Control of Malolactic Fermentation in Wine. A Review

R. Bauer and L.M.T. Dicks*

Department of Microbiology, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa

Submitted for publication: June 2004

Accepted for publication: October 2004

Key words: Malolactic fermentation, control

Malolactic fermentation (MLF) is conducted by lactic acid bacteria (LAB) and refers to the decarboxylation of L-malate to L-lactate. This secondary fermentation is difficult to control and is mainly driven by *Oenococcus oeni*. Uncontrolled MLF, especially in wines with a high pH, which are typical of warmer viticultural regions, may render the wine unpalatable or even cause spoilage. In this review we focus on wine compounds and emphasise factors that affect the growth of *O. oeni* and MLF, and discuss practical applications. We also explore alternative technologies that may enable better control over MLF.

INTRODUCTION

Winemaking normally involves two fermentation processes: an alcoholic fermentation conducted by yeast, and malolactic fermentation (MLF) performed by lactic acid bacteria (LAB) containing a malolactic enzyme (MLE). MLF plays an important role in determining the final quality of most red wines, but also certain white wines and classic sparkling wines. Apart from an increase in pH, additional sugars are fermented and aromatic compounds are produced which change the organoleptic profile of the wine. The cells gain energy from the uniport of monoanionic L-malate through the generation of a proton gradient across the cell membrane (Salema *et al.*, 1996b). Only strains of *Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus* resistant to low pH (<3.5), high SO_2 (50 ppm) and ethanol levels of ca. 10% (v/v), survive in wine (Van Vuuren & Dicks, 1993; Lonvaud-Funel, 1999). *Pediococcus damnosus*, *Leuconostoc mesenteroides* and *Oenococcus oeni* predominate during alcoholic fermentation (Lonvaud-Funel, 1999). However, towards the end of alcoholic fermentation spontaneous MLF is mainly driven by *O. oeni* (Van Vuuren & Dicks, 1993), a species formerly known as *Leuconostoc oenos* (Dicks *et al.*, 1995).

MLF is encouraged in cool viticultural regions where grapes may have high levels of malic acid, in wine aging in oak barrels, when long-time maturation in bottles is part of the process (e.g. Champagne), or when a specific organoleptic profile is required, as in Chardonnay, Burgundy white wines and Bordeaux red wines. In some wines MLF is considered spoilage, especially in warm viticultural regions with grapes containing less malic acid. In addition to undesirable organoleptic changes, the colour of red wine may be reduced by as much as 30% (Van Vuuren & Dicks, 1993), and biogenic amines may be produced (Lonvaud-Funel & Joyeux, 1994).

Spontaneous MLF is unpredictable, since it may occur any time during or several months after the completion of alcoholic fermentation. The wine may also become infected by bacteriophages, especially during extended fermentation (Henick-Kling, 1995). The use of starter cultures to induce MLF is often unsuccessful because of the rapid loss of cell viability after inoculation. Hence, studies on factors affecting the growth and survival of *O. oeni* in wine are important and methods to control MLF remain a priority.

Several excellent reviews of MLF and malolactic bacteria have been published (Radler, 1966; Kunkee, 1967; Amerine & Kunkee, 1968; Beelman & Gallander, 1979; Davis *et al.*, 1985; Wibowo *et al.*, 1985; Henick-Kling, 1988; Kunkee, 1991; Henick-Kling, 1993). This review focuses on the influence of physical and chemical factors on MLF, alternative technologies to promote MLF, and the role of bacteriocins (antimicrobial peptides) produced by lactic acid bacteria.

MALOLACTIC FERMENTATION AND THE MALOLACTIC ENZYME

LAB are strictly fermentative and, with the exception of a few streptococci, lack electron transfer chains (Salema *et al.*, 1996b). Therefore, generation of a proton motive force (PMF) can only be achieved by proton translocation via the membrane-bound F_0F_1 H^+-ATPase driven by the hydrolysis of ATP, or by some other chemiosmotic processes. Three chemiosmotic mechanisms for PMF generation have been described for LAB: (i) carrier-mediated excretion of fermentation end products in symport with protons (Ten Brink *et al.*, 1985), (ii) electrogenic precursor-product exchange (Poolman, 1990) and (iii) electrogenic uniport (Salema *et al.*, 1994) in combination with metabolic breakdown of the substrate in the cell. MLF (Salema *et al.*, 1994) and citrate metabolism (Ramos *et al.*, 1995b) are examples of the anion uniport mechanism in *O. oeni*. MLF is a PMF-generating process conducted by some LAB and, as a consequence, metabolic energy is conserved (Cox & Henick-Kling, 1989; 1990). The metabolic pathway is based on the electrogenic uptake of L-malate, its intracellular conversion to L-lactate plus CO_2, and the excretion of the end products (Salema *et al.*, 1994). The mechanism of metabolic energy generation by MLF in *O. oeni* was inferred from transport studies with membrane vesicles (Salema *et al.*, 1994). Monoprotonated L-malate (L-malate$^-$) is taken up by electrogenic uniport with a net negative charge being moved inwards, thereby

*Corresponding author: E-mail address: lmtd@sun.ac.za

creating an electrical potential, $\Delta\psi$ (inside negative relative to outside). Once inside the cell, L-malate is decarboxylated to L-lactate and carbon dioxide in a reaction that requires one proton. This alkalisation of the cytoplasm results in the creation of a pH gradient (ΔpH) that, together with the $\Delta\psi$, forms the proton motive force (expressed in Δp) across the cytoplasmic membrane. The PMF generated under such conditions is sufficient to drive ATP synthesis via the membrane-bound F_0F_1 ATPases (Olsen *et al.,* 1991; Poolman *et al.,* 1991). L-lactate and CO_2 appear to leave the cell as neutral species (Salema *et al.,* 1994). The latter mechanism of PMF generation was confirmed by *in vitro* reconstitution of the MLF pathway of *O. oeni* (Salema *et al.,* 1996a).

Decarboxylation of L-malate to L-lactate is catalysed by the malolactic enzyme (MLE) with the requirement of NAD^+ and Mn^{2+}, and does not generate intermediate nor cofactor reduction, which is different from the malic enzyme leading to pyruvate. MLE, the only enzyme involved in MLF, has been purified from several LAB (Lonvaud-Funel & Strasser de Saad, 1982; Caspritz & Radler, 1983; Spettoli *et al.,* 1984; Naouri *et al.,* 1990). The active form is composed of two or four identical subunits of 60-70 kDa and the protein is strongly homologous to malic enzymes from different organisms. Malic and malolactic enzymes are, however, distinct at the phylogenetic level, except for malic enzymes of yeast and *E. coli*, which are closer to malolactic enzymes than other malic enzymes (Groisilliers & Lonvaud-Funel, 1999). In the presence of NAD and Mn^{2+}, the activity of MLE is similar to the malic enzyme combined with lactate dehydrogenase, but without the release of intermediate products. The complete nucleic acid sequence of the *mle* gene has been determined for *Lactococcus lactis* (Denayrolles *et al.,* 1994), *O. oeni* (Labarre *et al.,* 1996) and *P. damnosus* (Bauer, 2003).

STARTER CULTURES AND GROWTH STIMULATION

O. oeni predominates at pH below 3.5 and is principally responsible for MLF (Kunkee, 1967; Wibowo *et al.*, 1985). *Pediococcus damnosus*, *Pediococcus pentosaceus*, *Pediococcus parvulus*, *Pediococcus inopinatus*, and several *Lactobacillus* spp. have been isolated from wines with a pH between 3.5 and 4.0 (Van der Westhuizen, 1980; Wibowo *et al.*, 1985).

Inoculation with starter cultures reduces the potential of spoilage by other lactic acid bacteria and/or bacteriophages, ensures a rapid onset of MLF, and provides better control over the production of aromatic compounds and thus wine flavour (Henick-Kling, 1988). A number of different starter cultures have been developed, most of which are marketed lyophilised or frozen. Viability as high as 95% has been recorded for freeze-dried cells (Henick-Kling, 1993). Although frozen concentrates have been used by some wineries in the United States, transport of the cultures and long-term storage in wineries is a problem. One possible alternative method of culture preparation is fluid bed drying, similar to the process developed to produce dried yeast. However, the technology has not been optimised for malolactic bacteria.

