Available online at www.sciencedirect.com
ScienceDirect
International Journal of Food Microbiology 125 (2008) 46–53
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Acetic acid bacteria in traditional balsamic vinegar: Phenotypic traits relevant for starter cultures selection

Maria Gullo *, Paolo Giudici

Department of Agricultural Sciences, University of Modena and Reggio Emilia, Via Amendola, 2, 42100 Reggio Emilia, Italy

Abstract

This review focuses on acetic acid bacteria in traditional balsamic vinegar process. Although several studies are available on acetic acid bacteria ecology, metabolism and nutritional requirements, their activity as well as their technological traits in homemade vinegars as traditional balsamic vinegar is not well known. The basic technology to oxidise cooked grape must to produce traditional balsamic vinegar is performed by the so called "seed-vinegar" that is a microbiologically undefined starter culture obtained from spontaneous acetification of previous raw material. Selected starter cultures are the main technological improvement in order to innovate traditional balsamic vinegar production but until now they are rarely applied. To develop acetic acid bacteria starter cultures, selection criteria have to take in account composition of raw material, acetic acid bacteria metabolic activities, applied technology and desired characteristics of the final product. For traditional balsamic vinegar, significative phenotypical traits of acetic acid bacteria have been highlighted. Basic traits are: ethanol preferred and efficient oxidation, fast rate of acetic acid production, tolerance to high concentration of acetic acid, no overoxidation and low pH resistance. Specific traits are tolerance to high sugar concentration and to a wide temperature range. *Gluconacetobacter europaeus* and *Acetobacter malorum* strains can be evaluated to develop selected starter cultures since they show one or more suitable characters.

Keywords: Acetic acid bacteria; Selected starter culture; Traditional balsamic vinegar

1. Introduction

Acetic acid bacteria (AAB) are well known for the ability to oxidise rapidly and incompletely carbon substrates, especially sugars and alcohols. This trait is used in several biotechnological processes among which include vinegar production, where acetic acid is produced from ethanol. Several types of vinegars are produced worldwide; they differ in raw materials, technologies and use (Giudici et al., 2006). Recently, new types of vinegar made with worthless raw materials or surplus products are produced and spread in the market, depending on the transformation of fermentescible worthless fruit and vegetable raw materials (Horiuchi et al., 1999). From a technological point of view, there are two well defined methods to produce vinegar: traditional (slow) and submerged (fast) processes (Tesfaye et al., 2002). Traditional methods are commonly referred to as "surface culture fermentations" and AAB grow abundantly on the media surface where oxygen concentration is higher. Submerged methods are usually used in semi-continuous processes for industrial vinegar production and they are characterized by a faster acetification process (Adams, 1998). However, as in traditional methods, oxidation is started by "seed-vinegar", the so-called "mother of vinegar" that is a microbiologically undefined starter culture obtained from previous vinegar.

Traditional balsamic vinegar (TBV) is homemade vinegar produced in Italy, by traditional method in surface culture fermentation. The raw material is cooked grape must having a content of soluble solids (above all glucose and fructose) ranging from 20 to 60°Bx and pH values of 2.3–3.2 (Solieri et al., 2006). As with other vinegars, it is obtained by a two-stage fermentation process. In the first one fermentable sugars are converted into ethanol by yeasts obtaining ethanol content between 4 and 10%; in the second one, AAB oxidise the ethanol to acetic acid. Both stages occur by spontaneous fermentation. The process is carried out through a set of 5/7 wood barrels arranged in a series of decreasing volume. A volume of final product is withdrawn from the smallest barrel (barrel 1) and replaced with equal volume of

* Corresponding author. Tel.: +39 0522 522063; fax: +39 0522 522027.
E-mail address: maria.gullo@unimore.it (M. Gullo).

0168-1605/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2007.11.076

product from the barrel immediately preceding in the set, the same is done for each intermediate barrel; the first one (barrel 5) receives new cooked must. This procedure, known as to "refilling", is done once per year, for the minimum period of 12 years (Fig. 1). Along the barrels set the sugar concentration increases due to the evaporation trough the opening on the top of the barrel and the wood; the correspondent loss of water, together with low pH, is responsible for restrictive conditions for AAB growth, consequently bacterial activity decreases from the first barrel to the last, where biological activity is not detectable. As established in an official TBV guideline (Production disciplinary of DOP, 2000), the final product must have a total acidity of 4.5% (expressed as acetic acid g/100g) and relative density higher than 1.240, no other quantitative parameters are defined.

Few in depth knowledge are available about TBV and technological traits of AAB able to optimise acetification process. Therefore the aim of this review is to underline the scientific gap and to propose a start point to select AAB strains for starter cultures improving a more reliable TBV technological process.

1.1. Acetic acid bacteria in TBV

AAB species that occur in TBV are not well known, and there are only few ecological studies and they are spread on a wide period of time (Sacchetti, 1970; Turtura and Benfanti, 1998; De Vero et al., 2006a; Gullo et al., 2006; Gullo and Giudici, 2006), with a significant gap between older papers and current AAB taxonomy.

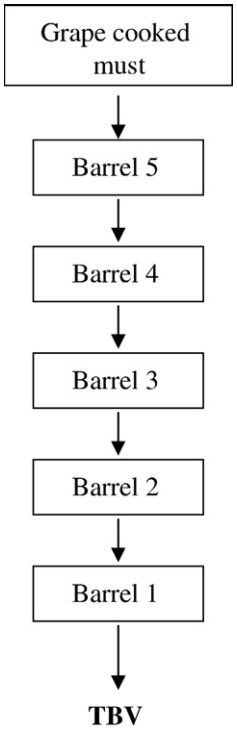

Fig. 1. Schematic representation of TBV production performed in a set of 5 wood barrels.

