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The occurrence, control and esoteric effect of acetic acid bacteria in winemaking

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Abstract – This review focuses on acetic acid bacteria in the winemaking process. The enumeration, isolation and identification of acetic acid bacteria from grapes and wines are discussed. This is followed by an outline of the conditions and measures that can assist the wine producer to inhibit the unwanted growth of acetic acid bacteria in wine, which include the ethanol concentration, low pH, minimum oxygen pick-up, temperature control, additives as well as clarification and filtration. The metabolism of acetic acid bacteria, which include ethanol, carbohydrate, organic acid and glycerol metabolism, and which can form spoilage products, are also reviewed, as well as the interaction between acetic acid bacteria and other wine-related microorganisms.

Key words: acetic acid bacteria, wine, spoilage.

INTRODUCTION

Acetic acid bacteria have long been considered to play little, if any, role in the winemaking process due to their aerobic nature (Joyeux *et al.*, 1984a). Sound winemaking practices were considered to be sufficient to inhibit the growth of these organisms. These practices include the maintenance of anaerobic conditions by blanketing the wine with an inert gas or filling containers completely, as well as the correct use of sulphur dioxide (SO_2) (Amerine and Kunkee, 1968). However, it has become increasingly evident that, in some cases, these organisms can survive and even multiply under the anaerobic or semi-anaerobic conditions found in winemaking. Their contribution to the chemical composition of the must and wine is now beginning to be understood and investigated (Drysdale and Fleet, 1989b). In recent years, there has been a renewed interest in wine-related acetic acid bacteria and important information has come to the fore (Drysdale and Fleet, 1989a, b; Kösebalan and Özilgen, 1992; Splittstoesser and Churney, 1992; Silva *et al*., 1995; Wilker and Dharmadhikari, 1997; Millet and Lonvaud-Funel, 2000; Poblet *et al*.,

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2000b; Barbe *et al.*, 2001; Du Toit and Lambrechts, 2002). Since the latest comprehensive overview concerning wine-related acetic acid bacteria was published more than a decade ago (Drysdale and Fleet, 1988), the latter information has therefore not been included in an updated review paper. Here we endeavour to summarise and critically assess the most recent findings against the backdrop of older publications, thereby presenting a timely and state-of-the-art overview on this important group of wine-related microorganisms. This review focuses on the role acetic acid bacteria can play in the winemaking process, their metabolism, which can affect wine quality, and ways of preventing or inhibiting the unwanted growth and spoilage of wine by acetic acid bacteria.

ENUMERATION, ISOLATION AND IDENTIFICATION OF ACETIC ACID BACTERIA FROM MUSTS AND WINES

Acetic acid bacteria belong to the family *Acetobacteraceae* and are Gram-negative, catalase-positive rods (De Ley *et al.*, 1984; Holt *et al.*, 1994). However, these parameters can change, with some strains being Gram-variable, spherically shaped and catalase negative (Ameyama, 1975; Gosselé *et al.*, 1983). Some strains of these bacteria are renowned for their variability and ability to change their characteristics (Carr and Passmore, 1979; Kittelman *et al.*, 1989), which further complicate classification. Numerous types of media have been reported for the isolation of acetic acid bacteria. These media normally contain a suitable carbon and nitrogen source, vitamins and other growth factors. The carbon sources include glucose, ethanol, mannitol, etc. Some of these media also incorporate $CaCO_3$ and bromocresol-green as acid indicators (Swings and De Ley, 1981; De Ley *et al.*, 1984; Drysdale and Fleet, 1988). In our laboratory we found GYC (consisting of 5% glucose, 1% yeast extract and 1.5% agar), YPM (consisting of 1% yeast extract, 0.5% peptone, 1.5% mannitol and 1.5% agar) and YPE (1% yeast extract, 0.5% peptones, 2% ethanol and 1.5% agar; pH 5.5) media to be most supportive for the growth of acetic acid bacteria. These agar plates are then incubated for at least five days at 30 ºC before the colonies are counted and the bacteria isolated. Other workers have reported that they incubate their plates for only two days, but we have found that certain strains grow too slowly to form a colony in this time (Drysdale and Fleet, 1988; Sanni *et al.*, 1999). Normally, we also include pimarisin in these agar plates to eliminate the growth of yeasts. Cycloheximide can also be used for this purpose, but certain fungi and non-*Saccharomyces* yeasts occurring on the grapes are resistant to this antibiotic (Drysdale and Fleet, 1988; Silva *et al.*, 1995). Nisin or penicillin can also be incorporated in these agar media to eliminate lactic acid bacteria, although these types of bacteria do not normally exhibit good growth on GYC, YPM and YPE media. Acetic acid bacteria can be stored on GYC slants at 4 ºC and freeze-dried, but the survival rate is also good during storage with glycerol at –80 ºC (Du Toit and Lambrechts, 2002).

The acetic acid bacteria are divided into the genera *Acetobacter, Acidomonas, Gluconobacter* and *Gluconacetobacter* (Yamada *et al.*, 1997; Ruiz *et al.*, 2000). Of these, *Gluconobacter oxydans* (*G. oxydans*), *Acetobacter aceti* (*A. aceti*), *Acetobacter pasteurianus* (*A. pasteurianus*), *Gluconacetobacter liquefaciens* (*Gl. liquefaciens* formerly known as *Acetobacter liquefaciens*) and *Gluconacetobacter hansenii* (*Gl. hansenii* formerly known as *Acetobacter hansenii*) are normally asso-

ciated with grapes and wine (Drysdale and Fleet, 1988). Other species, including *Acidomonas methanolica*, *Gluconacetobacter xylinus*, *Gluconacetobacter europeus*, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter sacchari*, *Gluconobacter asaii*, *Gluconobacter frateurii*, *Acetobacter oboediens*, *Acetobacter pomorum* and *Acetobacter intermedius* have been described, but it has not been reported whether they also occur on grapes and in wine (Yamada *et al*., 1997; Boesch *et al*., 1998; Sokollek *et al*., 1998; Franke *et al*., 1999).

One of the most common differences between *Acetobacter* and *Gluconobacter* is the ability of *Acetobacter* to oxidise ethanol to acetic acid and further to CO_2 and water. *Gluconobacter*, however, can only oxidise the ethanol to acetic acid. To further distinguish between these strains with biochemical and physiological tests, Holt *et al.* (1994) and Drysdale and Fleet (1988) may be consulted. Other, more sophisticated, tests include numerical analyses of phenotypical characteristics, electrophoretic protein profile analysis (Kerster and De Ley, 1975; Gosselé *et al.*, 1983), the occurrence and characterisation of plasmids (Fukaya *et al.*, 1985) and the sequencing and comparison of 16S rRNA and DNA (Yamada *et al.*, 1997; Sokollek *et al.*, 1998). The use of restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rDNA has been proposed by Poblet *et al.* (2000a) and Ruiz *et al.* (2000) as a rapid means to identify acetic acid bacteria occurring in wine.

Occurrence on grapes and in fresh must

The main species of acetic acid bacteria observed on unspoiled grapes is *G. oxydans*, which normally exists at cell counts of between 10^2-10^5 cells per ml (Joyeux *et al.*, 1984a; Du Toit and Lambrechts, 2002). In an investigation of sorbic acid-resistant *Gluconobacter* species occurring on grapes, Splittstoesser and Churney (1992) found 43 out of 65 isolates belonging to this species, but they also found ten isolates that did not correspond with the specific characteristics of the *Acetobacter, Gluconobacter* or *Frateuria* genera. Blackwood *et al.* (1969) found 73% of the species isolated from ripe Bordeaux grapes to be *G. oxydans*, while Passmore and Carr (1975) found this species only on dried-out grapes and on young shoots. These counts are the same for fresh must (Joyeux *et al.*, 1984a; Drysdale and Fleet, 1985). This is not surprising, since this species prefers a sugar-rich environment. In an investigation into the acetic acid bacteria status of South African red wine fermentations during 1998 and 1999, *G. oxydans* was found to also dominate in fresh must in six commercial wine fermentations, with *A. pasteurianus* and *Gl. liquefaciens* occurring at lower numbers. However, *A. pasteurianus* dominated in one of these musts (Du Toit and Lambrechts, 2002). Although *G. oxydans* is believed to die quite rapidly during alcoholic fermentations due to its low ethanol tolerance, cell counts of up to 10^4 cells per ml have been isolated from wine (Drysdale and Fleet, 1985). Normally, their numbers decrease to between zero and 10^2 cells per ml at the end of alcoholic fermentation (Joyeux *et al.*, 1984a; Du Toit and Lambrechts, 2002).