Preparation of starter cultures entails growth under controlled conditions, preferably below pH 4.5 (Lafon-Lafourcade, 1975; Henick-Kling, 1990) and at an incubation temperature with no more than 10°C delineation of wine-producing temperatures to prevent thermal shock (Henick-Kling, 1993). Direct inoculation of rehydrated starter cultures into wine leads to a decrease of at least three log-cycles in cell numbers (Rodriques *et al.*, 1990). To compensate for this reduction, cells have to be reactivated in media enriched with yeast extract and grape juice (Lafon-Lafourcade, 1970; Lafon-Lafourcade *et al.*, 1983). The optimal time of inoculation to ensure best growth of the starter culture depends on the type of wine (grape cultivar), SO_2 and alcohol content, pH and temperature (Henick-Kling, 1993).

Growth of *O. oeni* in wine is enhanced if grown in a medium supplemented with 40% to 80% wine (Davis *et al.*, 1985), or yeast (Kunkee, 1967). The effect of yeast on the growth of malolactic bacteria and *vice versa* has been reviewed by Alexandre *et al.* (2004). Nutrients produced during yeast autolysis may stimulate the growth of malolactic bacteria (Fornachon, 1968; Mascarenhas, 1984; Van Wyk, 1976). Gallander (1979), on the other hand, recorded poor growth in the presence of yeast extract, suggesting that the dependence on yeast extract may be strain specific.

Inoculation of bacteria during alcoholic fermentation is preferred by some winemakers (Davis *et al.*, 1985; Gallander, 1979). At this stage most of the free SO_2 is bound by organic acids produced during yeast growth (Davis *et al.*, 1985). Inoculation at the end of alcoholic fermentation may result in delayed MLF due to high ethanol concentrations (Lafon-Lafourcade *et al.*, 1983; Davis *et al.*, 1985).

INFLUENCE OF PHYSICAL AND CHEMICAL FACTORS ON MLF

Temperature

Temperature affects the growth rate and length of the lag phase of LAB, thus also the maximum population of malolactic bacteria. The optimal growth rate of strains of *O. oeni* is close to 25°C (Henick-Kling, 1993). Survival of *O. oeni* in wine and its ability to perform MLF was, however, improved by pre-incubation at 42°C (Guzzo *et al.,* 1994). The latter temperature induces synthesis of stress proteins in *O. oeni* (Guzzo *et al.,* 1997). Many of these proteins may function as molecular chaperones or proteases that participate in the refolding of proteins or the degradation of denatured cellular proteins (Craig *et al.,* 1993). At low growth temperatures (8°C), *O. oeni* became more resistant to pore-forming antimicrobial peptides, such as pediocin PD-1 (Bauer, 2003).

Tourdot-Maréchal *et al.* (2000) compared the kinetics of membrane fluidity variation of instantaneously stressed *O. oeni* cells with cells adapted to the stress factor by a pre-incubation in inhibitory growth conditions. Membrane fluidity of heat-adapted cells increased only slightly when exposed to 42°C and the rate of membrane fluidisation was five-fold lower than with non-adapted cells. To maintain optimal fluidity under various growth conditions, cells regulate the lipid composition in their cell membranes (Lehninger *et al.,* 1993). An increase in growth temperature induces an increase in the incorporation of saturated fatty acids, while unsaturated fatty acids decrease. A decrease in temperature has the opposite effect. The higher the proportion of saturated fatty acids, the higher the solid-to-fluid transition temperature of the cell membrane. According to Tourdot-Maréchal *et al.* (2000), the ability of *O. oeni* to regulate its membrane fluidity, as described here, represents a stress-tolerance mechanism. The decrease in pediocin PD-1-induced K^+ efflux observed at lower

temperatures (Bauer, 2003) may thus be due to changes in the lipid and protein content in the cell membrane of *O. oeni*.

Ethanol

Ethanol strongly interferes with the growth and metabolic activity of lactic acid bacteria. High ethanol concentrations decrease the optimal growth temperature of LAB and ethanol tolerance is decreased at elevated temperatures (Henick-Kling, 1993). Although ethanol concentrations found in wine (8-12%, v/v) is not inhibitory towards malolactic activity (Capucho & San Romão, 1994), the growth rate of *O. oeni* decreases linearly with increasing ethanol concentrations, with 14% (v/v) being the upper limit tolerated by most strains (Davis *et al.*, 1988; Henick-Kling, 1993). Growth is completely inhibited at 25°C and above in the presence of 10 to 14% (v/v) ethanol. Optimum growth (shortest lag time, fastest growth rate and highest cell yield) at these alcohol concentrations occurs between 18 and 20°C compared to 30°C at 0 to 4% (v/v) ethanol (Henick-Kling, 1993). Cell yield is less affected by ethanol and temperature than growth rate, with maximum cell yield in media containing 0 to 8% (v/v) ethanol at approx. 22°C. The degree of ethanol tolerance is, however, strain dependent and also depends on the pH and nitrogen status of the culture medium (Britz and Tracey, 1990). Strains of *Lactobacillus* and *Pediococcus* are in general more tolerant to high ethanol concentrations than *O. oeni* (Davis *et al.*, 1988).

The cell membrane is likely to be the primary site for the expression of an adaptive response to ethanol, with lipids being the main target (Jones, 1989). Changes in the membrane lipid composition induced by ethanol have been described for *Bacillus subtilis* (Rigomier *et al.,* 1980), *Escherichia coli* (Dombeck & Ingram, 1984), *Lactobacillus hilgardii* (Couto *et al.,* 1996), and *O. oeni* (Tracey & Britz, 1989a; Garbay *et al.,* 1995). The adaptive response to the presence of high concentrations of ethanol is aimed at maintaining the fluidity and integrity of the cell membrane (Couto *et al.,* 1996). Ethanol-induced changes in the fatty-acid profile of *Bacillus subtilis* cell membranes coincided with a decrease in membrane fluidity (Rigomier *et al.,* 1980). The model proposed for *E. coli* (Dombeck & Ingram, 1984) also predicts a decrease of membrane fluidity in cells grown in the presence of ethanol. On the other hand, the membrane fluidity of cells of *L. hilgardii* and *O. oeni* was increased in the presence of ethanol (Couto *et al.,* 1996; Tourdot-Marcéchal *et al.,* 2000; Teixeira *et al.,* 2002).

Tourdot-Maréchal *et al.* (2000) showed that the rate of membrane fluidisation after an ethanol shock was threefold lower with cells pre-incubated in ethanol than with non-adapted cells. The positive effect of adaptation was time-limited, since membrane fluidity was similar at the end of the treatment. Incubation in the presence of ethanol induced a rapid increase in membrane rigidity. Based on the hypothesis of 'homeoviscous adaptation' (Sinensky, 1974), the production of a more fluid membrane is a compensation for the increase in rigidity generated by ethanol stress.

Teixeira *et al.* (2002) studied the lipid and protein composition of the membrane of *O. oeni* in the presence of different ethanol concentrations. The percentage of membrane lactobacillic acid increased at the expense of *cis*-vaccenic acid when cells were grown in the presence of ethanol higher than 8% (v/v). Lactobacillic acid is a ring-containing fatty acid produced during late exponential to stationary phase growth and is formed by conversion of the unsaturated position of *cis*-vaccenic acid to a cyclopropane ring. Other than this, the membrane fatty-acid profile was similar along the cell growth cycle for all the ethanol concentrations assayed. The increase of lactobacillic acid in the membrane of *O. oeni* appears to provide protection against the toxic effect of ethanol, balancing the increase of membrane fluidity normally attributed to ethanol. By cyclising the unsaturated fatty acids, bacteria may stabilise their plasma membrane, particularly at stationary-phase. This could explain why bacteriocin-induced cell lysis of *O. oeni* was least prominent in stationary-phase cells (Bauer, 2003).