Table 1
Acetic acid bacteria species isolated from different kind of vinegars, wine and beer

Source	Specie	Reference
Rice vinegar	*A. pasteurianus*	Nanda et al. (2001)
		Haruta et al. (2006)
Industrial vinegar	*Ga. europaeus*	Sievers et al. (1992)
	A. oboediens; A. pomorum	Sokollek et al. (1998)
	A. intermedius	Boesch et al. (1998)
	Ga. entanii	Schüller et al. (2000)
Traditional balsamic vinegar	*Ga. xylinus; A. aceti*; *A. pasteurianus*	Gullo et al. (2006)
	Ga. europaeus; *Ga. hansenii*; *A. pasteurianus*; *A. malorum*	Gullo and Giudici (2006)
Wine	*A. pasteurianus*	Bartowsky et al. (2003)
	G. oxydans; Ga. hansenii; A. aceti	González et al. (2004)
	A. nitrogenifigens	Dutta and Gachhui (2006)
	A. oeni	Silva et al. (2006)
Beer	*A. cerevisiae*	Cleenwerck et al. (2002)
	Ga. sacchari	Franke et al. (1999)

A.: *Acetobacter*; G.: *Gluconobacter*; Ga.: *Gluconacetobacter*.

Among the ten genera of AAB now recognized, vinegar oxidation as well as spoilage of wine and beer is due mainly to strains belonging to the *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* species (Table 1). Recently, strains of AAB have been isolated from must for TBV production and identified by physiological and molecular methods. In particular strains belonging to the following species were detected: *Gluconacetobacter europaeus* (25 strains), *Gluconacetobacter hansenii* (1 strain), *Gluconacetobacter xylinus* (1), *Acetobacter pasteurianus* (2 strains), *Acetobacter aceti* (1 strain) and *Acetobacter malorum* (7 strains) (De Vero et al., 2006a; Gullo and Giudici, 2006). All these species were previously detected in vinegar, as showed in Table 1, except for *A. malorum*. The isolation procedure of AAB strains from TBV performed with different enrichment and isolation media, showed heterogeneous results, since not all media were able to support bacterial growth (Gullo et al., 2006). In particular, none or very slow growth was observed during isolation without enrichment media. After *ad hoc* set up of enrichment media containing sterile grape must at different concentration of ethanol, acetic acid, gluconic acid and sorbitol successful isolation of AAB was obtained (Gullo, 2004). Among isolation media, GYC (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar) and AE (0.5% glucose, 0.3% yeast extract, 0.4% peptone, 0.9% agar, 3 ml absolute ethanol, 3 ml glacial acetic acid) allowed the isolation of the higher number of strains, were YPM (0.5% yeast extract, 0.3% peptone, 2.5% mannitol, 1.2% agar) and MYA (1.5% malt extract, 0.5%, yeast extract 1,5% agar 60 ml ethanol) were less efficient. The AE was useful to isolate strains, but unsuitable for preservation because during long-time storage at +4 °C it affects the viability of cells. The difficulty of isolation and preservation of AAB is well known also for strains isolated from industrial vinegar (Sievers et al., 1992; Sokollek and Hammes, 1997; Schüller et al., 2000). Moreover Trcek (2005) reported that AAB strains involved in high acetic acid vinegar

production are rarely successfully isolated from an environment with an acetic acid concentration exceeding 10% and pH value as 2. Even after successful isolation, it is extremely difficult to handle the isolates and to preserve their high acetic acid resistance under laboratory conditions. High concentrations of un-ionized acetic acid exert more stress on the cell during preservation, because they are metabolically inactive. This is one of the reasons why strains need to be frequently transplanted in fresh media with calcium carbonate that reduce undissociated acetic acid increasing cell survival rates (Sokollek et al., 1998; Schüller et al., 2000).

Several studies performed on different AAB sources showed that in environments like wine, the nutritional requirement of AAB increases, for instance low pH (3.0–4.0) and high ethanol concentration (10%–15%), could alter their amino acid requirements (Gosselé et al., 1981; Drysdale and Fleet, 1988). Moreover the effect of nitrogen source and growth factors on AAB growth is critically influenced by the availability of carbon sources and energy (Rao and Stokes, 1953). In chemically defined media they are able to grow using inorganic ammonia as the sole source of nitrogen and there are not known requirements for essential amino acids. However among amino acids glutamate, glutamine, proline and histidine have stimulator effects, whereas other are inhibitory as valine for *Gluconobacter oxydans* and threonine and homoserine for *A. aceti* (Drysdale and Fleet, 1988). As described in the introduction, low pH and high sugar content are responsible for cooked must selectivity. Moreover, limited nutritional factors (above all growth factors) due to cooking process can negatively affects metabolic regulation and physiological activities resulting in uncultivable state of cells.

Alternative methods to study viable but not cultivable AAB are needed for in depth study of TBV ecosystem. In wine vinegar, for instance, Baena-Ruano et al. (2006) investigated the determination of AAB viability using an epifluorescence staining method. However this method showed the great disadvantage of not reliable discrimination of dead and live state of cells. More efficient methods are based on FISH (*Fluorescence in Situ Hybridisation*) techniques that are applied to study environmental, marine and food ecosystems (Zwirglmaier, 2005; Schmid et al., 2005) but until now never applied to TBV. These techniques allow to investigate AAB without handling them outside vinegar environment improving the determination of their effective occurrence, dominance and activity.

2. Relevant phenotypic traits of AAB in TBV acetification process

Phenotypical expression of AAB is strictly connected to physico-chemical composition of the medium. The oxidation of sugars and alcohols is carried out by two sequential reactions of membrane-bound quinohemoprotein alcohol dehydrogenase (ADH) and membrane-bound quinohemoprotein aldehyde dehydrogenase (ALDH) that have their active sites on the outer surface of the cytoplasmatic membrane (Matsushita et al., 2005; Saeki et al., 1997a). These two enzymes have a crucial role in the oxidation process. Furthermore the location of the active site makes these enzymes particularly sensitive to the medium composition.

The absence of process control together with the composition of cooked must are the main factors that affect AAB oxidation in TBV. Traits required can be summarized in ability to oxidise ethanol and carbohydrates, tolerance to acetic acid and high sugar concentration, as well as the capacity to grow at particular oxygen, temperature and pH conditions.