The number of acetic acid bacteria increases drastically on rotten or *Botrytis*-infected grapes, with cell counts increasing from a few cells per ml to 10^6 cells per ml after infection with this fungi (Barbe *et al*., 2001). When this happens, *Acetobacter* species can start to dominate. This may be due to the ethanol production by wild yeast occurring on the damaged grapes (Joyeux *et al.*, 1984a). *Acetobacter* species prefer ethanol as a carbon source (De Ley *et al.*, 1984). This may also

explain their dominance during the later stages of fermentation and in the wine (Du Toit and Lambrechts, 2002). *A. aceti* was reported to dominate in Spanish fresh must at the beginning of fermentation, with *G. oxydans, Gl. hansenii* and *A. pasteurianus* also occurring, while *A. aceti* also has been isolated in Portuguese grape pomace (Poblet *et al.*, 2000b; Silva *et al.*, 2000). Barbe *et al*. (2001), however, found *Gluconobacter* to dominate *Botrytis*-infected grapes, with *A. aceti* and *A. pasteurianus* occurring in lower numbers. It thus seems as if the ecology of acetic acid bacteria on grapes and in the fresh grape must can be diverse. Further in-depth studies on the occurrence of acetic acid bacteria in the vineyard and the effect that different viticultural practices have on these numbers may shed more light on this.

The process of cold soaking, when crushed red grapes are left with the skins for a few days at low temperatures, is applied to extract more colour before alcoholic fermentation starts (Ribéreau-Gayon *et al*., 2000b). During this time, the microbiological status of the must can change, despite the addition of SO_2 to prevent the unwanted growth of yeast and bacteria. Couasnon (1999) found a 60-fold increase in acetic acid bacteria in must that underwent maceration for 10 days. The pH of the must can also influence the number of acetic acid bacteria during this process, with cell counts increasing at higher pH values, at which less of the SO_2 is in the anti-microbial, molecular form, but staying at the initial numbers at lower pH values (pH< 3.5) (Du Toit and Lambrechts, 2002).

Occurrence during fermentation

Acetic acid bacteria are aerobic microorganisms, thus the production of carbon dioxide during alcoholic fermentation should inhibit their growth in must during fermentation. Joyeux *et al.* (1984a) found a drastic reduction (up to 10^3 cells per ml) in the number of acetic acid bacteria during alcoholic fermentation in white Sauterne-style wines, although certain *A. aceti* strains can acidify must during alcoholic fermentation (Vaughn, 1955). Drysdale and Fleet (1989b) found that *A. pasteurianus* and *G. oxydans* could grow in conjunction with *Saccharomyces cerevisiae* during alcoholic fermentation, from an initial inoculation of 10^3-10^5 cells per ml up to 10^6 to 10^8 cells per ml. An *A. aceti* strain tested was unable to grow in juice alone, but did grow in the presence of *S. cerevisiae*. This could be due to the weak ability of *A. aceti* to grow on sugar as sole carbon source and the possibility that the production of ethanol by the yeast stimulated its growth. In an investigation of the microbiological status of Nigerian palm wine, Okafor (1975) also found that *Acetobacter* grew from the third day of alcoholic fermentation. The growth of acetic acid bacteria during wine fermentations also seems to be dependent on the pH of the must. In musts with a lower pH (pH < 3.5), a reduction from an initial cell count of almost 10^5 to 10^2 cells per ml at the end of alcoholic fermentation was observed in South African commercial red wine fermentations (Du Toit and Lambrechts, 2002). In a higher pH fermentation (pH 3.75), the decrease was only 10 cells per ml, while in another (at pH 3.71), the cell counts actually increased from the beginning to the end of the fermentation (Du Toit and Lambrechts, 2002). Alcohol is known to be more toxic towards acetic acid bacteria at lower pH values, which may help to explain this effect (Dupuy and Maugenet, 1963). The effect of different fermentation temperatures on the growth of acetic acid bacteria has also not been investigated in detail. However, it seems that the differences in normal red wine fermentation temperatures (20 to 35 °C) do not play such a big role. The effect of SO_2 on the number of acetic acid bacteria, however, seems more pro-

nounced at the beginning of fermentation at a higher temperature (30 °C) than at lower temperatures (15 and 22 °C) (Du Toit and Lambrechts, 2002).

The growth of these bacteria during alcoholic fermentation may also be linked to the number of bacteria and yeast in the must at the start of fermentation, according to Watanabe and Iino (1984) as quoted in Drysdale and Fleet (1988). They found that the must into which only *S. cerevisiae* was inoculated contained only 0.54 g/l of acetic acid at the end of the alcoholic fermentation. This increased to 4.56 g/l of acetic acid when the same number of *S. cerevisiae* and *Acetobacter* species (about 10^6 cells per ml) were inoculated into the same must. Splittstoesser and Churney (1992) also found that the growth of *Gluconobacter* species isolated from grapes in the presence of different concentrations of ethanol and sorbic acid depended on the size of the inoculum used. It thus seems that the initial population of acetic acid bacteria, before the commencement of alcoholic fermentation, may also determine the number of cells surviving during fermentation.

A. aceti, *A. pasteurianus*, *Gl. liquefaciens* and, to a lesser extent, *Gl. hansenii*, normally start to dominate during the middle and later stages of the alcoholic fermentation (Joyeux *et al.*, 1984a; Poblet *et al.*, 2000b; Du Toit and Lambrechts, 2002) but Drysdale and Fleet (1985) found only *G. oxydans* in wine undergoing alcoholic fermentation. It is clear, however, that acetic acid bacteria can survive and even grow during alcoholic fermentation and it is of vital importance to the winemaker to keep these numbers as low as possible. This can be achieved by using healthy grapes, a high inoculum of yeast, the addition of SO_2 to the must, clarification of the must and the lowering of the pH by additions of acid. A high cell count for acetic acid bacteria at the end of fermentation could induce a sluggish or stuck fermentation (which will be addressed later in this review) or can lead to further growth of acetic acid bacteria and spoilage during subsequent winemaking operations.