Ethanol at concentrations up to 8% (v/v) induced an increase in membrane permeability in resting cells of *O. oeni*, but not in cells grown in the presence of 8% (v/v) ethanol (Teixeira *et al.*, 2002). The total membrane protein content of cells grown in the presence of 8% (v/v) or higher ethanol decreased (Teixeira *et al.,* 2002). However, the synthesis of low-molecular weight-stress proteins was induced and may be involved in cell adaptation (Guzzo *et al.,* 1997; Guzzo *et al.,* 2000; Tourdot-Maréchal *et al.,* 2000; Teixeira *et al.,* 2002). In conclusion, the development of ethanol resistance in *O. oeni* is a complex and multi-layered phenomenon, which depends on the severity and duration of the shock and on culture conditions such as medium composition, pH and temperature.

pH

Wine pH plays an important role in determining which LAB species will survive and develop as well as the growth rate of the bacteria. In terms of initiation and completion of MLF, wines of pH 3.3 and above generally exhibit few problems, whereas at lower pH, difficulties may be experienced (Kunkee, 1967). *O. oeni* usually represents the dominant species in wine below pH 3.5. At higher pH *Lactobacillus* and *Pediococcus* spp. may survive and grow. The pH strongly affects malolactic activity of the cell (Henick-Kling, 1993). Although sugar utilisation and growth of *O. oeni* are inhibited by low pH (Davis *et al.*, 1986), malolactic activity is the highest at pH 3.5 to 4.0. Also, malate transport activity in *L. plantarum* is higher in cells grown at pH 3.5 compared to cells grown at pH 6.0 (Olsen *et al.,* 1991).

Survival of *O. oeni* in wine improved when cells were subjected to an acid shock before inoculation, presumably due to the synthesis of specific stress proteins (Guzzo *et al.,* 1994, Guzzo *et al.,* 1997, 1998; Guzzo *et al.,* 2000). However, physiological studies concerning acid tolerance have mainly been focused on MLF. The energy-yielding MLF pathway explains the physiological benefits of MLF, particularly under very acid conditions. The fermentation of L-malate generates both a transmembrane pH gradient and an electrical potential gradient. Proton consumption during the decarboxylation of L-malate participates in the regulation of intracellular pH, while the PMF generated by MLF is used for additional ATP synthesis (Henick-Kling, 1995).

A mechanism that seems to be strictly linked to acid tolerance in LAB is ATP hydrolysis and proton extrusion by the membrane-bound H^+-ATPases (Tourdot-Maréchal *et al.,* 1999). Since bacteria extrude H^+ at acidic pH, this process plays an important role in PMF maintenance and pH homeostasis. In the case of anaerobic enterococci the only function of the membrane H^+-ATPase is to regulate the intracellular pH (pH_{in}) and maintain a ΔpH across the membrane (Shibata *et al.,* 1992). When the pH_{in} was lowered

below a certain threshold, the activity and synthesis of the H^+-ATPase increased. A study on the H^+-ATPase of *Enterococcus hirae* revealed a sub-unit composition identical to other bacterial F_0F_1 ATPases. Unfortunately, little is known about H^+ATPases and their role in pH homeostasis for other LAB. Drici-Cachon *et al.* (1996) have shown that the ATPase activity of an acidophilic *O. oeni* mutant significantly increases when grown at pH 2.6, which is usually lethal for the wild-type strain. The survival of LAB under acid conditions, therefore, depends on the activation of membrane-bound H^+-ATPase.

Tourdot-Maréchal *et al.* (1999) isolated *O. oeni* neomycin-resistant mutants as H^+-ATPase-deficient strains. The acid sensitivity of these mutants supported the hypothesis that the major role of H^+-ATPase is maintenance of intracellular pH. Surprisingly, all the mutants were devoid of malolactic activities. Since the growth rates of the mutant strains were also impaired when cultured under optimum conditions, acid sensitivity could not be the primary consequence of the lack of L-malate metabolism in energy production and intracellular pH homeostasis. The results suggested that the ATPase and malolactic activities of *O. oeni* are linked and play a crucial role in resistance to acid stress.

Another surprising observation was that no significant increase of ATPase activity was detected in wild-type *O. oeni* cells incubated at low pH. This absence of induction could be explained by the existence of several cation transport ATPase systems of which maximal activities depend on the pH of the media. Using inhibitors specific for different types of ATPases, Guzzo *et al.* (2000) demonstrated the existence of H^+-ATPase and K^+-translocating ATPase, which is also referred to as the P-type ATPase.

Sulfur dioxide

It is common practice to add SO_2 (50 to 100 mg/L) to must at the beginning of the vinification process to restrict the growth of indigenous yeast such as *Kloeckera* and *Henseniaspora* spp. and bacteria, mainly acetic acid bacteria (Fleet & Heard, 1993). Some yeast strains also produce relatively large quantities of SO_2 (King & Beelman, 1986). At low pH such as in wine (pH of 3 to 4), sulfite predominates as free SO_2 (Usseglio-Tomasset, 1992), consisting mainly of bisulfite anion (HSO_3^{-1}) and a small proportion of molecular SO_2 ($SO_2.H_20$) and sulfite anion (SO_3^{-2}). Molecular SO_2, the only form of SO_2 that can cross cell walls of yeast and bacteria, enters the cell by diffusion and is converted to HSO_3^{-1}. In the cell sulfite may react with proteins, nucleic acids and cofactors, affecting the growth of LAB (Carreté *et al.,* 2002) and yeast (Constantí *et. al.*, 1998). The majority of *O. oeni* cells died within 3 hrs in the presence of 15 mg/L free sulfite (Guzzo *et al.,* 1998). Levels of 5 mg/L free SO_2 resulted in complete MLF lasting longer than 40 days (Carreté *et al.,* 2002). The F_0F_1 ATPase activity of *O. oeni* cells was more than 50% inhibited in the presence of 20 mg/L free SO_2 (Carreté *et al.,* 2002). Malolactic activity is also influenced by SO_2 (Henick-Kling, 1993). Bound SO_2 at 20 mg/L reduces L-malate degradation by 13%, 50 mg/L reduces it by 50%, and 100 mg/L inhibits malolactic activity completely.

A number of carbonyl compounds (mainly acetaldehyde, α-ketoglutaric acid and pyruvic acid) bind with free SO_2 (especially HSO_3^{-1}) to form a complex compound (bound SO_2) which has only weak antimicrobial properties. Bound SO_2 at 30 mg/L delays the growth of LAB, whereas bound SO_2 at more than 50 mg/L may completely inhibit growth (Henick-Kling, 1993). Furthermore, free SO_2 released upon microbial metabolism of bound acetaldehyde may cause microbial inhibition resulting in stuck or sluggish MLF (Osborne *et al.,* 2000). Other SO_2-binding compounds, such as a-ketoglutaric acid and pyruvic acid, are also substantially reduced during MLF and may therefore lead to similar results (Nielsen & Riechelieu, 1999).

O. oeni developed a tolerance to sulfite as high as 30 mg/L and cells adapted to low pH survived better than non-adapted cells (Guzzo *et al.,* 1998). Addition of a sub-lethal concentration of sulfite (15 mg/L) during the adaptation step in acidic medium (pH 3.5) increased sulfite tolerance. Higher concentrations of sulfite (60 mg/L) induced the synthesis of Lo18, a small heat-shock protein. It appears, therefore, that several adaptation mechanisms, including pH homeostasis and stress protein synthesis, could be involved in the induction of sulfite resistance in *O. oeni*.

Carbohydrates

The major residual sugars in wine after completion of alcoholic fermentation are glucose and fructose, which may vary from 10 g/L to less than 0.5 g/L, depending on the style of wine. Fructose is always found in higher concentrations than glucose. Although glucose is preferred by *O. oeni*, fructose is the most efficiently metabolised sugar, leading to maximum biomass levels during co-metabolism with glucose (Maicas *et al.,* 1999a). Fructose is not only metabolied via the heterofermentative pathway, but is also reduced to mannitol by mannitol dehydrogenase (Fig. 1). Sugars other than glucose and fructose may be present in wine at concentrations as high as 1.3 g/L (Henick-Kling, 1995). The ability of these sugars to support growth of *O. oeni* is strain specific.