2.1. Ethanol, oxidation and tolerance

The ability to oxidise ethanol is a general feature of AAB, but there is a wide variability among strains and species regarding the amount of acetic acid produced.

Among AAB genera ethanol in some cases is preferred to sugars e.g. for *Acetobacter* and *Gluconacetobacter* genera, whereas *Gluconobacter* spp. oxidise sugars more easily than ethanol.

Carbon sources preference has ecological consequences, for instance *G. oxydans* grows widely on unspoiled grape, while *A. aceti* and *A. pasteurianus* prevail in spoiled grapes and became predominant during alcoholic fermentation (Joyeux et al., 1984). The effect of ethanol on AAB has been evaluated in wine where cells remain viable with 10 and 14% (v/v) of ethanol (Du Toit and Pretorius, 2002). However ethanol tolerance is a species and strain-dependent trait that is conditioned by temperature, pH and oxygen (Drysdale and Fleet, 1988; Du Toit and Pretorius 2002).

48 AAB strains isolated from fermented cooked must were tested for their growing ability at different ethanol concentrations (Gullo, 2004). *Ga hansenii, A. pasteurianus* and *Ga. europaeus* strains grew up to 10% of ethanol in 5% of yeast extract medium, but variability among strains of the same species was observed. For instance among 25 *Ga. europaeus* strains 11 of them were able to grow at 5% of ethanol but not at 10%. It is a common practice in TBV to use fermented cooked must with less than 8% (v/v) of ethanol (Gullo et al., 2006) for this reason the ethanol tolerance is not considered a limiting growth factor in the existing production system.

2.2. Acetic acid, tolerance and oxidation

During TBV making acetic acid concentration rarely overcomes 5%, increasing in the period of intense AAB activity. This acid is both the main product of AAB ethanol oxidation and a limiting factor for their growth. Requirement and tolerance to high concentrations of acetic acid are distinctive traits of isolates from industrial vinegar productions, where acetic acid content is around 12% (Adams, 1998). *Ga. europaeus*, which strains require acetic acid, to grow is the main species recovered from industrial vinegar (Sievers and Teuber, 1995). In this species, high ADH activity and stability enable strains to grow and stay metabolically active at high concentration of acetic acid (Trcek et al., 2006). In other AAB species (e.g. *A. pasteurianus* and *Gluconacetobacter intermedius*), high acetic acid concentration causes cell stress due to

faster decrease of ADH activity. In general, strains tolerance range is correlated with media conditions and substrates. For instance, *A. aceti* in continuous industrial process evolves the ability to grow with acetate concentrations exceeding 5%, but this phenotype is stable for several generations only in the absence of selective pressure (Steiner and Sauer, 2003). Higher tolerance to acetic acid (11.5–12.0%) was observed in semi-continuous industrial vinegar process carried out by seed-vinegar as starter, due the occurrence of strains with different abilities to adapt to medium condition occur (Fregapane et al., 1998).

2.3. Carbohydrates oxidation and sugar tolerance

As mentioned in the introduction, sugar concentration of barrels changes according to the dimension and position in the cask set. The largest barrel (the first of the set), where biological activity is observed, has the lowest sugar concentration. After alcoholic fermentation of cooked must, substantial quantities (20–40%) of sugar still remain unfermented mainly represented by glucose and fructose (Solieri et al., 2006). The large sugar range depends on the alcoholic fermentation process that is not monitored and no official guideline to conduct the fermentation has been proposed until now. In spite of sugars as excellent carbon sources for AAB, high concentration found in TBV (40%) inhibits AAB growth (Gullo et al., 2006). Among AAB genera there are osmotolerant species and one osmophilic genus: *Saccharibacter*, recently described by Jojima et al. (2004) as able to grow at 40% (w/v) of glucose. The species generally involved in industrial vinegar production do not show high sugar tolerance, where this trait is required in TBV production. We tested AAB strains isolated from cooked must for growing at increasing sugar concentration: among 48 strains all of them grown at 20% of glucose in broth with 5% of yeast extract but only 4 at 25% of glucose (Gullo, 2004). 25 *Ga. europaeus* strains showed growth at 20%. Among more sugar tolerant species, 7 strains of *A. malorum* where detected in samples of TBV (De Vero et al., 2006a,b). This species was first described by Cleenwerck et al. (2002) as AAB of spoiled apples and differ phenotipically from other *Acetobacter* species for the ability to grow on 30% of D-glucose.

From a biochemical point of view, there are different metabolic pathways of carbohydrates oxidation. For instance, the genus *Acetobacter* utilises glucose through both the hexose monophosphate pathway (De Ley et al., 1984; Drysdale and Fleet, 1988), as well as through the Embden-Meyerhof-Parnas and Entner-Doudoroff pathways (Attwood et al., 1991). Successively metabolites can be further oxidised to CO_2 and H_2O through the tricarboxylic acid pathway.

In the *Gluconobacter* genus sugar metabolism is largely studied in consequence of the several biotechnological applications, e.g. vitamin C and gluconic acid production for food, pharmaceutical and hygienic applications in industries (Ramachandran et al., 2006). The oxidative potential, as well as the mechanism of incomplete oxidation in *G. oxydans*, has been recently well elucidated by Prust et al. (2005) that have sequenced the complete genome. They observed that *G. oxydans* contains many membrane-bound dehydrogenases that allow to thrive and to survive in nutrient-rich environment, promoting the rapidly formation of sugars or sugar acids that are difficult to assimilate for most microorganisms. Furthermore, the incomplete oxidation of glucose and other aldoses lead to the formation of sugar acids and to decrease in pH value, thereby prevent growth of other microorganisms. Among sugar acids in TBV, gluconic acid has been studied by several authors. Glucose availability and very low nitrogen and phosphorus sources are optimal conditions for gluconic acid production and concentrations of about 3.0% were observed by Plessi et al. (1989) and Giudici (1993). Furthermore the highest values (3.0%) were detected in barrels containing the oldest and most concentrated product. Lower concentrations (0,37 and 0,28%) were found in wine vinegar, balsamic vinegar (obtained by fast acetification of a blend of cooked grape must and wine vinegar) and cider vinegar. The occurrence of gluconic acid has been proposed as an indicator of TBV quality, differentiating it from other balsamic vinegars (Giudici et al., 1994).