Occurrence during maturation of wine

Joyeux *et al.* (1984a) found that, in red wine, the number of acetic acid bacteria increased from 20 cells per ml after alcoholic fermentation to about 3×10^4 cells per ml after this wine had been drained from the fermentation tank. During malolactic fermentation, cell counts remained at 10^2-10^3 cells per ml and consisted mainly of *A. pasteurianus*. These numbers were also found when the wine was aged in barrels, but at this stage *A. aceti* started to dominate. *Gluconobacter* strains were isolated until a few days after the barrels had been topped up, but disappeared soon afterwards, suggesting that these bacteria can be incorporated into the wine by contaminated wine or equipment. An investigation by Drysdale and Fleet (1985) showed that *A. pasteurianus* dominated in wine stored in tanks and barrels and that these counts were between 10 and 10^5 cells per ml. *A. aceti* was also present, but to a lesser extent than *A. pasteurianus*. These bacteria were isolated from the top, middle and bottom parts of these tanks and barrels, suggesting that acetic acid bacteria can actually survive under the anaerobic or semi-anaerobic conditions occurring in wine containers. *Gl. hansenii* was also isolated around the openings of the barrels. The pumping over and racking of wine may lead to the uptake of small amounts of oxygen. This may lead to the renewed growth, or at least the survival, of acetic acid bacteria in the wine. It is clear from Fig. 1 that the number of acetic acid bacteria can increase drastically (between 10^2 and 10^3 cells per ml) after racking and fining, which are both operations that can introduce some oxygen into the wine. It is

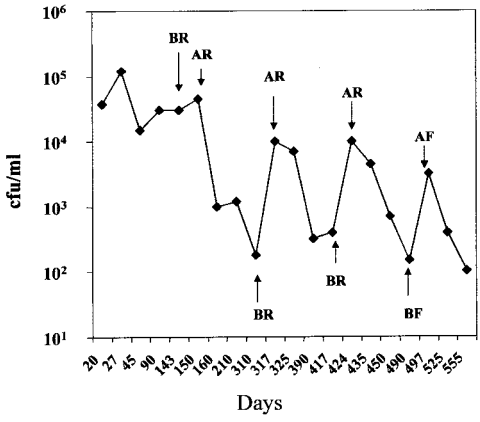

FIG. 1 – The effect of racking and fining on acetic acid bacteria numbers in red during ageing oak barrels (Millet and Loncaud-Funel, 2000).

believed that the number of acetic acid bacteria normally decreases rapidly after bottling, due to the relatively anaerobic conditions in a bottle. However, excessive addition of oxygen during bottling can lead to an increase in the number of acetic acid bacteria (Millet and Lonvaud-Funel, 2000). The high frequency at which we have isolated high cell counts of acetic acid bacteria from spoiled bottled wine confirms this (Du Toit, 2000).

CONDITIONS AND MEASURES THAT CAN PREVENT/INHIBIT THE GROWTH OF ACETIC ACID BACTERIA IN WINE

Ethanol concentration

Acetic acid bacteria are well known for their ability to oxidise ethanol to acetic acid. Ethanol is thus a good carbon source for acetic acid bacteria, but is also inhibiting at concentrations that are too high. De Ley *et al.* (1984) stated that, in media containing 5% ethanol, only 58% of the *Acetobacter* strains tested grew and that this was reduced to only 13% in media containing 10% ethanol. Of these, only 20% of the *A. pasteurianus* strains and no *A. aceti* strains that were tested were able to grow. Only 5% of the *Gluconobacter* strains tested were able to grow in media containing 5% ethanol. However, the ability of these bacteria to remain viable and even to grow in wine containing between 10 and 14% (v/v) alcohol is well known. In different experiments, Joyeux *et al.* (1984a), Drysdale and Fleet (1989a) and Kösebalan and Özilgen (1992) were all able to grow acetic acid bacteria at these alcohol concentrations. Vaughn (1955) stated that the maximum alcohol concentration tolerated by these bacteria is between 14 and 15% (v/v). An alcohol concentration of 15.5% (v/v) is recommended for sherry stock to inhibit the growth of acetic acid bacteria (Cruess, 1948; as quoted in Drysdale and Fleet, 1988). Thermotolerant acetic acid bacteria were able to grow and oxidise ethanol at 9% (v/v) without a lag phase. When this concentration was increased to 10% (v/v), the bacteria overcame this inhibitory effect and oxidised the ethanol after an initial lag

phase, which suggests that these bacteria have acquired ethanol tolerance (Saeki *et al.*, 1997b). Acetic acid bacteria have even been isolated from saké and tequila, which have a higher alcohol concentration than wine (Swings and De Ley, 1981). It is thus clear that the ethanol tolerance of these bacteria is dependent on the strain and that some of these strains can grow under the normal alcohol concentrations found in wine. However, with an increase in alcohol concentration the probability for acetic acid bacteria to prevail decreases.

Low pH

The optimum pH for the growth of acetic acid bacteria is 5.5-6.3 (Holt *et al.*, 1994). However, these bacteria can survive at the low pH values of between 3.0 and 4.0 found in wine. Vaughn (1955) states that a pH of 3.3 and lower is inhibitory to most lactic acid bacteria in wine, but not to acetic acid bacteria. Acetic acid bacteria were isolated from different Australian cellars in wines with pH values ranging from 3.02 to 3.85 (Drysdale and Fleet, 1985). It is believed, however, that the growth of these bacteria in wine is inhibited at lower pH values. Joyeux *et al.* (1984a) found that cell numbers of an *A. aceti* strain decreased faster at pH 3.4 than at pH 3.8 under strict anaerobic conditions. The ethanol sensitivity of these bacteria may also differ at different pH values. Dupuy and Maugenet (1963) found that an *A. pasteurianus* strain was able to survive at pH 3.4 at a maximum ethanol concentration of 12.5%, but this decreased to 8.2% ethanol at pH 3.0. The growth of acetic acid bacteria in commercial South African red wine fermentations also correlated with the pH value of the must, as mentioned in a previous section (Du Toit and Lambrechts, 2002). At a lower pH in wine more SO_2 is also in the free molecular form, which is the active form against microorganisms (Ribéreau-Gayon *et al.*, 2000a). However, acetic acid bacteria have been isolated that could grow at pH values of as low as 2.0-2.3 in media containing acetate while being aerated. In this regard, Kittelman *et al.* (1989) postulated that there are three groups of strains that might exist in a vinegar fermentation, namely acetophilic strains that only grow at a pH value of about 3.5, acetophobic strains that only grow at pH levels higher than 6.5, and acetotolerant strains that can grow at both these pH values. There may be a gradual development from acetophobic to acetotolerant strains and, with prolonged exposure to low pH and high acetic acid concentrations, to acetophilic strains. This suggests the development of a gradual acid resistance in these bacteria (Kittelman *et al.*, 1989). The prolonged survival of acetophilic strains was also observed by Kösebalaban and Özilgen (1992), who also suggested a gradual development from acetophobic to acetophilic strains. The growth of selected *A. aceti*, *A. pasteurianus*, *G. oxydans*, *Gl. hansenii* and *Gl. liquefaciens* strains did not, however, differ significantly in Chenin blanc grape juice at pH 3.4, 3.6 and 3.8 (Du Toit, 2000). Lower pH values were not tested and should be investigated in the future. The maintenance of a lower pH (< 3.5) throughout the winemaking process should, however, assist the winemaker to inhibit the growth of acetic acid bacteria. This process must be initiated in the vineyard, where fertilisation, irrigation, pruning and other viticultural practices all can contribute to the pH of the must and the wine. Winemaking techniques, such as acid additions, blending, cold stabilisation and malolactic fermentation, which can all change the pH of the wine, must be managed by the winemaker to obtain a lower pH in the wine. This could help not only to inhibit spoilage by microorganisms, but could also contribute to providing the wine with a longer ageing potential (Zoecklein *et al.*, 1995).

Minimum oxygen pick-up

Acetic acid bacteria use oxygen as the terminal electron acceptor during respiration (Matsushita *et al.*, 1994). As mentioned before, the maintenance of anaerobic conditions and the use of SO_2 have always been believed to be sufficient to inhibit the growth of aerobic acetic acid bacteria in wine (Amerine and Kunkee, 1968). Although it is true that these actions could help prevent the growth of these bacteria, it is becoming clear that acetic acid bacteria can survive and even grow under these unfavourable conditions. The isolation of high numbers of acetic acid bacteria from tanks and wooden barrels confirms this statement (Joyeux *et al.*, 1984a; Drysdale and Fleet, 1985). The exposure of wine to air, even if it is only for a very short period of time, enhances this process. This could happen when wine is being pumped over or transferred. Joyeux *et al.* (1984a) found a 30 to 40-fold increase in the number of acetic acid bacteria growing in wine in which about 7.5 mg/l oxygen had dissolved after exposure to air. The acetic acid concentration also increased significantly. During the ageing of wine in wooden barrels, about 30 mg/l oxygen penetrates through the wood into the wine in a year. This could sustain a viable population of acetic acid bacteria in the wine.