MLF is reduced by 50% in the presence of 2 mM glucose (Miranda *et al.*, 1997). At 5 mM or higher approx. 70% inhibition was observed. The activity of acetaldehyde dehydrogenase is very low compared to the activity of NAD(P)H-forming enzymes in the early steps of glucose metabolism (Veiga-da-Cunha *et al.,* 1993). This prevents efficient NAD(P)H disposal during glycolysis, leading to a high intracellular concentration of NAD(P)H. Consequently, glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are inhibited, which results in the accumulation of glucose-6-phosphate and 6-phosphogluconate, respectively (Fig. 1). Nuclear magnetic resonance (NMR) spectroscopic analysis revealed the accumulation of phosphorylated intermediates during glucose-malate co-metabolism (Miranda *et al.,* 1997). The data showed that NADH, which is expected to accumulate during glucose catabolism as a result of inefficient NAD(P)H disposal, causes glucose-induced inhibition of malolactic activity. NADH at a concentration of 25 µM resulted in 50% inhibition of the malolactic enzyme purified from *O. oeni*, whereas NADPH had no inhibitory effect. Although slightly lower than glucose, galactose, trehalose, maltose and mannose inhibited the malolactic activity in whole cells in a manner similar to that observed for glucose.

Ribose did not affect the rate of malolactic activity (Miranda *et al.,* 1997). This observation was explained by the fact that ribose does not undergo oxidative-decarboxylation, since it enters the heterofermentative pathway at the level of xylose-5-phosphate (Fig. 1). Fructose is partially converted to mannitol via mannitol dehydrogenase, thus providing an extra route for the reoxidation

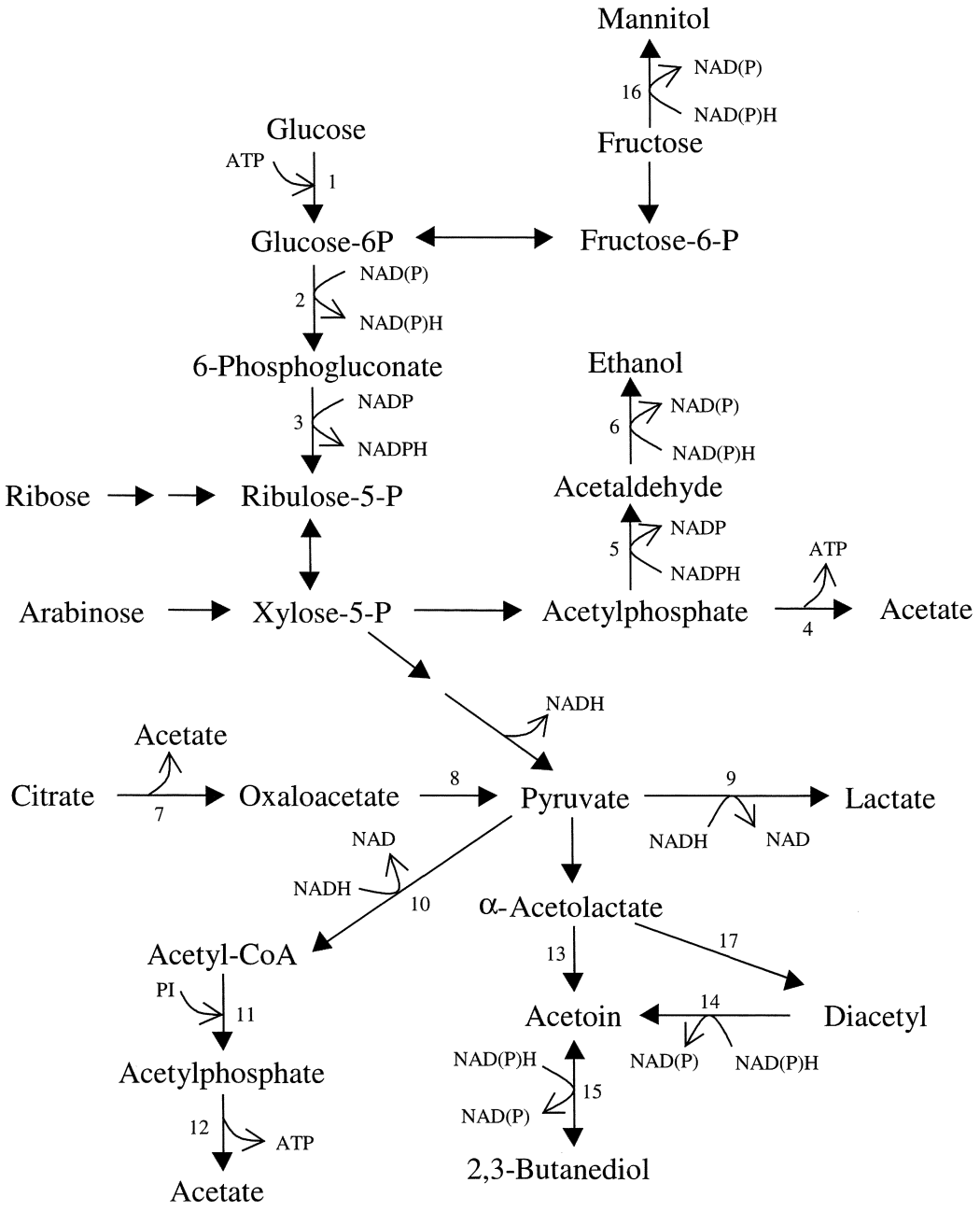

FIGURE 1

Metabolic pathways in *O. oeni*. 1, hexokinase; 2, glucose-6-phosphate dehydrogenase; 3, 6-phosphogluconate dehydrogenase; 4, acetate kinase; 5, acetaldehyde dehydrogenase; 6, alcohol dehydrogenase; 7, citrate lyase; 8, oxaloacetate decarboxylase; 9, lactate dehydrogenase; 10, pyruvate dehydrogenase complex; 11, phosphotransacetylase; 12, acetate kinase; 13, α-acetolactate decarboxylase; 14, diacetyl reductase; 15, acetoin reductase; 16, mannitol dehydrogenase; 17, nonenzymatic decarboxylative oxidation of α-acetolactate.

of NAD(P)H (Salou *et al.*,1994). This provides cells with additional oxidised redox power compared to that obtained from glucose alone, hence the increase in biomass production when both sugars are present (Maicas *et al.*, 1999a). Moreover, the addition of fructose completely relieved glucose-induced inhibition of MLE (Miranda *et al.*, 1997). The same was observed in the presence of citrate (see section on citrate metabolism). The intracellular pool of NAD(P)H decreases during the co-metabolism of citrate and glucose, due to pyruvate being increasingly converted to lactate and 2,3-butanediol, with a concomitant regeneration of $NAD(P)^+$ (Ramos & Santos, 1996).

L-malate

Grape juice contains between 1 and 8 g/L malate (Henick-Kling, 1993). The concentration of malate decreases during grape maturation. In cool viticultural regions final concentrations in grape must are typically 2-5 g/L, while the malate content is much lower in warm climates (typically <2 g/L). LAB metabolise L-malate by one of three different enzymatic pathways, converting

it to L-lactate and CO_2 (Radler, 1986). Some LAB possess an active MLE, which decarboxylates L-malate directly to L-lactate without free intermediates. *L. casei* and *Enterococcus faecalis* possess a malic enzyme that converts L-malate to pyruvate, which is in part reduced to L-lactate, and enables growth on malate as carbon source. A third pathway has been described for *L. fermentum*, where L-malate is reduced by malate dehydrogenase to oxaloacetate, followed by decarboxylation to pyruvate.