2.4. Oxygen requirement

Oxygen is a limiting factor in acetification processes because it is only sparingly soluble in aqueous media and both temperature and solute content condition its solubility (Adams, 1998). In industrial wine vinegar produced by submerged culture in semi-continuous processes, the concentration of dissolved oxygen is the most important parameter that allows bacterial growth, so that air is supplied. However, it has been established that a high dissolved oxygen value can inhibit AAB growth and that the optimum concentration in semi-continuous processes is 1–3 mg kg^{-1} (Rubio-Fernández et al., 2004).

Differently from submerged culture, surface traditional methods have not forced oxygen solubilization system. TBV making is performed in wood barrels and oxygen transfer is favoured by the span on the top of the barrels, filled in a 3/4 volume ratio. The headspace between the liquid surface and the top of the barrels allows aerobic conditions for AAB that produce a surface film. Besides oxygen penetrates through the wood at a rate of about 30 mg l^{-1} per year (Joyeux et al., 1984). This evidence derives from wine production field, where this oxygen support prevents the complete destruction of AAB population, resulting in wine spoilage. Also in bottled wine, AAB occurrence has been described (Bartowsky et al., 2003). This is due to AAB ability to survive under anaerobic conditions, using electron acceptors other than oxygen (Drysdale and Fleet, 1989).

2.5. Optimal growth temperature

TBV manufacturing is performed in locals called "acetaia" where no physical parameters are controlled, so the product is subjected to climate variation. In general AAB activity is prevailing in spring and summer, but stuck of oxidations can take place as consequence of the increase of temperature that

can reach 40 °C. AAB are mesophilic microorganisms and their optimum growth temperature is between 25 and 30 °C. Above optimum temperature, bacterial deactivation processes occur since essential enzymes are denatured, membrane damage causes cellular constituents scattering and the organisms become more sensitive to the toxic effect of acetic acid (de Ory et al., 1998). Minimum and maximum growth temperatures are more difficult to define both for the variability among the species and for the influence of medium composition. De Ory et al. (1998) demonstrated that in discontinuous culture, *A. aceti* cannot grow below 8 °C. About upper temperature limits, several studies showed the occurrence of thermotolerant AAB strains in industrial vinegar production (Saeki et al., 1997b; Moonmangmee et al., 2000). These thermotolerant strains were able to oxidise ethanol at high temperatures (38–40 °C) and ethanol concentrations (up to 9%) without any appreciable lag time; they worked rapidly with a higher fermentation rate, where mesophilic strains were unable to do it. Recently, Ndoye et al. (2006) have selected two strains of *A.tropicalis* and *A. pasteurianus* species for the ability to grow at 40 and 45 °C and proposed them to produce an artisanal spirit vinegar.

Since oxidation is an exothermic reaction and causes a substantial temperature increase [around 8.4 MJ for every litre of ethanol oxidised (Adams, 1998)], thermotolerance is an advantageous AAB trait for industrial vinegar production because it reduces cooling expenses (Adachi et al., 2003). Also in TBV the thermotolerance is a positive trait to avoid breakdown of oxidation during summer.

2.6. Low pH growth

Despite the optimal pH growth of AAB is between 5.0–6.5 (De Ley et al., 1984; Drysdale and Fleet 1988), they are also able to grow at lower pH values, as in TBV, where bacterial activity has been detected for pH values lower than 3 (De Vero et al., 2006b). Also in wine with pH values from 3.02 to 3.85 AAB growth was well documented (Drysdale and Fleet, 1985). Kittelmann et al. (1989) isolated AAB strains from aerated acetate media at pH of 2.0–2.3. However the tolerance to low pH is strongly dependent on other parameters, such as ethanol concentration and oxygen availability. In particular, high concentration of ethanol (12.5%) increases pH sensitivity, as observed in *A. pasteurianus* (Dupuy and Maugenet, 1963 as quoted in Du Toit and Pretorius, 2002). When pH is below 3.4, low oxygen concentration causes a fast decrease of viable cell number of *A. aceti* (Joyeux et al., 1984).

3. TBV defects

Total or partial inactivation of AAB during the production of TBV results in low acidification levels producing a high-pH that causes a lack of preservation properties and hence allows the growth of spoiling microorganisms. Two causes of acidification slowness are bacteriophage infection and overoxidation of acetic acid; other source of defect is the extracellular polysaccharides production.

3.1. Bacteriophage infection

Bacteriophage infection can cause problems in vinegar production by inducing lysis of AAB cells and is considered a cause of unexplained fermentation breakdown. Although the exact quantification of bacteriophage infection is affected by the difficulty to keep and handle AAB under laboratory conditions, some authors have observed a high presence of phages in vinegar production with a spontaneously developed culture of AAB (Teuber et al., 1987, Stamm et al., 1989; Sellmer et al., 1992). The problem is more serious in submerged acetification than in quick vinegar generators where a diversity of strains differing in phage-susceptibility can be found; so that acetification can be slowed as a result of phage infection but does not stop completely. The use of pasteurized substrate, sterile filtered air and the strict separation of submerged fermenters from seed-vinegar are used as precautionary measures to avoid bacteriophage infections in the industrial field (Sellmer et al., 1992).