Drysdale and Fleet (1989a) investigated the effect of different oxygen concentrations in wine on the growth and survival of acetic acid bacteria. Wine that was fully aerated (100% dissolved oxygen) sustained the rapid growth of *A. aceti* and *A. pasteurianus* from an initial cell count of 10^4-10^5 cells per ml to 10^8 cells per ml within a few days. Both these species also grew in wine held at 70% dissolved oxygen, but the final cell counts were lower, ranging from 10^6 to 10^7 cells per ml. The *A. aceti* strain did not grow in 50% dissolved oxygen, whereas *A. pasteurianus* only exhibited limited growth at this oxygen concentration. Importantly, the bacteria did not grow, but survived at low numbers in the wine containing 50% oxygen. This was accompanied by an increase in the acetic acid concentration. Since wine at 20 ºC, when it is saturated with air, contains 7.2 mg/l oxygen, the occurrence of these bacteria in wine stored in wooden barrels into which oxygen can dissolve can be a problem for the winemaker. The prolonged occurrence of these bacteria can thus, over a period of time, increase the volatile acidity of the wine, especially at higher temperatures and pH values (Ribéreau-Gayon, 1985).

Acetic acid bacteria can also use compounds, such as quinones and reducible dyes, as electron acceptors and this may also contribute to their occurrence in wine. *G. oxydans* exhibits a four-fold higher oxidation reaction rate of glycerol with ρ-benzoquinone as electron acceptor than it does with oxygen. The byproduct formed from this, hydroquinone, can be oxidised to ρ-benzoquinone for re-use (Adlercreutz and Mattiasson, 1984). These phenolic compounds can occur in wine. The production of 2,5-diketogluconic acid from glucose by *G. oxydans* (Qazi *et al.*, 1993) is also enhanced by a high dissolved oxygen concentration. It thus seems as if acetic acid bacteria can survive and even grow in the anaerobic or semi-anaerobic conditions imposed on them in wine. The winemaker should thus strive for the minimum pick-up of air when wines that have a high acetic acid bacteria count are being moved during racking, pumping over, fining and bottling. The addition of SO_2 and the maintenance of cellar equipment, such as pumps and tanks, to prevent excessive oxygen pick-up during winemaking operations can be used to achieve this. Wine in barrels should also be filled up regularly, due to the process of evaporation of water and ethanol from the barrel (Ribéreau-Gayon *et al.*, 2000e). Failure to do this could present the acetic acid bacteria with a surface to grow on in the

wine. The addition of oxygen in small amounts over a long period of time, however, contributes to the polymerisation of tannins and other phenolic compounds, which are essential for the sensory enhancement and stability of red wine in particular (Ribéreau-Gayon *et al*., 2000c).

Temperature control

According to Holt *et al.* (1994), the optimum growth temperature for *Acetobacter* and *Gluconobacter* is 25-30 ºC, with no growth observed for the latter strain at 37 ºC. De Ory *et al.* (1998) found the maximum temperature for the growth of *A. aceti* to be about 35 ºC. Thermotolerant acetic acid bacteria that are able to grow at 37-40 ºC have also been isolated (Saeki *et al*., 1997b). These bacteria were able to oxidise ethanol at 38-40 ºC at the same rate that mesophilic strains do at 30 ºC, as well as being able to oxidise ethanol more rapidly than the mesophilic strains at the higher temperatures Lu *et al.* (1999) also found that a thermotolerant *Acetobacter* strain produced more acetic acid (up to 41 g/l) in comparison with two non-thermotolerant *Acetobacter* strains. This thermotolerant strain still retained 97 and 68% of its acetic acid production at 35 ºC and 37 ºC, respectively, compared to at 30 ºC. The ability of these bacteria to grow at higher temperatures may be a consequence of their increased tolerance to ethanol and acetic acid (Ohmori *et al.*, 1980).

At lower temperatures, acetic acid bacteria can still be active, with Joyeux *et al.* (1984a) observing a 30 to 40-fold increase in cell numbers of *A. aceti* in wine stored at 18 ºC for one week. Weak growth was observed even at 10 ºC. Drysdale and Fleet (1989b) found that experiments done on the effect of acetic acid bacteria upon the growth and metabolism of fermenting yeast in grape juice produced the same conclusions from results obtained at 18 and 25 ºC. It thus seems that these bacteria can survive at lower cellar temperatures but lowering the wine storage temperatures to 10-15 ºC seems to inhibit their growth to a large extent (Joyeux *et al.*, 1984a). It still has to be determined at which minimum temperature these bacteria can grow (Drysdale and Fleet, 1988), although De Ory *et al*. (1998) found that *A. aceti* could not grow below 8 ºC.

Additives

As mentioned before, the correct use of SO_2 should prevent the growth of acetic acid bacteria in wine (Amerine and Kunkee, 1968). SO_2 in wine consists of the free and the bonded form. The free form consists of molecular SO_2, bisulphate and sulphite ions. The molecular form is the most active anti-microbial form, but at normal wine pH only about 5% of the free SO_2 occurs in the molecular form (Ribéreau-Gayon *et al*., 2000a). Acetic acid bacteria can grow in wine containing 20 mg/l of free SO_2 (Joyeux *et al.*, 1984a). Thus, it has been concluded that the levels of SO_2 used in wine are not always adequate to inhibit these bacteria and that temperature and pH have a more pronounced effect on the bacteria. Watanabe and Iino (1984), as quoted in Drysdale and Fleet (1988), found that up to 100 mg/l of SO_2 was needed to inhibit the growth of an *Acetobacter* species in grape must. Drysdale and Fleet (1985) isolated more than 10^5 *A. pasteurianus* cells per ml from a red wine containing 81.6 mg/l of SO_2 and which had a pH of 3.46, concluding that higher occurrences of acetic acid bacteria did not necessarily correlate with low SO_2 concentrations and high pH values. According to Boulton *et al.* (1995), *A. aceti* can be controlled by 0.8 mg/l of molecular SO_2. They also state that the recent trend to make wine with a lowered SO_2 content may contribute to the higher incidence of

Acetobacter pasteurianus found in wine. The aforementioned value correlates with the molecular SO_2 concentration needed to inhibit *Gl. hansenii* from growing in grape juice in a study undertaken to determine the SO_2 resistance of five representative species normally found in wine (Du Toit, 2000). However, this strain was the most SO_2 resistant. The other four strains were more sensitive to SO_2, with 0.6, 0.2, 0.1 and 0.05 mg/l molecular SO_2 eliminating *A. pasteurianus*, *A. aceti*, *Gl. liquefaciens* and *G. oxydans*, respectively. The SO_2 resistance of these strains also correlated with their growth in the grape juice, with the two strains exhibiting the most rapid growth being the most SO_2 resistant. However, it is clear that strain variation also influences the SO_2 resistance (Du Toit, 2000).

The storage of wine in wooden barrels is a practice being used all over the world. These barrels are used two to three times and, due to the high cost of these items, an effective cleaning operation is necessary between usage of the barrels to eliminate bacteria from them (Ribéreau-Gayon *et al*., 2000e). In tests conducted to determine which treatment is the most successful for eliminating acetic acid bacteria from mini wooden staves contaminated with *A. aceti* and *A. pasteurianus*, Wilker and Dharmadhikari (1997) found that even 250 mg/l of free SO_2 was not enough to completely eliminate these bacteria from the wooden staves. Acetic acid bacteria were detected two weeks after the treatment of the staves with SO_2. These authors also treated the staves with potassium carbonate, chlorine and hot water. Of these, only the hot water treatment (85-88 ºC for 20 minutes) was effective in eliminating the acetic acid bacteria. The pores in the wood, as well as the film produced by the bacteria, might have kept the chemical treatments from coming into direct contact with the bacteria, thus rendering them ineffective. *Gluconobacter* species were found to be resistant to up to 1000 mg/l of sorbic acid (Splittstoesser and Churney, 1992). Very little, if any work has been done on the effect of other preservatives, such as fumaric acid, sorbic acid, benzoic acid and dimethyl dicarbonate, on acetic acid bacteria.

