach method, are focused on the use of inert materials, such as bacterial supports, to mimic the air–liquid interface created in the traditional method to allow direct contact with atmospheric air. The Schützenbach method uses wood shavings as a bacterial support material, and to increase the oxygen accessibility, the acetifying liquid is pumped through the wood shavings, achieving relatively high acetification rates (Llaguno 1991).

The use of well-defined starter cultures in vinegar elaboration to date has been limited or nonexistent. Few studies have tested the use of a selected AAB culture as a starter for the production of vinegar by the traditional method (Gullo *et al.* 2009; Hidalgo *et al.* 2010b, 2013) or the submerged method (Saeki *et al.* 1997; Sokollek and Hammes 1997; Hidalgo *et al.* 2010b), and no studies have tested such starters with the Schützenbach method. Therefore, more inoculation studies should be carried out in order to better understand the AAB population dynamic and the efficiency of the inoculated strains.

The aim of this work was to study the AAB population during the production of blueberry vinegar by the Schützenbach method. The acetifications were carried out spontaneously and after inoculation with an *Acetobacter cerevisiae* strain that was previously isolated from grapes (Prieto *et al.* 2007).

Results and discussion

Acetification kinetics

The kinetics of the spontaneous and inoculated processes was followed by measuring the increase in acidity over time (Fig. 1). The blueberry wine was produced spontaneously at room temperature and it had an initial alcohol concentration of 7·3% (v/v), acetic acid concentration of 0·6% (w/v) and a pH of 3·15. During vinegar production, no variation in the pH was observed. The three spontaneous acetifications had similar kinetics, reaching maximum acidity [5·5% (w/v)] after 28 days. However, the three samples inoculated with *Acet. cerevisiae* reached higher acidity values [6·6 to 6·9% (w/v)], and the highest acidity levels were reached sooner than the highest levels for the spontaneous process, but the exact time needed to reach the highest level of acidity varied among the triplicates (9, 17 and 24 days).

In this study, the Schützenbach method was used successfully to produce blueberry vinegar. This system was designed to achieve acetification rates that are faster than those obtained with traditional methods (Llaguno

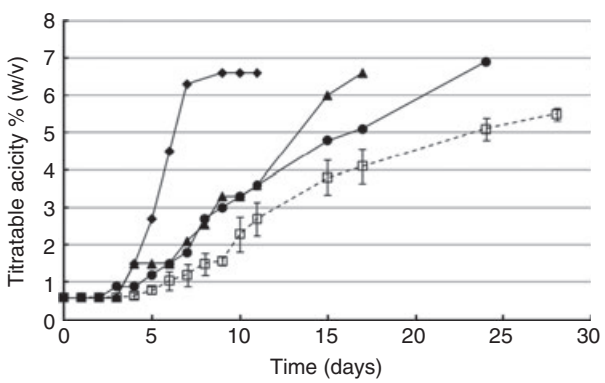

Figure 1 Kinetics of acetification represented by the titratable acidity. Average of spontaneous acetifications (--□--) and individual inoculated acetifications [replicates 1 (♦), 2 (▲) and 3 (●)].

Table 1 Enumeration of acetic acid bacteria by plating (culturability, CFU ml^{-1}) and microscopy (total cells, Cell ml^{-1}) during blueberry acetification processes

Type of process	Samples	Cell ml^{-1}	CFU ml^{-1}
Spontaneous acetification	Initial	8·00 ± 0·15E+06	2·50 ± 0·21E+06
	Mid	1·42 ± 0·17E+07	4·57 ± 0·32E+06
	Final	7·64 ± 0·89E+07	1·83 ± 0·68E+04
Inoculated acetification	Initial	3·68 ± 0·85E+08	1·30 ± 0·10E+08
	Mid	3·39 ± 0·47E+08	3·60 ± 0·70E+06
	Final	4·89 ± 0·83E+08	8·70 ± 0·85E+04

1991). The elaboration of vinegar from grape wine or from other fruit wines using traditional methods requires more than 30 days for the complete process (Hidalgo *et al.* 2010a,b, 2012; Vegas *et al.* 2010). Therefore, the Schützenbach method can be used to produce fruit vinegars, such as blueberry vinegar, in a shorter time than that required for the traditional method.