3.2. Overoxidation

In addition to their ability to oxidise ethanol, *Acetobacter* and *Gluconacetobacter* species can further oxidise acetic acid to CO_2 and H_2O, generating the so-called acetate overoxidation, that is carried out by the tricarboxylic acid cycle when there is a high level of dissolved oxygen and no ethanol in the medium. Strains of *Gluconobacter* are not able to overoxidise because of nonfunctional α-ketoglutarate dehydrogenase and succinate dehydrogenase of tricarboxylic acid cycle; they can only oxidise ethanol to acetic acid (Greenfield and Claus, 1972; as quoted in Du Toit and Pretorius 2002). As reported by Matsushita et al. (2005), *A. aceti* in ethanol culture have three growth phases; firstly, cells grow by oxidizing the ethanol to acetic acid completely (ethanol oxidation phase; then the growth stops and the viable cell number remains stationary for a long time (first stationary phase); finally the growth starts again using the acetic acid accumulated through overoxidation. In vinegar, overoxidation reactions are documented by several authors (Mariette et al., 1991, Saeki et al., 1997a; Sokollek et al., 1998). In particular, Saeki et al. (1997a) reported that the overoxidation is a serious problem during vinegar making without temperature control in tropical and temperate countries. Reasons for overoxidation could be changes in the population or in the physiology of strains, stimulated by the lack of alcoholic substrate.

3.3. Extracellular polysaccharides

Dextrans, levans and cellulose are the main extracellular polysaccharides produced by AAB glucose metabolism. Occurrence of polysaccharides is a disadvantage in vinegar industry because it negatively affects the filterability of the product. Although TBV is not a filtered vinegar, polysaccharides are undesired since their occurrence is detrimental for the sensorial quality of the final product. In TBV high polysaccharides production is often observed as a thick layer on the barrel surface, caused by high-cellulose-producing strains, which increase this ability in static condition (Hwang et al., 1999).

Among AAB species, *Ga. xylinus* is used as model organism in studying the mechanism of cellulose production (Swings, 1992). This trait is largely used for biological synthesis of the polymer and several studies have been done about the influence of substrates availability on cellulose synthesis. Ramana et al. (2000) observed a higher productivity using mannitol as carbon substrate followed by glucose and sucrose. Other carbon sources, such as sorbitol, galactose, maltose, starch and acetic acid, proved to be poor substrates yielding relatively low amounts of cellulose. Various nitrogen sources (peptone, ammonium sulphate or caseine hydrolysate) were found to be suitable for cellulose synthesis associated to sucrose, glucose or mannitol.

4. Selection criteria for AAB starter culture development

Selecting strains with interesting properties to be used as new starter cultures may lead to an improved fermentation process and an enhanced quality TBV respect to fermentation processes by indigeneous cultures, where optimization of expressing strains properties as well as elimination of undesirable microbial side effects are not possible. Selected starter culture are the result of advanced technological applications and they are widely diffused in several fermented food productions, such as wine, beer, bread, dairy products, fermented meat sausages and various vegetables (Buckenhüskes, 1993; Leroy and De Vuyst, 2004), but they are rarely applied to vinegar production (Sokollek and Hammes, 1997). Up to now, all the attempts to isolate and maintain strains for this task have not produced successful results. The main obstacle has been found in the recovery of cells in culture media. (Mariette et al., 1991; Sokollek and Hammes, 1997). Then, the first requirement is to set up procedures to overcome isolation, cultivation, and preservation problems of strains. In this way it will be possible to have an AAB strains collection to perform the selection by a multi step screening procedure. Once selected, it is possible to further modify a strain's characteristic in order to improve its suitability by transfer of genetic material from one strain to another. Some techniques, such as conjugation between cells are regarded as natural events and widely accepted (GRAS status–Generally regarded as safe); others, such as gene cloning are more controversial and less accepted techniques. (Stanley, 1998).

With the purpose to develop AAB starter cultures for TBV making, selection criteria have to take in account the characteristics of raw material, desired AAB metabolic activities, applied technology and desired characteristics of the final product. In this way, the evaluation of AAB traits with technological significance as well as those involved in the final product quality is to be considered. To satisfy these requirements, strains selection must be based on the ability to achieve a precise function or perform a specific task (Giudici et al., 2005). About TBV, some required traits are supported by sufficient information, some other have to be studied. Suitable basic traits of AAB for vinegar production are generally known as well as some parameters which influence AAB growth mainly reported during the last years in several papers that focused AAB contamination in wine (Joyeux et al., 1984; Millet and Lonvaud-Funel, 2000; Drysdale and Fleet 1988, 1989; Du Toit and Pretorius, 2002; Bartowsky et al., 2003).

A proposed list of AAB traits is presented in Table 2: they are divided into basic traits common to industrial vinegar production and specific for TBV making. Basic phenotypical AAB traits include those that favourite their persistence and dominance over the whole oxidation process. For example, ethanol preferred and efficient oxidation is strictly joined to a quick production of acetic acid that prevents the restarting of alcoholic fermentation and avoids spoilage by other organisms such as moulds. The fast increasing of acetic acid concentration requires an adequate tolerance of this acid. Both preference to oxidise ethanol rather than sugars and the ability to grow in acetic acid medium highlights the suitability of *Ga. europaeus* strains to achieve these technological tasks. At the same time other characters (low polysaccharide production and bacteriophage resistance) are needed to avoid defects in the final products mainly represented by undesired sensorial profile and low acidity.

Specific AAB traits for TBV are not completely known, so that new scientific knowledge will add other technological requirements. However, on the basis of the available information, some of them can be suggested, such as tolerance to sugars and to temperature range. Sugar concentration is the main selective pressure source in TBV, a similar role to ethanol and acetic acid in industrial vinegar production. Among isolates from TBV strains *A. malorum* shows resistance to 30% of glucose: this suggests that it can be useful to carry out oxidation in the long run of the process. Further investigations are needed to evaluate the suitability of *A. malorum* strains in TBV environment. Generally, osmotolerant selectable strains have to be evaluated also for other specific traits like a wide range of temperature tolerance.