The correct usage of SO_2 can inhibit acetic acid bacteria. It thus seems that a molecular SO_2 concentration of between 0.7 to 1 mg/l can achieve this. The worldwide trend to use less of this preservative should not prevent the winemaker from utilising this preservative at appropriate levels and in a responsible manner (Du Toit, 2000). However, due to mounting consumer bias against chemical preservatives other alternatives, such as bacteriocins, are currently under investigation to further assist the wine producer to eliminate acetic acid bacteria from wine (Du Toit and Pretorius, 2000).

Clarification and filtration

The clarification of white must in particular before fermentation is a practice that is generally used in the winemaking process. The yeast generally produces more esters during fermentation in clearer juice. Oxidative enzymes and elemental sulphur from the vineyard can also be removed with clarification. The natural microflora occurring on grapes, including acetic acid bacteria, can be reduced in this way. Techniques to clarify the must include natural settling at lower temperatures with or without fining agents, flotation, as well as clarification with centrifugation and filtration (Boulton *et al*., 1995). By reducing their numbers in the fresh must, the probability of acetic acid bacteria growing during fermentation can be decreased. This would also decrease the numbers being “brought over” to subse-

quent winemaking operations (Fugelsang, 1997). Very little work has been done on the effect of wine clarification techniques (e.g., fining), on acetic acid bacteria counts. However, the microbial count of a wine can be reduced by racking and this should also include a reduction in acetic acid bacteria.

Filtration can also be applied to reduce suspended solids and microorganisms in wine. At present there is a trend in certain wine-producing countries to use minimal filtration during red wine production, due to the possible loss of flavour and colour during the filtration process. This can lead to the development of acetic acid bacterial spoilage in the bottle, especially if excessive oxygen was picked up during the bottling procedure (Baldwin and Wollan, 1999). The different filtration systems can include diatomaceous earth, pad, cross-flow and membrane filters. Diatomaceous earth and pad filtrations, which are depth filtrations, are normally applied to remove larger suspended particles from the wine, while cross-flow and membrane filtrations, which are normally finer filtrations, can be used to filter a wine sterile. Certain suppliers claim to be able to filter a wine sterile with depth filters, but these filters do not have a uniform pore size, as is the case with the membrane filters that normally are used for this purpose (Boulton *et al*., 1995). The use of diatomaceous earth with different permeabilities can assist the wine producer to reduce the number of bacteria in the wine. In an experiment, the number of bacteria were reduced from 180 000 viable cells per 100 ml to 7700, 3000 and 1500 cells per 100 ml with coarse (1.5 darcys), average (0.35 darcys) and fine (0.06 darcys) diatomaceous earth filtrations, respectively. Filter sheets can also differ regarding their ability to reduce the number of bacteria (Ribéreau-Gayon *et al*., 2000d). The smallest pore size of a sterile membrane filter used in the wine industry is 0.45 μm. This should retain acetic acid bacteria, which have a cell size of 0.6-0.8 μm by 1.0-4.0 μm (Holt *et al*., 1994). However, it appears that acetic acid bacteria that have survived a long period in wine under conditions of sulphating can undergo a reduction in cell size, which can cause them to pass through a 0.45 μm filter. This condition is reversed when the bacteria re-enter the active growth phase under more favourable conditions, allowing them to be retained by the filter (Millet and Lonvaud-Funel, 2000). Ubeda and Briones (1999) also observed acetic acid bacteria in unfiltered and filtered bottled wines, but did not specify the types of filtration used. Nevertheless, filtration should be applied to reduce or remove microorganisms, including acetic acid bacteria, from wine. There is no scientific evidence that filtration removes flavour and colour compounds from the wine, as they are in a soluble state in wine, and it appears that this assumption is a marketing strategy only (Boulton *et al*., 1995; Baldwin and Wollan, 1999).

METABOLISM AND METABOLITES PRODUCED THAT CAN INFLUENCE WINE QUALITY

Ethanol oxidation and the formation of acetaldehyde and acetic acid

The oxidation of ethanol to acetic acid is the most well-known characteristic of acetic acid bacteria. Due to the economic importance of this process in the production of vinegar, the biochemical processes involved have been studied extensivel (Drysdale and Fleet, 1988) Two enzymes play a critical role in this oxidation process, namely a membrane-bound alcohol dehydrogenase and a membrane-

bound aldehyde dehydrogenase, both of which have their active sites on the outer surface of the cytoplasmic membrane (Adachi *et al.*, 1978, 1980; Saeki *et al.*, 1997b). These dehydrogenase enzymes consist of quinoproteins and flavoproteins, which have pyrroloquinoline quinone and covalently-linked flavin adenine dinucleotide as prosthetic groups, respectively. The alcohol dehydrogenase oxidises ethanol to acetaldehyde, which is further oxidised to acetate by aldehyde dehydrogenase (Matsushita *et al.*, 1994; Saeki *et al.*, 1997b). The alcohol dehydrogenase consists of two or three subunits, which include the dehydrogenase and cytochrome *c* subunits that are essential for the activity of the enzyme. The three-component-type alcohol dehydrogenase, consisting of a 72-78 kDa dehydrogenase, a 48 kDa cytochrome-*c* and a 20 kDa subunit, were found in *A. aceti* and *A. pasteurianus*. The two-component-type alcohol dehydrogenase was found in *Acetobacter polyoxgenes*. The two larger subunits play a role in the intramolecular transport of electrons from the alcohol dehydrogenase to ubiquinone, and further to the terminal oxidase during the oxidation of ethanol. The smaller one helps the two functional subunits with their association with the membrane (Kondo and Horinouchi, 1997b; Saeki *et al.*, 1997b). This membrane-bound alcohol dehydrogense has pyrroloquinoline as a cofactor and is independent of $NAD(P)^+$, although a cytoplasmic $NAD(P)^+$-dependent alcohol dehydrogenase has also been identified. The latter, however, has a much lower specific activity than the membrane-bound alcohol dehydrogenase and a higher optimal pH of 6-8, which limits its contribution to the oxidation process of ethanol (Adachi *et al.*, 1978; Takemura *et al.*, 1993; Matsushita *et al.*, 1994). The $NAD(P)^+$ independent enzyme has an optimal pH of 4, but the enzyme is still active on intact cells or cell homogenate at a pH of 2. When the enzyme is removed from the cell membrane, no activity is observed at pH levels lower than 3 (Adachi *et al.*, 1978; Nomura *et al.*, 1997). The alcohol dehydrogenase activity of *Acetobacter* is more stable under acetic conditions than that of *Gluconobacte*r, explaining the higher production of acetic acid by *Acetobacter* (Matsushita *et al.*, 1994). However, an insertion sequence can inactivate this ethanol oxidising ability in *A. pasteurianus*, which can explain the inability of spontaneously derived mutants to oxidise ethanol (Kondo and Horinouchi, 1997a). It nevertheless is clear that these enzymes are active in the environment imposed on them by wine. The different enzymes and intermediates involved in the utilisation of glucose and ethanol are shown in Fig. 2 (Saeki *et al.*, 1997a).