Several studies have shown that L-malate stimulates the growth and biomass production of *O. oeni* (Tracey & van Rooyen, 1988; Champagne *et al.,* 1989; Firme *et al.,* 1994). At low pH, L-malate is metabolised at a high rate, whereas carbohydrate metabolism proceeds very slowly. The resulting increase in pH allows an increase in carbohydrate utilisation, which explains malate-induced growth (Miranda *et al.,* 1997). L-malate degradation also stimulates growth in a pH-independent fashion (Pilone and Kunkee, 1976) by generating a PMF that drives ATP synthesis (Cox & Henick-Kling, 1989; 1990).

L-lactate

Lactate (0.1 to 7 g/L in wine) can only be metabolised aerobically by LAB and will result in wine spoilage (Henick-Kling, 1993). L-lacate at 0.5 g/L reduced the growth of *O. oeni* in synthetic medium (pH 3.5) and at 3 g/L growth was completely inhibited (Henick-Kling, 1995). High lactate concentrations in wine may also limit the level of energy obtained from MLF by slowing the export of lactate from the cell.

Citrate

Citrate (0.1 to 0.7 g/L) is a major component in must and wine (Henick-Kling, 1993). During MLF *O. oeni* metabolises citrate (1 to 5 mM) and the residual carbohydrates present after alcoholic fermentation (Ramos & Santos, 1996). *O. oeni* is not able to grow on citrate as sole energy source (Salou *et al.,* 1994; Ramos & Santos, 1996). However, in the presence of glucose, the specific growth rate and biomass production yields of *O. oeni* are enhanced (Salou *et al.,* 1994). Since citrate catabolism is also of importance in the production of flavor compounds, such as diacetyl and acetoin, several studies have dealt with the co-metabolism of citrate and sugars (Salou *et al.,* 1994; Ramos & Santos, 1996; Miranda *et al.,* 1997).

Ramos and Santos (1996) used ^{13}C nuclear magnetic resonance spectroscopy (NMR) to distinguish between end products derived from the metabolism of citrate and glucose. In the presence of glucose, the metabolic flux from pyruvate was mainly directed towards the production of 2,3-butanediol and lactate, whereas acetoin was the main product of citrate metabolism (Fig. 1). The use of additional pathways for re-oxidation of NAP(P)H, in the presence of citrate, allows for the diversion of sugar carbon to reactions in which ATP is synthesised. Not only did the intracellular NAD(P)H/NAD(P)$^+$ ratio decrease during citrate-glucose co-metabolism, but the intracellular concentration of glucose-6-phosphate also decreased (Ramos & Santos, 1996). Moreover, in the presence of citrate the rate of glucose consumption increased. This is due to the relief of inhibition of NAD(P)H on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Veiga-da-Cunha *et al.,* 1993).

Citrate-induced growth enhancement is in part due to the increased ATP yield from glucose during co-metabolism with citrate (Ramos & Santos, 1996). ATP is formed via substrate-level phosphorilation in the reaction catalysed by acetate kinase, with consequent formation of acetate at the expense of ethanol. Although acetate formation via acetate kinase is negligible in the absence of glucose and at low pH (Ramos *et al.,* 1995a), more ATP is derived from citrate metabolism than from glucose metabolism (Ramos & Santos, 1996). Uniport of the monoanionic species of citrate and further metabolism generate a PMF composed of a transmembrane electrical potential and a pH gradient (Ramos *et al.,* 1994). The generated PMF is high enough to drive ATP synthesis. During growth of *O. oeni* on citrate-glucose mixtures, the energy generated by the secondary transport of citrate supplements the energy obtained from glucose by substrate-level phosphorilation, which in turn renders competitiveness to *O. oeni* (Ramos & Santos, 1996). Moreover, the addition of citrate completely relieved glucose-induced inhibition of malate utilisation caused by the inhibition of MLE by NADH (Miranda *et al.,* 1997), which is expected to accumulate during glucose catabolism as a result of inefficient NAD(P)H disposal (see section on carbohydrates). The relief of inhibition of MLF is due to the regeneration of NAD(P)$^+$ in the presence of citrate (Ramos & Santos, 1996).

Production of diacetyl and acetoin by *O. oeni* is stimulated by increased citrate concentrations (Nielsen & Riechelieu, 1999). Diacetyl is considered one of the most important flavours produced during MLF. When present at a concentration above the sensory threshold, which varies from 0.2 mg/L in Chardonnay wine to 2.8 mg/L in Cabernet Sauvignon wine, diacetyl gives the wine an aroma characterised as buttery or nutty (Martineau *et al.,* 1995). An unstable compound, α-acetolactic acid (ALA), is the only source of diacetyl in wine (Hugenholtz, 1993). At high redox potential and O2 concentrations, and at low pH, ALA decarboxylates spontaneously to diacetyl (Richelieu *et al.,* 1997). At low redox potential and O_2 concentration, ALA is converted, either chemically or by bacterial ALA decarboxylase, to acetoin. However, during MLF the degradation of citric acid is delayed compared to the degradation of L-malate (Nielsen & Riechelieu, 1999). This may be due to an inhibitory action of malate on the synthesis of citrate permease, since citric acid transport is inhibited in the presence of malate (Martineau & Henick-Kling, 1995). As a result, the maximum concentration of diacetyl coincides with the exhaustion of L-malate. This is then followed by degradation by *O. oeni* and yeast to acetoin and 2,3-butandiol, which in normal concentrations has no influence on wine aroma (Nielsen & Riechelieu, 1999). If the buttery note from diacetyl is overpowering after depletion of L-malate, it is advisable to delay the addition of sulfite until the diacetyl concentration has been reduced to acceptable levels. SO_2 binds rather strongly with diacetyl and thereby reduces the buttery flavor. In contrast to microbial reduction, this reaction is reversible. It is therefore important to take into consideration that the diacetyl concentration will increase again as the concentration of SO_2 decreases during storage of the wine. The levels of diacetyl and acetoin produced during MLF varies considerably from wine to wine and also during ageing, depending on the level of excess pyruvate, redox potential and the metabolic activity of yeast (Kandler, 1983; Postel & Meier, 1983).

Other organic acids

L-malate and tartaric acid (2 to 10 g/L) are the predominant organic acids in wine. Unlike malate, tartrate can only be

metabolised aerobically by LAB, which means wine would have to be exposed to air (Henick-Kling, 1993).

Succinate (0.2 to 2 g/L) is produced by yeast during alcoholic fermentation and is not metabolised by LAB (Henick-Kling, 1993), while acetic acid (0.05 to 0.2 g/L in dry wine) is produced during bacterial growth due to sugar and citric acid metabolism (Krieger *et al.,* 1992). Low concentrations of gluconate (0.05 to 1.1 g/L) and pyruvate (0.03 to 0.3 g/L) are present in wines. These acids may be metabolised by LAB under winemaking conditions via the hexose monophosphate (HMP) pathway to lactate, acetate and CO_2. Since pyruvate binds SO_2, removal through growth of LAB may decrease the need to add SO_2 for protection against oxidation and microbial spoilage.

Other acids, such as fumarate and sorbate, are only present in significant amounts if added after alcoholic fermentation to prevent growth of LAB (Henick-Kling, 1993). Fumarate is bactericidal against LAB at concentrations between 0.4 to 1.5 g/L, and the effect is synergistic with decreasing pH (Cofran & Meyer, 1970; Pilone *et al.*, 1977). Bacteria may overcome inhibition by converting fumarate to malate through a reaction catalysed by fumarase. Sorbate is effective against yeast in wine at concentrations ranging from 150 to 250 mg/L and may be added to wines in the USA at concentrations up to 300 mg/L (Splittstoesser & Stoyla, 1989). *O. oeni* metabolises sorbate to a geranium off-odour (Splittstoesser & Stoyla, 1989).

Apart from the antimicrobial action of organic acids, the pH of the wine is lowered. Although organic acids have no known effect on specific malolactic activity, malate degradation is the highest at low pH (Henick-Kling, 1993).