Another main task is to preserve desired expressing traits of AAB in vitro as well as in the starter culture propagation medium. In this respect, some authors observed the loss of physiological activities in AAB as consequence of spontaneous mutations. For instance, Kondo and Horinouchi, (1997) studied spontaneous high-frequency mutations resulting from ISs family insertions, that are responsible for genetic instability

Table 2
Basic and specific phenotypical trait of AAB for vinegar production

AAB basic phenotypical traits for vinegars
Ethanol preferred and efficient oxidation
Fast production of acetic acid
Tolerance to acetic acid
No overoxidation
Low pH resistance
Bacteriophage resistance
No cellulose production
No undesidered aroma
AAB specific phenotypical traits for TBV
Osmotolerance
Tolerance to temperature range

leading to deficiencies in various physiological properties of AAB, such as ethanol oxidation and cellulose production. In *A. pasteurianu*s it was found an insertion sequence element associated with the inactivation of the ADH by insertion in the *adhA* of *A. aceti*, which can explain the inability of spontaneously derived mutants to oxidise ethanol.

5. Conclusions

The acetic oxidation of TBV occurs through the use of seed-vinegar. This practice, although is widely used in vinegar production, causes lack in the reliability and standardisation of the process. These evidences have been confirmed by studies showing the difficulty to have efficient oxidation by spontaneous acetification. Many variables affect the growth and activity of AAB during acetification, above all sugar concentration among substrates and temperature among physical parameters. AAB current knowledge can guide the application of selected starter cultures in TBV production. However, applied studies are still needed to implement the starter culture in the current technology. The optimisation of isolation and cultivation procedures may contribute to perform more quantitative ecological studies of AAB contributing to strain selection and process set up. This may result in better process control, enhancing the quality of TBV.

Acknowledgements

This work was supported by a grant from the European Communities (Winegar–Craft. Co-operative Research 6FP).

References

Adachi, O., Moonmangmee, D., Toyama, H., Yamada, M., Shinagawa, E., Matsushita, K., 2003. New developments in oxidative fermentation. Applied Microbiology and Biotechnology 60, 643–653.

Adams, M.R., 1998. Vinegar. In: Wood, Brian, J.B. (Ed.), Microbiology of Fermented Foods. Blacki Academic and Professional, London, pp. 1–44.

Attwood, M.M., Van Dijken, J.P., Pronk, J., 1991. Glucose metabolism and gluconic acid production by *Acetobacter diazotrophicus*. Journal of Fermentation and Bioengineering 72, 101–105.

Baena-Ruano, S., Jiménez-Ot, C., Santos-Dueñas, I.M., Cantero-Moreno, D., Barja, F., García-García, I., 2006. Rapid method for total, viable and non-viable acetic acid bacteria determination during acetification process. Process Biochemistry 41, 1160–1164.

Bartowsky, E.J., Xia, D., Gibson, R.L., Fleet, G.H., Henschke, P.A., 2003. Spoilage of bottled red wine by acetic acid bacteria. Letters and Applied Microbiology 36, 307–314.

Boesch, C., Trcek, J., Sievers, M., Teuber, M., 1998. *Acetobacter intermedius*, sp. nov. Systematic and Applied Microbiology 21, 220–229.

Buckenhüskes, H.J., 1993. Selection criteria for lactic acid bacteria to be used as starter for food commodities. FEMS Microbiology Reviews 12, 252–272.

Cleenwerck, I., Vandemeulebroecke, K., Janssens, D., Swings, J., 2002. Re-examination of the genus *Acetobacter*, with descriptions of *Acetobacter cerevisiae sp. nov.* and *Acetobacter malorum sp. nov.* International Journal of Systematic and Evolutionary Microbiology 52, 1551–1558.

De Ley, J., Gillis, M., Swings, J., 1984. In: Holt, J.G. (Ed.), Family VI. Acetobacteraceae. Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins Baltimore, Baltimore, pp. 267–278.

de Ory, I., Romero, L.E., Cantero, D., 1998. Modelling the kinetics of growth of *Acetobacter aceti* in discontinuous culture: influence of the temperature of operation. Applied Microbiology and Biotechnology 49, 189–193.

De Vero, L., Gala, E., Gullo, M., Solieri, L., Landi, S., Giudici, P., 2006a. Application of denaturing gradient gel electrophoresis (DGGE) analysis to evaluate acetic acid bacteria in traditional balsamic vinegar. Food Microbiology 23, 809–813.

De Vero, L., Gullo, M., Gala, E., Giudici, P., 2006b. Aspetti microbiologici nella gestione di una batteria di Aceto Balsamico Tradizionale. Industrie Delle Bevande XXXV, 443–448.

Drysdale, G.S., Fleet, G.H., 1985. Acetic acid bacteria in some Australian wines. Food Technology in Australia 37, 17–20.

Drysdale, G.S., Fleet, G.H., 1988. Acetic acid bacteria in winemaking: a review. American Journal of Enology and Viticulture 39, 143–154.

Drysdale, G.S., Fleet, G.H., 1989. The growth and survival of acetic acid bacteria in wines at different concentrations of oxygen. American Journal of Enology and Viticulture 40, 99–105.

Dupuy, P., Maugenet, J., 1963. Métabolisme de l'acid lactique par *Acetobacter rancens*. Annales Technologie Agricole 12, 5–14.

Du Toit, W.J., Pretorius, I.S., 2002. The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. Annals of Microbiology 52, 155–179.

Dutta, D., Gachhui, R., 2006. Novel nitrogen-fixing *Acetobacter* nitrogenifigens sp. nov., isolated from Kombucha tea. International Journal of Systematic and Evolutionary Microbiology 56, 1899–1903.

Franke, I.H., Fegan, M., Hayward, C., Leonard, G., Stackebrandt, E., Sly, L.I., 1999. Description of *Gluconacetobacter sacchari* sp. nov., a new species of acetic acid bacterium isolated from the leaf sheath of sugar cane and from the pink sugar-cane mealy bug. International Journal of Systematic Bacteriology 49, 1681–1693.

Fregapane, G., Rubio-Fernández, H., Nieto, J., Salvador, M.D., 1998. Wine vinegar production using a noncommercial 100-litre bubble column reactor equipped with a novel type of dynamic sparger. Biotechnology and Bioengineering 63, 141–146.