The other enzyme involved in the oxidation of ethanol to acetic acid is aldehyde dehydrogenase. This $NAD(P)^+$-independent enzyme is also located in the cytoplasmic membrane and has an optimum pH of between 4 and 5. It is, in addition, also able to catalyse the oxidation of acetaldehyde to acetate at lower pH values (Adachi *et al.*, 1980). Saeki *et al.* (1997b) found during the characterisation of thermotolerant acetic acid bacteria that there was little difference in the thermostability of the alcohol dehydrogenases and the aldehyde dehydrogenases from thermotolerant and mesophilic strains. The latter enzyme, however, is more thermostable than the alcohol dehydrogenase. The aldehyde dehydrogenase is more sensitive to the alcohol concentrations found in wine than the alcohol dehydrogenase and this may lead to an accumulation of acetaldehyde in the wine at the expense of acetic acid formation (Muraoka *et al.*, 1983). Drysdale and Fleet (1989a) also found increased concentrations of acetaldehyde in wine with a lower dissolved oxygen concentration in which acetic acid bacteria had grown. They postulated that conditions that may lead to a higher alcohol dehydrogenase activity

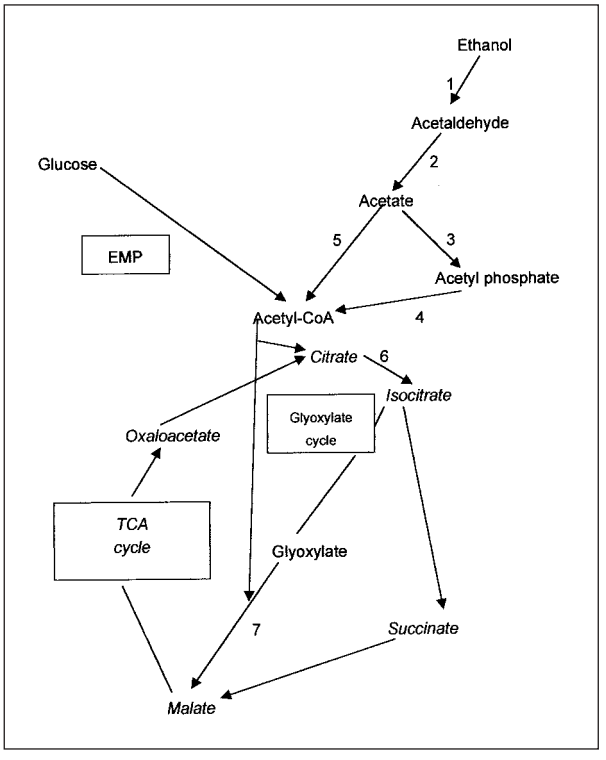

FIG. 2 – Pathways (the TCA and glyoxylate cycles) and enzymes involved in the utilisation of glucose and acetic acid in *Acetobacter*. Different enzymes involved: 1, alcohol dehydrogenase; 2, aldehyde dehydrogenase; 3, acetate kinase; 43, phosphotransacetylase; 5, acetyl-CoA synthetase; 6; isocitrate lyase and 7, malate synthetase (Saeki *et al.*, 1997a).

compared to that of the aldehyde dehydrogenase, may lead to higher acetaldehyde concentrations when lower oxygen concentrations are available. Acetic acid bacteria thus can produce acetaldehyde at concentrations of up to 250 mg/l, which exceeds the sensory perception threshold value of 100-125 mg/l. Acetaldehyde can give the wine an oxidised character and levels that are too high are unwanted, especially in delicate table wines. Acetaldehyde also binds SO_2 very effectively, rendering it ineffective against microorganisms.

Acetic acid bacteria are able to produce very high concentrations of acetic acid. This characteristic has made them very important in the vinegar industry. Some strains easily can produce more than 50 g/l and up to 150 g/l acetic acid in a vinegar fermentation (Sievers *et al.*, 1997; Lu *et al.*, 1999). These high concentrations are unlikely to be produced in wine during normal winemaking practices due to the lack of oxygen, but it is undoubtedly true that these bacteria can significantly increase the acetic acid concentration in wine, which may lead to spoilage. Acetic acid concentrations are considered to be detrimental to wine quality at concentrations ranging from 0.7-1.2 g/l and higher, depending on the wine style, although it

can be perceived at even lower concentrations (Drysdale and Fleet, 1988). In a survey of 7311 Australian wines, Eglinton and Henschke (1999) found that a high percentage of these wines (up to 33% of the red wines tested) had a volatile acidity level that should give the winemakers cause for concern. A small increase in acetic acid concentration (10-50 mg/l) by *A. aceti* and *A. pasteurianus* has been reported, while *G. oxydans* increases this concentration by 1.64 g/l (Drysdale and Fleet, 1989b). The increase in volatile acidity by South African strains of acetic acid bacteria in grape juice was more dramatic in the case of *Acetobacter* species, especially *A. pasteurianus* and *Gl. hansenii*, with the latter producing up to 4 g/l in five days. The *G. oxydans* strain tested produced very little volatile acidity (< 0.2 g/l) (Du Toit, 2000). The increase in acetic acid concentration in fully aerated wine was between 1.28 and 3.75 g/l after the growth of *A. aceti* and *A. pasteurianus*, with the latter being the strongest producer of acetic acid. This increase also correlated with the weaker growth of these bacteria in wine that has not been fully aerated, emphasising the important role oxygen plays in this process (Drysdale and Fleet, 1989a).

In an investigation to determine when acetic acid bacteria produce most of the acetic acid in wine, Kösebalan and Özilgen (1992) found that an *Acetobacter* strain produced most acetic acid during the stationary and death phases and not during active growth. Their extended occurrence in the wine may thus also lead to an increase in acetic acid concentration and this spoilage is related to bacterial cell counts. The abovementioned authors postulated that wine contaminated with acetic acid bacteria could be saved from being spoiled by removing the bacteria prior to their death phase. The production of volatile acidity during the growth of acetic acid bacteria in grape juice also established that more acetic acid was produced during the stationary phase (Du Toit, 2000). The autolysis of these bacteria after cell death may also release more of the acid into the wine. Joyeux *et al.* (1984a), however, found a significant increase in acetic acid concentration during the growth of these bacteria in wine Lu *et al.* (1999) found that a thermotolerant *Acetobacter* strain steadily oxidised ethanol to acetic acid during the exponential growth phase. The production of acetic acid then reached a maximum during the stationary phase, indicating that most acetic acid was produced during the active growth phase. Since these bacteria can survive until the end of fermentation and can increase after exposure to air during racking, pumping over, etc., it is highly likely that they can increase the volatile acidity and acetic acid concentrations of wine during further storage and maturation. Small amounts of acetic acid formed by acetic acid bacteria or even by other microorganisms, such as yeast or lactic acid bacteria can enhance the growth of these bacteria on ethanol, as was found by Nanba *et al.* (1984).

Acetobacter strains can further oxidise acetic acid to CO_2 and water through the tricarboxylic acid cycle (De Ley *et al.*, 1984; Drysdale and Fleet 1989b). Strains of *Gluconobacter* are unable to do this, as a result of a nonfunctional tricarboxylic acid cycle. This is due to two enzymes of this cycle, α-ketoglutarate dehydrogenase and succinate dehydrogenase, being nonfunctional (Greenfield and Claus, 1972). The difference in the ability of these two genera to oxidise acetic acid is an important characteristic by which to distinguish them. This oxidisation is unwanted in the vinegar industry because of the loss of acetic acid. The enzyme acetyl-CoA synthethase is responsible for the formation of acetyl-CoA from acetic acid. The acetyl-CoA enters the tricarboxylic acid cycle to be converted to the intermediates of this cycle, or can also be metabolised further through the glyoxylate cycle

(Fig. 2; Saeki *et al.*, 1997a). An increase in the activity of acetyl-CoA synthethase, isocitrate lyase and malate synthethase occurs in the presence of acetic acid (Saeki *et al.*, 1997a). Joyeux *et al.* (1984b) found that *Acetobacter* strains produced very small amounts of acetic acid in must, while *G. oxydans* produced up to 0.92 g/l of the acid in the same must. They postulated that this could be due to the break down of acetic acid by the *Acetobacter* strains. However, it seems unlikely that acetic acid bacteria metabolise acetic acid under normal winemaking conditions, because these bacteria only utilised acetic acid when all other alternative carbon sources, such as ethanol and glucose, had been exhausted completely (Saeki *et al.*, 1997a).