Fatty acids

Tween 80 (polyoxyethylene-sorbitan-mono-oleate) is often included in synthetic culture media for LAB, since it enhances bacterial growth (Johnsson *et al.,* 1995) and may improve the production of antimicrobial peptides (Nel *et al.,* 2002). According to Lonvaud-Funel and Desens (1990), cells of *O. oeni* grown in the presence of Tween 80 incorporate oleic acid (C18:1Δ9) into their cell membranes and form the methylated derivate, dihydrosterculic acid (C19:0cy9). Cells grown without Tween 80 lack both these acids, but contain higher levels of the cyclic lactobacillic acid (C19:0cy11). Cyclopropane acids originate from a methylation of the corresponding octadecenoic acids, explaining the inability of *O. oeni* to synthesise oleic acid.

Strains of *O. oeni* differ in their ability to assimilate oleic acid from a culture medium (Bastianini *et al.,* 2000; Guerrini *et al.,* 2002). Strains possessing higher percentages of oleic acid and dihydrosterculic acid revealed higher cell viability and conducted complete MLF after inoculation into wine without oleic acid (Guerrini *et al.,* 2002). In wines supplemented with Tween 80, oleic acid acted as a survival factor for strains with low capacity to assimilate oleic acid and acted as a growth factor for strains with high assimilative capacity. Survival factors are unable to affect total growth, but maintain viability of resting cells and their metabolic activities. Growth factors increase biomass without affecting population viability during the decline phase.

Since MLF depends on the ability of the malolactic starter culture to maintain high cell viability in wine, the presence of oleic acid is recommended. The success of MLF is influenced by the ability of the strain to assimilate oleic acid. If a wine lacks oleic acid, which could be due to must clarification practices, the success of MLF, unless inoculated at very high cell densities, will depend on the level of C18:1Δ9 + C19:0cyΔ9 acids present in the strain.

Antagonism between yeast and LAB during alcoholic fermentation may be, at least in part, explained by the production of medium-chain fatty acids (C_6 to C_{12}), derived from yeast metabolism (Alexandre *et al.*, 2004; Edwards *et al.,* 1990). Decanoic (0.6 to 14 mg/L) and dodecanoic acids are the most common fatty acids in wine (Lafon-Lafourcade *et al.,* 1984). Decanoic acid up to 12.5 mg/L and dodecanoic acid up to 2.5 mg/L act as growth factors and stimulate malolactic activity in the presence of 4% (v/v) ethanol (Capucho & San Romão, 1994). At higher concentrations these acids exerted an inhibitory effect and the toxicity increased when the pH of the media decreased from 6 to 3, indicating that the undissociated molecule is the toxic form. This form is highly soluble in membrane phospholipids and enters the cell by passive diffusion. A fraction of these fatty acids may be incorporated into the plasma membrane and modify its composition and permeability. An increase in L-malate degradation at low concentrations of fatty acids may be due to an increase in passive transport of L-malate into the cell as a result of increased membrane permeability. In the presence of decanoic acid (20 mg/L) and dodecanoic acid (5 mg/L) the ATPase activity of *O. oeni* was reduced by approx. 5% and 42%, respectively (Carreté *et al.,* 2002). Longer chain fatty acids are more toxic due to their higher liposolubility (Sá-Coreia, 1986). The toxicity of decanoic acid increased significantly in the presence of ethanol (Carreté *et al.,* 2002). Although ATPase activity was only slightly inhibited by 12% (v/v) ethanol, it was reduced to approx. 65% in the presence of decanoic acid. The synergistic inhibition by ethanol and fatty acids has also been shown in yeast (Sá-Coreia, 1986).

The growth of certain LAB in wine could be encouraged by the presence of fungal polysaccharides produced by *Botrytis cinerea.* These polysaccharides could act by protecting LAB against the inhibitory action of some fatty acids (Henick-Kling, 1993).

Amino acids

The efficiency of MLF is influenced by the nutrient composition of the wine and free amino acids appear to be of great significance. However, only a few studies have focused on the amino acid requirements of *O. oeni* and their effect on malolactic conversion (Garvie 1967; Tracey & Britz 1989b; Fourcassie *et al.,* 1992). Fourcassie *et al.* (1992) demonstrated the absolute requirement for four amino acids (arginine, glutamic acid, tryptophan and isoleucine), while six others (valine, methionine, cysteine, leucine, aspartic acid and histidine) are required for optimum growth of *O. oeni.*

Vasserot *et al.* (2001) studied the effect of high concentrations of the non-essential amino acid, L-aspartic acid, on the growth of *O. oeni* and MLF. Bacterial growth in a medium without L-aspartic acid was reduced by 30 to 50%, depending on the strain of *O. oeni* studied (Fourcassie *et al.,* 1992; Vasserot *et al.,* 2001). The favourable effect of L-aspartate on bacterial growth may be due to the ability of *O. oeni* to metabolise it to the essential amino acid L-isoleucine (Saguir and Manca De Nadra, 1995). On the other hand, high concentrations of L-aspartate almost completely inhibited bacterial growth and reduced D-glucose fermentation and L-

malic consumption (Vasserot *et al.,* 2001). L-aspartate interacted with the essential amino acid L-glutamic acid and, as a result, L-glutamic acid transport is competitively inhibited. Such antagonistic interactions between amino acids could explain some of the difficulties experienced with the induction of MLF in wine.

O. oeni grows poorly under aerobic conditions with glucose as the only carbohydrate (Maicas *et al.,* 2002). When cysteine is added, glucose consumption in aerobic conditions reaches rates similar to those found in anaerobic conditions. Cysteine acts as an electron acceptor, scavenging oxygen, and suppresses inactivation of the ethanol-forming pathway enzymes by molecular oxygen, allowing the regeneration of NAD(P)H (see section on oxygen and carbon dioxide).

Arginine, being one of the most important amino acids in grape must and wine, represents a potential source of energy and increases the viability of *O. oeni* (Tonon & Lonvaud-Funel, 2000). In wine, heterofermentative LAB may degrade arginine during MLF via the arginine deiminase (ADI) pathway, leading to the formation of ammonia, ornithine, citrulline, ATP and CO_2 (Liu *et al.,* 1996). Arginine degradation by LAB has several enological implications. The production of ammonia increases pH and, therefore, increases the risk of growth of spoilage microorganisms (Mira de Orduña *et al.,* 2001). Formation of ATP may give arginine-positive LAB, including spoilage LAB, an ecological advantage. Two major precursors for the formation of carcinogenic ethyl carbamate (EC) in wine are citrulline (Liu *et al.,* 1994) and urea (Kodama *et al.,* 1994). Ethyl carbamate is formed from a non-enzymatic and spontaneous reaction between alcohol and excreted citrulline. The reaction is favoured upon wine storage in warm cellars. Urea, however, is formed by yeast arginase. Since alcoholic fermentation by yeast is traditionally conducted before MLF, control of EC formation has been focused on the reduction of arginine levels in must and wine and the selection of low-urea-producing yeast or yeast that reutilise most of the produced urea (Mira de Orduña *et al.,* 2001).

Although most arginine is degraded by yeast during alcoholic fermentation, some wines have arginine levels as high as 2 to 5 g/L after alcoholic fermentation (Lehtonen, 1996). Oenococci were able to degrade arginine at pH 3.9 and partially at pH 3.6, but not at pH 3.3 (Mira de Orduña *et al.,* 2001). Lactobacilli degraded arginine at all pH values tested, excreting considerable amounts of citrulline. In addition to higher minimum pH requirements, arginine degradation by oenococci was delayed in comparison to L-malate degradation. In practice, this would allow the winemaker to avoid arginine degradation by carefully monitoring L-malate degradation and removing cells or inhibiting cell activity after L-malate depletion. Pure cultures of *O. oeni* and non-arginine degrading strains should be used to induce MLF.

Many LAB strains in wine are able to decarboxylate amino acids, producing high concentrations of biogenic amines (Lonvaud-Funel, 2001). This reaction favours growth and survival in acidic media, since it results in an increase in pH. If biogenic amine-producing strains are present, the winemaker is encouraged to inoculate with selected malolactic starter cultures to replace the indigenous microflora.