Giudici, P., 1993. Acido gluconico: criterio di genuinità dell'aceto balsamico tradizionale. Industrie Delle Bevande 22, 123–125.

Giudici, P., Barbagallo, R.N., Altieri, C., Masini, G., 1994. Origine ed evoluzione degli acidi organici durante l'invecchiamento dell'aceto balsamico tradizionale. Industrie Delle Bevande XXIII, 569–574.

Giudici, P., Solieri, L., Pulvirenti, A., Cassanelli, S., 2005. Strategies and perspectives for genetic improvement of wine yeasts. Applied Microbiology and Biotechnology 66, 622–628.

Giudici, P., Gullo, M., Solieri, L., De Vero, L., Landi, S., Pulvirenti, A., Rainieri, S., 2006. Gli aceti del mondo. In: Diabasis (Ed.), Le fermentazioni dell'aceto balsamico tradizionale. Reggio Emilia, Italy, pp. 7–13. ISBN: 888103421.

González, Á., Hierro, N., Poblet, M., Rozès, N., Mas, A., Guillamón, J.M., 2004. Application of molecular methods for the differentiation of acetic acid bacteria in a red wine fermentation. Journal of Applied Microbiology 96, 853–860.

Gosselé, F., den Mooter, M., Van Verdonck, L., Swings, J., De Ley, J., 1981. The nitrogen requirements of *Gluconobacter, Acetobacter* and *Frateuria*. Antonie van Leeuwenhoek 47, 289–296.

Greenfield, S., Claus, G.W., 1972. Nonfunctional tricarboxylic acid cycle and the mechanism of glutamate biosynthesis in *Acetobacter suboxydans*. Journal of Bacteriology 112, 1295–1301.

Gullo, M., 2004. Tesi di dottorato: "I batteri dell'aceto balsamico tradizionale: caratteristiche fenotipiche e molecolari".

Gullo, M., Giudici, P., 2006. Isolation and selection of acetic acid bacteria strains for traditional balsamic vinegar. Industrie Delle Bevande 35, 345–350.

Gullo, M., Caggia, C., De Vero, L., Giudici, P., 2006. Characterization of acetic acid bacteria in "traditional balsamic vinegar". International Journal Food Microbiology 106, 209–212.

Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., Ishii, M., Igarashi, Y., 2006. Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. International Journal of Food Microbiology 109, 79–87.

Horiuchi, J.I., Kanno, T., Kobayashi, M.I., 1999. New vinegar production from onions. Journal of Bioscience and Bioengineering 88, 107–109.

Hwang, J.W., Yang, Y.K., Hwang, J.K., Pyun, Y.R., Kim, Y.S., 1999. Effects of pH and dissolved oxygen on cellulose production by *Acetobacter xylinum* BRC5 in agitated culture. Journal of Bioscience and Bioengineering 88, 183–188.

Jojima, Y., Mihara, Y., Suzuki, S., Yokozeki, K., Yamanaka, S., Fudou, R., 2004. *Saccharibacter floricola* gen. nov., sp. nov., a novel osmophilic acetic acid bacterium isolated from pollen. International Journal of Systematic and Evolutionary Microbiology 54, 2263–2267.

Joyeux, A., Lafon-Lafourcade, S., Ribereau-Gayon, P., 1984. Evolution of acetic acid bacteria during fermentation and storage of wine. Applied and Environmental Microbiology 48, 153–156.

Kittelmann, M., Stamm, W.W., Follmann, H., Truper, H.G., 1989. Isolation and classification of acetic acid bacteria from high percentage vinegar fermentations. Applied Microbiology and Biotechnology 30, 47–52.

Kondo, K., Horinouchi, S., 1997. Characterization of the genes encoding the three-component membrane-bound alcohol dehydrogenase from *Gluconobacter suboxydans* and their expression in *Acetobacter pasteurianus*. Applied and Environmental Microbiology 63, 1131–1138.

Leroy, F., De Vuyst, L., 2004. Lactic acid bacteria as functional starter cultures fro the food fermentation industry. Trends in Food Science & Technology 15, 67–68.

Mariette, I., Schwarz, E., Vogel, R.F., Hammes, W.P., 1991. Characterization by plasmid profile analysis of acetic acid bacteria from wine, spirit and cider acetators for industrial vinegar production. Journal of Applied Bacteriology 71, 134–138.

Matsushita, K., Inoue, T., Theeragool, G., Trcek, J., Toyama, H., Adachi, O., 2005. Acetic acid production in acetic acid bacteria leading to their 'death' and survival. In: Yamada, M. Kerala (Ed.), Survival and Death in Bacteria. Research Signpost, Kerala, India, pp. 169–181.

Millet, V., Lonvaud-Funel, A., 2000. The viable but non-culturable state of wine micro-organisms during storage. Letters in Applied Microbiology 30, 136–141.

Moonmangmee, D., Adachi, O., Ano, Y., Shinagawa, E., Toyama, H., Theeragool, G., Lotong, N., Matsushita, K., 2000. Isolation and characterization of thermotolerant *Gluconobacter* strains catalyzing oxidative fermentation at higher temperatures. Bioscience, Biotechnology and Biochemistry 64, 2306–2315.

Nanda, K., Taniguchi, M., Ujike, S., Ishihara, N., Mori, H., Ono, H., Murooka, Y., 2001. Characterization of acetic acid bacteria in traditional acetic acid fermentation of rice vinegar (Komesu) and unpolished rice vinegar (Kurosu) produced in Japan. Applied and Environmental Microbiology 67, 986–990.

Ndoye, B., Lebecque, S., Dubois-Dauphin, R., Tounkara, L., Guiro, A.T., Kere, C., Diawara, B., Thonart, P., 2006. Thermoresistant properties of acetic acids bacteria isolated from tropical products of Sub-Saharan Africa and destined to industrial vinegar. Enzyme and Microbial Technology 39, 916–923.