Acetic acid can also inhibit acetic acid bacteria, but these organisms are generally far more resistant to this effect than other microorganisms associated with winemaking. This resistance is also strain dependent, with ethanol functioning synergistically with acetic acid to inhibit the bacteria (Nanba *et al.*, 1984). The enzyme citrate synthase plays a key role in this resistance, which detoxicates acetic acid by incorporation into the tricarboxylic or glyoxylate cycles. Citrate synthethase could also supply the large amounts of ATP necessary to overcome the toxic effect of the acid (Fukaya *et al.*, 1990; Sievers *et al.*, 1997). Menzel and Gottschalk (1985) reported that an *Acetobacter* strain lowered its internal pH in response to a lower external pH. The bacterium was still able to grow with a small ΔpH existing over the cell membrane. However, an adaptation to high acetate concentrations seems to be a prerequisite for high tolerance (Lasko *et al.*, 2000).

Another product of the metabolism of acetic acid bacteria that could affect wine quality is ethyl acetate. This ester of acetic acid could contribute positively to wine aroma at low concentrations, but is considered unwanted at higher concentrations, due to its low flavour threshold of 10 mg/l (Berg *et al.*, 1955). Kashima *et al.* (1998) isolated two esterases responsible for the production of ethyl acetate in *A. pasteurianus*, which are activated by ethanol and are still active at pH 3. The growth of acetic acid bacteria can increase the ethyl acetate concentration by up to 140 mg/l in wine and up to 30 mg/l in must (Drysdale and Fleet, 1989a). Ethyl acetate is also produced during alcoholic fermentation by the yeast and the growth of acetic acid bacteria could further increase the amount of this ester over and above the concentration considered to be detrimental to wine quality (Drysdale and Fleet 1989a, b). Lactic acid bacteria can also produce this ester, but, according to Henick-Kling (1993), only at very low levels, often spoiling the wine in a sensorially different manner from acetic acid bacteria, which can produce higher levels. Acetic acid bacteria can also oxidise higher alcohols, like isoamyl alcohol, 1-propanol and 2-phenylethanol to the corresponding aldehyde and carboxylic acid (Molinari *et al.*, 1997, 1999).

Carbohydrate metabolism and its products

Acetic acid bacteria can metabolise different carbohydrates as a carbon source. As in the case of many other microorganisms, glucose is a good carbon source for most strains of acetic acid bacteria. These bacteria can utilise glucose through different metabolic pathways. *Acetobacter* species can use this sugar through 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). From here it is further metabolised to CO_2 and water through the tricarboxylic acid pathway. Flücker and Ettlinger (1977) postulated that some *Acetobacter* strains use the pentose phosphate pathway to metabolise

glucose. However, it seems as if not all strains of *Acetobacter* can utilise glucose effectively, as was found by De Ley (1961). De Ley *et al.* (1984) also state that some *A. pasteurianus* strains cannot grow on media containing glucose as a sole carbon source. This could be due to the inability of these strains to phosphorylate this sugar upon entry into the cell (De Ley, 1959). However, the ease and regularity with which *A. pasteurianus* has been isolated from glucose-containing media (Drysdale and Fleet, 1985; Du Toit and Lambrechts, 2002) confirms the fact that this characteristic is probably strain dependent.

Sugar is normally preferred as a carbon source more by *Gluconobacter* than by *Acetobacter*. This is also reflected in the fact that glucose is a better carbon source for this genus than for *Acetobacter* (De Ley *et al.*, 1984). The glucose metabolism in *Gluconobacter* has been the topic of numerous studies because of the production of metabolites with industrial importance by the bacteria during growth on glucose (Olijve and Kok, 1979; Weenk *et al.*, 1984; Buse *et al.*, 1991; Qazi *et al.*, 1991; Qazi *et al.*, 1993; Velizarov and Beschkov, 1994). These metabolites include gluconic, 2-ketogluconic, 5-ketogluconic and 2,5-diketogluconic acid. As does the alcohol dehydrogenase enzyme, the acetic acid bacteria also possess an $NAD(P)^+$-dependent glucose dehydrogenase in the cytoplasm and a membrane-bound $NAD(P)^+$-independent glucose dehydrogenase, with the latter being responsible for most of the glucose conversion. According to Qazi *et al.* (1991), glucose is oxidised to glucono-δ–lactone and from there to gluconic, 2-ketogluconic and 2,5-diketogluconic acid, respectively. *Gluconobacter* can also use the pentose phosphate pathway to generate energy. The route through which acetic acid bacteria oxidise glucose is dependent on the pH and the glucose concentration. Olijve and Kok (1979) found that a pH lower than 3.5 and a glucose concentration of between 0.9-2.7 g/l inhibit glucose oxidation through the pentose phosphate pathway and it gets oxidised directly to gluconic acid. According to Weenk *et al.* (1984), the bacterium starts to utilise gluconic acid at a glucose concentration lower than 1.8 g/l. The optimum temperature for this direct oxidation is between 30 and 33 ºC (Stadler-Szöke *et al.*, 1980).

G. oxydans can produce up to 120 g/l of gluconic acid, while *Acetobacter* strains are also able to produce high levels (Seiskari *et al.*, 1985; Attwood *et al.*, 1991). The production of these sugar acids in grape musts can thus be attributed mainly to the oxidation of glucose by acetic acid bacteria and not by the growth of the fungus *Botrytis cinerea*, as previously thought (Sponholz and Dittrich, 1984; Sponholz and Dittrich, 1985; Eschenbruch and Dittrich, 1986). The production of gluconic and ketogluconic acids can also influence the winemaking process, as a result of the ability of these acids to bind SO_2, thus rendering it ineffective against microorganisms. This could then lead to a higher total SO_2 concentration in the must or wine to maintain the desired level of free SO_2 (Eschenbruch and Dittrich, 1986). Barbe *et al.* (2001) found a total SO_2 content of 3000 mg/l being needed to obtain a free SO_2 level of 50 mg/l in synthetic musts in which certain *Gluconobacter* strains had grown, due to the production of high concentrations of gluconic acid (51 g/l), 5-oxofructose (5833 mg/l) and dihydroxyacetone (2032 mg/l) from glucose, fructose and glycerol, respectively. The latter two compounds can also bind SO_2 very efficiently. Another important byproduct of glucose metabolism is the production of extracellular polysaccharides by acetic acid bacteria (Kouda *et al.*, 1997). Tayama *et al.* (1986) found one of these polysaccharides to consist of β-(1-4)-linked D-glucose residues with side chains consisting of L-rhamnosyl-(1-6)-D-

glucosyl-(1-6)-D-glucosyl-(1-4)-D-glucuronosyl-(1-2)-D-mannose. Drysdale and Fleet (1989b) also reported on an *A. pasteurianus* strain producing large quantities of an extracellular gum-like material in grape must in the presence of *S. cerevisiae*. The production of this gum was surprisingly not observed in the absence of the yeast. The production of these polysaccharides could affect the filterability of wine. Tahara *et al.* (1998) described an exo-1,4-β-glucosidase isolated from *Acetobacter xylinum* that breaks down some of these extracellular oligosaccharides.

Acetic acid bacteria can also utilise other carbohydrates, such as arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol and xylose (De Ley *et al.*, 1984). Joyeux *et al.* (1984b) found that *G. oxydans* and *A. aceti* preferred glucose over fructose in grape must, but started to utilise some of the fructose when there was still some glucose left in the must. Fructose is oxidised to 5-oxofructose by a fructose dehydrogenase, which is linked to the membrane of *Gluconobacter*. This bacterium also has a cytoplasmic 5-oxofructose reductase, which reduces the oxidised sugar (Avigad *et al.*, 1966; Barbe *et al.*, 2001). Arabitol, erythritol, mannitol and sorbitol can occur in *Botrytis*-infected grape must at low concentrations (Barbe *et al.*, 2001), but it remains to be seen if it could support the growth of acetic acid bacteria in must or wine. Drysdale and Fleet (1989a), however, found that *A. aceti* and *A. pasteurianus* are able to utilise residual sugar completely in two red wines during their growth. *S. cerevisiae* is unable to metabolise these sugars during primary fermentation, which therefore could leave the residual sugars in the wine, allowing the acetic acid bacteria to grow.