AAB enumeration

The bacteria were counted by microscopy and plating (Table 1). The initial population size determined by microscopy showed that the cell population in spontaneous acetification samples was approximately 10^6 cells ml^{-1} and increased by one order of magnitude by the end of the process. In contrast, the number of bacteria in the inoculated acetification samples remained constant at 10^8 cells ml^{-1} throughout the entire process.

Poor AAB recovery on plates relative to the enumeration by microscopy during vinegar elaboration has been previously reported (Entani *et al.* 1985; Sievers *et al.* 1992; Sokollek *et al.* 1998; Trcek 2005; Ilabaca *et al.* 2008). In this study, this effect was observed, especially at the end of processes when the acetic acid concentration was higher, decreasing the level of plate recovery to the order of 10^4 CFU ml^{-1} for both acetification conditions. Different explanations have been proposed for the limited AAB recovery on plates, among them, the possible entrance of AAB into a viable but nonculturable state due to the adverse conditions (Millet and Lonvaud-Funel 2000). Culture-independent molecular methods may be an adequate tool to understand the composition at species level of the microbial community in this extreme medium.

AAB typing and species identification

The both fingerprinting methods used [ERIC-PCR and $(GTG)_5$-PCR] showed identical results. Three different genotypes were detected (Fig. 2), one of which was the inoculated genotype (Ac0). The inoculated strain (Ac0) belonged to *Acet. cerevisiae*, which was the only species isolated in the original ecological study on grapes cultivated in the north of parallel 35 (latitude S) (Prieto *et al.* 2007). Among all of the strains isolated, Ac0 was selected for its good performance during the ethanol resistance test and the acetic acid production test (data not shown).

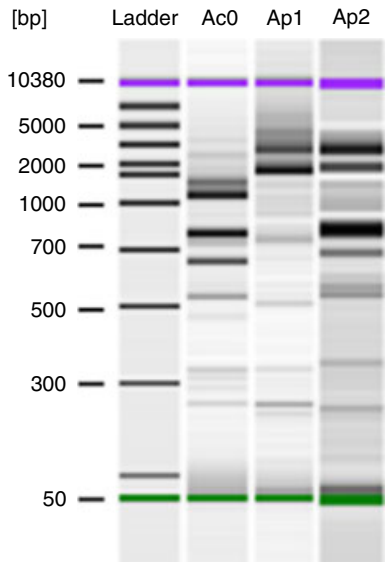

Figure 2 Different ERIC-PCR profiles isolated from highbush blueberries vinegar process: Ac0, the inoculated genotype of *Acetobacter cerevisiae*. Ap1 and Ap2, genotypes of *Acetobacter pasteurianus* detected in both acetification processes (spontaneous and inoculated).

Several isolates of each genotype were analysed to identify the bacteria at species level by RFLP-PCR based on the 16S rRNA gene and by sequencing. The genotype Ac0 was correctly identified as the expected species, *Acet. cerevisiae*, and the other two genotypes (Ap1 and Ap2) were identified as *Acet. pasteurianus*. Because these species are closely related to *Acet. malorum* and *Acetobacter pomorum*, respectively, further identification by sequencing the 16S-23S ITS rRNA gene region was performed.

The presence of these genotypes along the processes is detailed in Table 2. The diversity isolated from the blueberry vinegar samples was low; only two genotypes of *Acet. pasteurianus* were identified throughout the blueberry spontaneous acetifications. The profile Ap1 was the sole genotype identified at the beginning of the acetification processes and was replaced by Ap2, which became the predominant genotype in the middle and end of the process. The more restrictive conditions of berry fruits may be a tool to select micro-organisms because the more stringent bacterial growth results in a greater selective pressure exerted on the indigenous micro-organisms (Solieri and Giudici 2009).