Oxygen and carbon dioxide

LAB have a fermentative metabolism and do not usually grow well under absolutely aerobic conditions. However, some strains of *Leuconostoc* yielded higher biomass production when cultured aerobically, due to the presence of inducible NAD(P)H oxidases. These enzymes enable the cells to gain an ATP molecule from the transformation of acetyl phosphate to acetate (Lucey and Condon, 1986; Plihon *et al.,* 1995; Sakamoto & Komagata, 1996). Other LAB, such as *L. plantarum* and *Lactococcus lactis*, do not benefit from O2, but they are not inhibited by its presence (Cogan *et al.,* 1989; Murphy & Condon, 1984).

Growth of *O. oeni* is stimulated under strict anaerobic conditions (Henick-Kling, 1993). Cells did not grow under aerated conditions with glucose as the only carbohydrate (Maicas *et al.,* 2002). Oxygen inactivates the enzymes of the ethanol-forming pathway, acetaldehyde dehydrogenase and alcohol dehydrogenase (Fig. 1), thus stopping the reoxidation of cofactors produced in the first steps of heterolactic sugar catabolism. Moreover, *O. oeni* lacks significant NAD(P)H-oxidase activities under aerobic conditions. These results suggest that the regeneration of cofactors is the limiting factor for aerobic metabolism of glucose.

The addition of fructose or pyruvate, which act as external electron acceptors, stimulated the growth of *O. oeni* slightly (Gottschalk, 1986; Krieger *et al.*, 1992). Fructose was converted to mannitol, oxidising two molecules of NAD(P)H, and pyruvate was transformed to lactate, enabling the regeneration of NAD^+. In the presence of cysteine, the metabolism of glucose under aerobic conditions reached similar rates to those under anaerobic conditions (Kandler, 1983). Cysteine suppressed the oxygen-induced inactivation of the ethanol-forming pathway enzymes (Kandler, 1983). Improved growth in the presence of added substrates that act as electron acceptors is important if high biomass levels are needed, as in the preparation of commercial starters for MLF.

Acetaldehyde

Acetaldehyde is one of the most important sensory carbonyl compounds formed during vinification, constituting more than 90% of the total aldehyde content in wine, and originates mainly from yeast metabolism (Liu & Pilone, 2000). Variable levels of acetaldehyde have been described, ranging from 4 to 212 mg/L in red wine and 11 to 493 mg/L in white wine, with average values of about 30 mg/L and 80 mg/L for red and white wine, respectively. Acetaldehyde is highly volatile and has a sensory threshold value of 100 to 125 mg/L in wine. At low levels, acetaldehyde gives a pleasant fruity aroma, but results in an undesirable aroma described as green, grassy, or apple-like when present in excess (Zoecklein *et al.,* 1995). The aroma can be masked by the addition of SO_2. Binding of SO_2 to acetaldehyde reduces its effectiveness as an antimicrobial compound and its antioxidative effect. The interaction of acetaldehyde with phenolics improves red wine color by forming stable polymeric pigments resistant to SO_2 bleaching, but it may also induce phenolic haze and eventual deposition of condensed pigments (Liu & Pilone, 2000).

The impact of free acetaldehyde on wine LAB such as *O. oeni* has not been defined. Since acetaldehyde (<100 mg/L) stimulates the growth of heterofermentative dairy LAB (e.g. *Leuc. mesenteroides*), it has been suggested that acetaldehyde acts as an electron receptor during heterofermentation with the formation of additional energy (Liu & Pilone, 2000). However, high levels (>100 mg/L) inhibit the growth of LAB.

The inhibitory effect of acetaldehyde-bound SO_2 on LAB

growth has been well-documented (Fornachon, 1963; Hood, 1983). Nielsen & Riechelieu (1999) measured a decrease in the concentration of acetaldehyde in Chardonnay wine from 17 mg/L before MLF to 1.5 mg/L after MLF. Subsequently, it was shown that oenococci and lactobacilli are able to convert free and SO_2-bound acetaldehyde to mainly ethanol and acetate (Osborne *et al.,* 2000). Free SO_2 released from the degradation of SO_2-bound acetaldehyde by SO_2-sensitive strains of *O. oeni* may cause inhibition, resulting in stuck or sluggish MLF. By using efficient acetaldehyde-degrading strains to conduct MLF, the addition of SO_2 to reduce acetaldehyde aroma can be minimised.

Phenolic compounds

Red wines contain large quantities of phenolic compounds, such as carboxylic acids (240 to 500 mg/L); anthocyanins (40 to 470 mg/L); flavonols (65 to 240 mg/L), e.g. quercetin (1 to 30 mg/L); and flavan-3-ols (25 to 560 mg/L), e.g. catechin (15 to 390 mg/L) (De Beer *et al.,* 2002). Carboxylic/phenolic acids belong to the non-flavanoid group of phenolics in wine and are derivatives of benzoic and cinnamic acids. The most common carboxylic acids are gallic (3,4,5-trihydroxy-benzoic acid), caffeic (3,4-dihydroxy-cinnamic acid), ferulic (3-methoxy-4-hydroxy-cinnamic acid) and r-coumaric acid (4-hydroxy-cinnamic acid). In red cultivars of *Vitis vinifera* grapes, anthocyanins occur only as monoglucosides. Flavonols are reduced products of anthocyanins. Flavan-3-ols differ from other flavanoids, in that they do not generally occur as glycosides. Phenolic compounds may influence growth and metabolism of bacteria and the rate of MLF. The antimicrobial properties of tannins, polymers of carboxylic acids and flavanoid phenols are well documented (Scalbert, 1991). Some phenolic compounds may be involved in the release of fermentable sugars, or serve as oxygen scavengers and thereby reduce the redox potential of wine.

At high concentrations hydroxycinnamic acids are inhibitory against growth of wine-spoilage LAB (Stead, 1993) and *O. oeni* (Reguant *et al.,* 2000). Since the pKa of these compounds is in the 5 to 7 range, a low pH would produce greater proportions of the undissociated form, which is inhibitory towards growth because of its ability to enter the cell and acidify the cytoplasm. For some *Lactobacillus* spp. a stimulatory effect on growth at low concentrations has been described (Stead, 1993). These species are able to metabolise hydroxycinnamic acids by reduction to ethyl phenols, a non-inhibitory form. *O. oeni* is unable to metabolise hydroxycinnamic acids (Reguant *et al.,* 2000).

Gallic acid (3 OH in *ortho* position) is metabolised by *O. oeni* and stimulates growth (Reguant *et al.*, 2000; Vivas *et al.,* 1997). Vivas *et al.* (1997) not only observed an increase in the rate of MLF in the presence of gallic acid, but also an increase in specific malolactic activity. Two other phenolic acids of the benzoic series, prorocatechuic acid (2 OH in *ortho* position) and vanillic acid (1 OH and 1 OCH_3 in *ortho* position), displayed no effect and a slight inhibiting effect, respectively, on MLF (Vivas *et al.,* 1997).

Anthocyanins are metabolised by *O. oeni*, stimulating both growth and MLF (Vivas *et al.,* 1997). The increase in the rate of MLF is, however, not due to an increase in specific malolactic activity, but rather to an increase in growth rate. The bacteria use the glucose moiety of the anthocyanins as an energy source. Both the flavonoid compounds catechin and quercetin stimulated MLF, although only catechin stimulated the growth of *O. oeni* (Reguant *et al.,* 2000). It remains unclear how phenolic compounds such as quercetin and gallic acid increase the specific activity of the malolactic enzyme.

Pesticides

Chemical treatment against fungi, such as mildew and *Botrytis*, can lead to pesticide residues in the must and wine (Garcia-Cazorla & Xirau-Vayreda 1994). These residues not only affect yeast but also LAB in wine, and delay MLF (Cabras *et al.,* 1994). Vidal *et al.* (2001) examined the inhibitory effect of two commonly used pesticides, copper and dichlofluanid, on several strains of *O. oeni* and on MLF in simulated wine. Sensitivity to these pesticides varied and was enhanced by the presence of ethanol. Inhibition was due to a decrease in cell number and not to a decrease in malolactic activity. Carreté *et al.* (2002) recorded an approx. 25% reduction in F_0F_1 ATPase activity of *O. oeni* in the presence of 20 mg/L copper.