Organic acids metabolism and its products

Acetic acid bacteria are also able to metabolise different organic acids. This is achieved through the tricarboxylic acid cycle through which these acids are oxidised to CO_2 and water. It is not surprising then that *Gluconobacter*, which lacks a functional tricarboxylic acid cycle, is unable to oxidise most organic acids (Holt *et al.*, 1994). These organic acids include acetic, citric, fumaric, lactic, malic, pyruvic and succinic acids. *Acetobacter* strains were able to significantly decrease the malic and citric acid concentrations in must (4.7 to 1.8 g/l and 230 to 147 mg/l, respectively), while the succinic acid concentration increased, especially in the must in which *Gluconobacter* had grown. A decrease in tartaric acid concentrations due to the growth of acetic acid bacteria has also been reported. The cumulative effect of these changes can influence the wine quality (Joyeux *et al.* 1984b; Drysdale and Fleet, 1989a). De Ley and Schell (1959) found that an *Acetobacter* strain degraded D-lactate four times faster than the L-isomer. Another acid that is also formed is propionic acid, which has a threshold value of 20 parts per million, and acetic acid bacteria can produce between 10 and 30 mg/l of this acid in wine, which could further influence wine quality due to its unpleasant smell (Drysdale and Fleet, 1989a).

Another important metabolic byproduct of lactate metabolism is acetoin. De Ley (1959) reported on an *Acetobacter* strain that converted most of the lactate in a culture medium into acetoin. This formation could be via the formation of a-acetolactate. The buttery aroma of this compound is considered to be an unwanted flavour in wine, in which it has a detection limit of 150 mg/l (Romano and Suzzi, 1996; Du Toit and Pretorius, 2000).

Glycerol metabolism and dihydroxyacetone formation

Glycerol is also a good carbon source for acetic acid bacteria (De Ley *et al.*, 1984).

Most of the glycerol is converted into dihydroxyacetone and a small part is utilised via the phosphorylating oxidative pathway for biomass and energy synthesis (Œvitel and Œturdik, 1994). Certain *G. oxydans* and *Acetobacter* strains can produced high levels of dihydroxyacetone in grape or synthetic must, ranging from 259 mg/l to 2543 mg/l, which could influence wine quality (Sponholtz and Dittrich, 1985; Barbe *et al.*, 2001). Eschenbruch and Dittrich (1986) found that acetic acid bacteria produced more dihydroxyacetone in grape must in the presence of yeast. This could be due to the production of glycerol by the yeast, which is converted to dihydroxyacetone by the acetic acid bacteria. Dihydroxyacetone is also known to bind SO_2, which could decrease the free SO_2 concentration in must and wine. The enzyme responsible for this conversion, glycerol dehydrogenase, is situated on the plasma membrane, and sufficient oxygen is required for this reaction (Claret *et al.*, 1994; Œvitel and Œturdik, 1994). The ability of acetic acid bacteria to use glycerol as a carbon source could further enhance their survival and growth in wine. Glycerol is considered to enhance the mouth feel of a wine and is therefore a wanted compound in wine. Drysdale and Fleet (1989a) reported on an *A. aceti* strain that reduced the glycerol concentration in a wine from 6.43 g/l to 2.39 g/l, which is below the taste threshold value of 4 to 5 g/l. Acetic acid bacteria can also use electron acceptors other than oxygen, such as ρ-benzoquinone, when growing on glycerol (Adlercreutz and Mattiasson, 1984), which could further support their growth in wine.

INTERACTIONS WITH OTHER WINE-RELATED MICROORGANISMS

The microbial ecology of grape must can be complex, with different species and strains of non-*Saccharomyces* yeasts, *S. cerevisiae*, lactic acid bacteria and acetic acid bacteria occurring in the same fermentation. Yeast and lactic acid bacteria are generally more sensitive to acetic acid than to acetic acid bacteria. Therefore, it is not surprising that acetic acid bacteria can influence these other microorganisms. Gilliland and Lacey (1964) found an *Acetobacter* strain capable of inhibiting *S. cerevisiae* and other wild yeasts. Grossmann and Becker (1984) and Joyeux *et al.* (1984b) also observed that these bacteria have an inhibiting effect on yeast. In this work, the yeast cells were inoculated after the initial growth of the acetic acid bacteria had taken place. Drysdale and Fleet (1989b) studied the effect of inoculating yeast and acetic acid bacteria simultaneously, as would normally happen in a wine cellar when yeast is inoculated after the grapes are crushed. They found that the acetic acid bacteria did not inhibit the growth of the yeast to a great extent, but that they did affect the ability of the yeast to ferment the must to dryness. The *A. pasteurianus* strains tested inhibited the fermentation ability of the yeast the most, with between 24-30 g/l of glucose and 45-55 g/l of fructose being left after the control fermentations were fermented dry. In the control fermentations, no acetic acid bacteria were inoculated prior to alcoholic fermentation. The inhibition of *S. cerevisiae* by acetic acid bacteria also correlates with the production of acetic acid by these bacteria, with *Gl. hansenii* and *A. pasteurianus* inhibiting the yeast the most. However, some other unknown inhibition mechanisms could also exist (Du Toit, 2000). Most strains of *S. cerevisiae* are glucophilic and therefore prefer to take up glucose faster than fructose. This could lead to an imbalance in the glucose/fructose ratio, which can lead to a stuck fermentation (Schütz and Gafner, 1993). Joyeux *et*

al. (1984b) reported that the acetic acid bacteria tested metabolised glucose faster than fructose. This could also lead to an imbalance in the glucose/fructose ratio, which could further contribute to a stuck fermentation. It is therefore clear that any delay preventing the onset of alcoholic fermentation could result in these bacteria being able to grow and produce compounds inhibitory to the yeast. Saeki *et al.* (1997b) isolated thermotolerant acetic acid bacteria able to grow at temperatures between 37-40 ºC, and it is likely that thermotolerant acetic acid bacteria can also occur under oenological conditions. These temperatures can easily be reached in an alcoholic fermentation if efficient temperature control is not applied and could be detrimental to the yeast. Bacteria are less likely to suffer the harmful effects of temperatures exceeding their optimal temperature than yeast, due to a higher energy requirement for multiplication and denaturation activation by the yeast. Excessively high fermentation temperatures thus could promote the growth of these thermotolerant acetic acid bacteria, while making the yeast more sensitive to ethanol and acetic acid (De Ory *et al*. 1998).

Acetic acid bacteria can also influence lactic acid bacteria, but very little work has been done in this regard. Gilliland and Lacey (1964) found an *Acetobacter* strain that inhibits a *Lactobacillus* species, but Joyeux *et al.* (1984b) found acetic acid bacteria to stimulate malolactic fermentation.

CONCLUSION

The ability to survive in wine, which is normally an anaerobic or semi-anaerobic medium and which, in addition, has a low pH, a high alcohol content and is characterised by the presence of SO_2, imposes challenges to the survival of aerobic acetic acid bacteria. It is clear, however, that acetic acid bacteria can survive and even grow in this harsh environment if left unchecked by the wine producer. Sound winemaking practices should be able to assist the wine producer in preventing the unwanted process of wine spoilage by acetic acid bacteria. These practices include the elements discussed in this review, as well as good cellar hygiene. Knowledge of the occurrence, metabolism, interactions with other microorganisms and methods of inhibition of acetic acid bacteria in the winemaking process in particular are still limited, however, and need further investigation. At present, acetic acid bacteria in winemaking are still very much an unknown factor. Fortunately, new developments in analytical chemistry, microbiology and molecular biology can help to change this situation.

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