The three inoculated acetifications did not have the same microbial population. The fastest acetification

Table 2 Isolation, identification and typing of acetic acid bacteria during acetification

Samples	Replicate	Number of profiles	Species (%)	GTG_5/ERIC profiles (%)
Spontaneous Acetification				
Initial	1	1	*Acetobacter pasteurianus* (100)	Ap1 (100)
Mid	1	1	*Acet. pasteurianus* (100)	Ap2 (100)
	2	1		
	3	1		
Final	1	2	*Acet. pasteurianus* (100)	Ap2 (60), Ap1 (40)
	2	1		Ap2 (100)
	3	2		Ap2 (82), Ap1 (18)
Inoculated Acetification				
Initial	1	1	*Acetobacter cerevisiae* (100)	Ac0 (100)
Mid	1	1	*Acet. cerevisiae* (100)	Ac0 (100)
	2	2	*Acet. cerevisiae* (80), *Acet. pasteurianus* (20)	Ac0 (80), Ap1 (20)
	3	3	*Acet. cerevisiae* (82), *Acet. pasteurianus* (18)	Ac0 (82), Ap1 (9), Ap2 (9)
Final	1	1	*Acet. cerevisiae* (100)	Ac0 (100)
	2	3	*Acet. cerevisiae* (25), *Acet. pasteurianus* (75)	Ac0 (25), Ap1 (42), Ap2 (33)
	3	2	*Acet. cerevisiae* (58), *Acet. pasteurianus* (42)	Ac0 (58), Ap2 (42)

(9 days) was carried out by the inoculated *Acet. cerevisiae* strain (Ac0), which accounted for 100% of the identified bacteria. However, in the other two acetifications, although Ac0 was also the main genotype detected, the two *Acet. pasteurianus* genotypes (Ap1 and Ap2) identified in the spontaneous process samples were also isolated. The presence of these *Acet. pasteurianus* genotypes in the inoculated processes reveals adaptation of these micro-organisms to the blueberry conditions.

Therefore, the two genotypes of *Acet. pasteurianus*, which were present in most of the processes and took over the spontaneous acetification samples, could be good candidates for starter cultures. Furthermore, *Acet. pasteurianus*, which has always been linked to traditional wine vinegar production (Vegas *et al.* 2010) and has also been identified in cereal vinegar production by solid-state fermentation (Wu *et al.* 2010), could be suitable for use in alternative vinegar production methods such as the Schützenbach method.

Despite the presence of the two *Acet. pasteurianus* genotypes in the inoculated processes, there was a clear dominance of the *Acet. cerevisiae* inoculated strain (Ac0) throughout the acetifications, resulting in a considerable reduction in the production time. The studies of AAB inoculation carried out recently using traditional methods to produce Traditional Balsamic Vinegar (Gullo *et al.* 2009) and wine or fruit vinegar (Hidalgo *et al.* 2010b, 2013) reported that although the inoculated strain used in the vinegar mother production was not the dominant one at the end of the process, inoculation clearly improved the vinegar production process. In the present study, a high AAB population (10^8 cells ml^{-1}) was directly inoculated, unlike other processes where the inoculated AAB population was lower (approximately 10^6 cells ml^{-1}) or not controlled. Although this inoculation (10^8 cells ml^{-1}) was successful, it is not easy to implement this technique in plant vinegar, as it is difficult (high amount of material, long time, contamination risk) to obtain the volume necessary with this high population to inoculate industrial acetators of a vinegar plant. Hence, an in-depth study to find an adequate amount of pure culture on the production of a vinegar mother must be carried out to optimize this important part of the vinegar elaboration process.