Plessi, M., Monzani, A., Coppini, D., 1989. Quantitative determination of acids and derivatives in balsamic and other vinegars. Sciences Des Aliments 9, 179–183.

Production disciplinary of DOP, 2000. Aceto balsamico tradizionale di Modena. Provv. May 15, 2000, published in Gazzetta Ufficiale Italiana, p. 124 (May 30).

Prust, C., Hoffmeister, M., Liesegang, H., Wiezer, A., Fricke, W.F., Ehrenreich, A., Gottschalk, G., Deppenmeier, U., 2005. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nature Biotechnology 23, 195–200.

Ramachandran, S., Fontanille, P., Pandey, A., Larroche, C., 2006. Gluconic acid: properties, applications and microbial production. Food Technology and Biotechnology 44, 185–195.

Ramana, K.V., Tomar, A., Singh, L., 2000. Effect of various carbon and nitrogen sources on cellulose synthesis by *Acetobacter xylinum*. World Journal of Microbiology and Biotechnology 16, 245–248.

Rao, M.R., Stokes, J.L., 1953. Nutrition of the acetic acid bacteria. Journal of Bacteriology 65, 405–412.

Rubio-Fernández, H., Salvador, M.D., Fregapane, G., 2004. Influence of fermentation oxygen partial pressure on semicontinuous acetification for wine vinegar production. European Food Research and Technology 219, 393–397.

Sacchetti, M., 1970. Sull'Aceto Balsamico Modenese. Edagricole, Bologna.

Saeki, A., Taniguchi, M., Matsushita, K., Toyama, H., Theeragool, G., Lotong, N., Adachi, O., 1997a. Microbiological aspects of acetate oxidation by acetic acid bacteria, unfavorable phenomena in vinegar fermentation. Bioscience, Biotechnology, and Biochemistry 61, 317–323.

Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lotong, N., Adachi, O., 1997b. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. Bioscience, Biotechnology, and Biochemistry 61, 138–145.

Schmid, M.W., Lehner, A., Stephan, R., Schleifer, K.H., Meier, H., 2005. Development and application of oligonucleotide probes for in situ detection of thermotolerant *Campylobacter* in chicken faecal and liver samples. International Journal of Food Microbiology 105, 245–255.

Schüller, G., Hertel, C., Hammes, W.P., 2000. *Gluconacetobacter entanii* sp. nov., isolated from submerged high-acid industrial vinegar fermentations. International Journal of Systematic and Evolutionary Microbiology 50, 2013–2020.

Sellmer, S., Sievers, M., Teuber, M., 1992. Morphology, virulence and epidemiology of bacteriophage particle isolated from industrial vinegar fermentations. Systematic and Applied Microbiology 15, 610–616.

Sievers, M., Teuber, M., 1995. The microbiology and taxonomy of *Acetobacter europaeus* in commercial vinegar production. 1995. Journal of Applied Bacteriology-Symposium Supplement 79, 84–95.

Sievers, M., Sellmer, S., Teuber, M., 1992. *Acetobacter europaeus* sp. nov., a main component of industrial vinegar fermenters in central Europe. Systematic and Applied Microbiology 15, 386–392.

Silva, L.R., Cleenwerck, I., Rivas, R., Swings, J., Trujillo, M.E., Willems, A., Velázquez, E., 2006. *Acetobacter oeni* sp. nov., isolated from spoiled red wine. International Journal of Systematic and Evolutionary Microbiology 56, 21–24.

Sokollek, S.J., Hammes, W.P., 1997. Description of a starter culture preparation for vinegar fermentation. Systematic and Applied Microbiology 20, 481–491.

Sokollek, S.J., Hertel, C., Hammes, W.P., 1998. Description of *Acetobacter obiediens sp. nov.* and *Acetobacter pomorum sp. nov.*, two new species isolated from industrial vinegar fermentations. International Journal of Systematic and Bacteriology 48, 935–940.

Solieri, L., Landi, S., De Vero, L., Giudici, P., 2006. Molecular assessment of indigenous yeast population from traditional balsamic vinegar. Journal of Applied Microbiology 101, 63–71.

Stamm, W.W., Kittelmann, M., Follmann, H., Trüper, H.G., 1989. The occurrence of bacteriophages in spirit vinegar fermentation. Applied Microbiology and Biotechnology 30, 41–46.

Stanley, G., 1998. Cheeses. In: Wood, Brian, J.B. (Ed.), Microbiology of Fermented Foods. Blacki Academic and Professional, London, pp. 285–286.

Steiner, P., Sauer, U., 2003. Long-term continuous evolution of acetate resistant *Acetobacter aceti*. Biotechnology and Bioengineering 84, 40–44.

Swings, J., 1992. The Genera *Acetobacter and Gluconobacter*. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes. Springer-Verlag, New York, NY, pp. 2268–2286.

Tesfaye, W., Morales, M.L., García-Parrilla, M.C., Troncoso, A.M., 2002. Wine vinegar: technology, authenticity and quality evaluation. Trends in Food Science & Technology 13, 12–21.

Teuber, M., Andresen, A., Sievers, M., 1987. Bacteriophage problems in vinegar fermentations. Biotechnology Letters 9, 37–38.

Trcek, J., 2005. Quick identification of acetic acid bacteria based on nucleotide sequences of the 16S–23S rDNA internal transcribed spacer region and of the PQQ-dependent alcohol dehydrogenase gene. Systematic and Applied Microbiology 28, 735–745.

Trcek, J., Toyama, H., Czuba, J., Misiewicz, A., Matsushita, K., 2006. Correlation between acetic acid resistance and characteristics of PQQ-dependent ADH in acetic acid bacteria. Applied Microbial and Cell Physiology 70, 366–373.

Turtura, G.C., Benfanti, L., 1998. Caratteristiche microbiologiche e chimiche dell'aceto balsamico naturale. Studio del prodotto. Annals of Microbiology 38, 51–74.

Zwirglmaier, K., 2005. Fluorescence *in situ* hybridisation (FISH)—the next generation. FEMS Microbiology Letters 246 (2), 151–158.