For the first time, blueberry vinegar produced by the Schützenbach method based on spontaneous acetification and inoculation of an *Acet. cerevisiae* strain was studied. Although the spontaneous acetifications finished within an acceptable length of time, the use of the AAB starter culture resulted in a reduction in the time for acetification required of up to 66%. The identities and genetic variability of the AAB strains involved in the acetification processes were determined, and two genotypes of *Acet. pasteurianus* were isolated from both types of acetification. These results suggest that these *Acet. pasteurianus* genotypes can be used to perform an in-depth study to determine their possible technological potential as starter cultures in the production of blueberry vinegar or in studies of other vinegar production methods.

Materials and methods

Acetification conditions

Spontaneous and inoculated highbush blueberry wine acetifications were performed in triplicate and monitored daily for acetic acid production. The study was conducted under laboratory conditions in the Instituto de Nutrición y

Tecnología de los Alimentos (INTA) (Santiago de Chile, Chile). The AAB strain used as a starter was isolated from grapes from the northern Chilean valleys (Prieto *et al.* 2007) and was selected because of its ethanol resistance and high level of acetic acid production. This strain belongs to the *Acet. cerevisiae* species (Ac0) and was inoculated at a concentration of 1×10^8 cell ml^{-1}. The inoculum was prepared by growing this strain on glucose medium (GY medium) [10% glucose (Cultimed, Barcelona, Spain) and 1% yeast extract (Cultimed) w/v]. Cells were recovered by centrifugation (5 min, 11 200 ***g***) and used to inoculate blueberry wine.

The six acetification processes were carried out using the Schützenbach method under laboratory conditions. French oak shavings (1 g l^{-1}), serving as a bacterial support material and five litres of blueberry wine were used to carry out the acetification processes. A system of PVC pipes of 4 cm in diameter with a submersible 300 l h^{-1} pump was designed to move the acetifying liquid through the bed of oak shavings arranged in two chambers at different heights. The acetification temperature was controlled at 23°C.

The following samples were taken during acetification for microbiological analysis: initial mixture (T0); mid-acetification (when the ethanol was half consumed); and final acetification [when the ethanol concentration had fallen below 1% (v/v)]. Samples of the vinegar mother and wines were also analysed.

The titratable acidity was determined by titration with 0·1 mol l^{-1} NaOH and phenolphthalein as the indicator (Ough and Amerine 1987). The levels of ethanol and residual sugars (glucose and fructose) were measured with enzymatic kits (Boehringer, Mannheim, Germany).

AAB isolation and molecular analysis

Bacteria were counted by light microscopy using an improved Neubauer counting chamber (0·0025 mm^2 and 0·02 mm deep) and plated at an appropriate dilution on GY agar [GY medium with 1·5% agar (Cultimed, Barcelona, Spain)] supplemented with natamycin (100 mg l^{-1}) (Delvocid, DSM, Delft, The Netherlands) to suppress fungal growth. After incubation at 28°C for 3–5 days, between ten and fifteen colonies were randomly isolated at each point and plated on GYC (GY agar medium supplemented with 2% $CaCO_3$). Each bacterial colony that produced a clear halo on GYC was subjected to a catalase test, and the positive colonies were considered putative AAB isolates. A total of 203 isolates were analysed by molecular methods in the Universitat Rovira i Virgili (Tarragona, Spain).

Total DNA was extracted by the modified CTAB (cetyltrimethylammonium bromide) method described by Ausubel *et al.* (1992).

AAB genotyping was carried out using ERIC-PCR (González *et al.* 2004) and $(GTG)_5$-PCR (De Vuyst *et al.* 2008). In both cases, the molecular profiles were determined by both electrophoresis on 1·5% (w/v) agarose and analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany) with the 7500 Labchip and 12 000 Labchip kits (Panaro *et al.* 2000).

AAB identification was performed by amplifying and sequencing the 16S rRNA gene and 16S-23S rRNA gene internal transcribed spacer (ITS) region (Ruiz *et al.* 2000). The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea). The DNA sequences were compared with those in the GenBank database. Identity was established by 100% sequence homology with available sequences in all the cases.

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