Pre-culture conditions

Most LAB grown in rich and synthetic media do not survive in wine without a preculturing or a reactivation process. A limiting medium with composition close to that of wine is recommended (Nault *et al.,* 1995). The rate of MLF in wine is directly linked to cell density and to the specific malolactic activity of the cell, with malolactic activity at its highest during the early stages of growth (Krieger *et al.,* 1992). However, survival of a culture of *O. oeni,* and consequently malolactic activity following inoculation into wine, was the highest when the pre-culture was harvested 18-24 hrs after it entered stationary phase. Establishing an arbitrary duration of the reactivation process is not that simple and following the growth phase of bacteria under conditions in a winery is not always possible. A more practical approach to determine the best moment for starter collection would be to follow L-malate degradation. If this is the method of choice, inoculation into wine should only commence after all the L-malate of the medium is degraded (Nault *et al.,* 1995). Furthermore, the cell numbers in the pre-culture medium should be between 10^6 and 10^7 cfu/mL after inoculation to ensure that L-malate degradation follows bacterial growth. Higher cell numbers leads to high malate decarboxylation by non-proliferating cells. Survival of *O. oeni* in wine and its ability to perform MLF was also improved by pre-treating the cells at 42°C for 1h (Guzzo *et al.,* 1994). The positive effect of a heat shock may be attributed to the synthesis of stress proteins, which are induced in stationary growth phase (Guzzo *et al.,* 1997). This is in agreement with the observation that stationary phase cells survive better in wine after direct inoculation (Krieger *et al.,* 1992).

Contamination with yeast and other bacteria during reactivation and cultivation of a starter culture is difficult to avoid in a winery. Starter cultures developed for direct inoculation after simple rehydration in water will improve the management of MLF in wine. Freeze-dried cultures of *O. oeni* are commercially available (Henick-Kling, 1995) and modifications of freeze-drying techniques have resulted in improved cell viability (Nielsen *et al.,* 1996).

ALTERNATIVE TECHNOLOGIES FOR PROMOTING MLF

Bioreactors based on high biomass of free cells

High cell numbers of *O. oeni* have long been used to improve MLF (Gao and Fleet, 1994; Maicas *et al.,* 2000). At high cell den-

sities (approx. 10^7 to 10^8 cfu/mL) the inhibition of MLF by low pH is diminished, as bacterial development is not essential to perform MLF. Approaches to increase productivity in high cell density fermentations by using bioreactors have been explored and recently reviewed (Maicas, 2001). Cell-recycle bioreactors use a tangential flow or hollow-fibre filter to separate the cells from the wine. Cells remain in the vessel and reach high cell densities, with the wine being constantly removed to prevent inhibition of cell growth by lactic acid production and low pH. Limitations include stress on cells entering the filtration unit, potential difficulties in up-scaling due to the filtration system, and a drastic decrease in malolactic activity after only a few days. Recently, Maicas *et al.* (1999b) made use of free *O. oeni* cells in a continuous stirred tank reactor to control continuous fermentation. The system was successfully operated for 2 to 3 weeks and MLF was successfully conducted. Contrarily to cell-recycle bioreactors, no NAD^+ depletion and inhibition by lactic acid were recorded.

Bioreactors based on immobilised cells

Several studies demonstrated the possibility of achieving control over MLF by immobilised bacteria (Diviès *et al.,* 1994). Immobilisation may increase productivity due to greater packing density or by providing a more protective environment, and also improves subsequent cell separation. Starter cultures may be reused and the fermentation induced and halted at any moment.

Immobilisation techniques applied to induce MLF in wine include entrapment and adsorption/attachment (reviewed by Maicas, 2001). In the case of entrapment, cells are held either within the interstices of porous materials, such as a sponge of fibrous matrix, or by the physical restraints of membranes or encapsulating gel matrices. Entrapment of *O. oeni* for wine deacidification has been studied using alginates, polyacrylamide and κ-carrageenan. Immobilisation via adsorption begins with a sterilised support inoculated with cell suspensions. A biofilm subsequently develops upon exposure to a/the growth medium. Recently, Maicas *et al.* (2001) reported on the adsorption of *O. oeni* on positively charged cellulose sponges.

Although these techniques proved to be successful in decreasing L-malate, most of the materials are rejected by wine producers due to toxicity, pre-fermentation preparation, requirements of additional chemicals, or mechanical instability in the presence of medium components. Other disadvantages include a decrease in cell viability and malolactic activity upon prolonged use, infection by phages, and the risk of modifying the organoleptic properties of wine.

Bioreactors based on enzymes

A cell-free membrane reactor consisting of free *O. oeni* MLE and cofactors was developed by Formisyn *et al.* (1997). Complete and rapid consumption of L-malate was, however, not efficiently achieved. The efficiency of the conversion is furthermore dependent on strict pH regulation, leading to wine dilution.

Malate degradation by recombinant strains of *S. cerevisiae*

The ability of genetically engineered yeast strains to conduct MLF has been studied by various research groups (Denayrolles *et al.,* 1995; Ansanay *et al.* 1996; Bony *et al.,* 1997; Volschenk *et al.,* 1997a,b; Bauer, 2003). Wild-type strains of *S. cerevisiae* metabolise insignificant amounts of malate during alcoholic fermentation due to the absence of an active transport system for malate (Van Vuuren *et al.,* 1995) and the low substrate affinity of its malic enzyme (Fuck *et al.,* 1973). On the other hand, efficient malo-ethanolic fermentation by *Schizosaccharomyces pombe* is accomplished under anaerobic conditions through the constitutive synthesis of malate permease, encoded by the *mae1* gene (Grobler *et al.,* 1995), and the malic enzyme, encoded by the *mae2* gene (Viljoen *et al.,* 1994). Volschenk *et al.* (1997a) constructed a malolactic yeast strain by co-expressing the *mae1* gene and the *Lactococcus lactis* malolactic gene (*mleS*) in *S. cerevisiae.* This recombinant strain showed rapid growth at very low pH, at conditions even the acid tolerant *O. oeni* are unable to survive (Kunkee, 1967). The strain completed MLF within three days in Cabernet Sauvignon and Shiraz grape musts at 20°C (Volschenk *et al.,* 1997a). At 15°C MLF in Chardonnay grape must was completed within seven days. Apart from a more rapid MLF, compared to the bacterial process, the use of malolactic strains of *S. cerevisiae* as starter cultures should prevent stuck or sluggish MLF, the production of biogenic amines and unwanted flavours. However, compared to fermentation by *O. oeni*, such wines would contain high levels of micronutrients, rendering the wine microbiologically unstable. Aromatic compounds derived from bacterial metabolism would also be missing. Replacement of malolactic bacteria with genetically engineered yeast in all cases is thus doubtful.

Bauer (2003) co-expressed the *S. pombe mae1* gene with the malolactic gene of either *P. damnosus* NCFB 1832 (*mleD*), *Lactococcus lactis* (*mleS*) or *O. oeni* (*mleA*) in *S. cerevisiae* and compared the efficiency of malolactic conversion. Rapid conversion of 4.5 g/L of L-malate to L-lactate, reaching l-malate concentrations of below 0.3 g/L within 3 days under fermentative conditions in synthetic grape must media, was achieved with all three malolactic enzymes. However, the strain with the *mleD* gene produced significantly lower levels of L-lacate (LA). After four days 2.8 g/L L-lacate was produced with the recombinant yeast strain harbouring *mleD*, compared to 3.3 g/L produced by the same strain containing *mleS* or *mleA*.

Volschenk *et al.* (2001) investigated an alternative pathway to reduce the levels of L-malate in wines. The malic enzyme of *S. pombe* decarboxylates L-malate to pyruvate and CO_2 intracellularly. Under fermentative conditions, pyruvate is further metabolised to ethanol and CO_2 resulting in the so-called malo-ethanolic fermentation. However, strains of *S. pombe* produce off-flavours. This and the fact that *S. pombe* requires higher growth temperature, renders this yeast unsuitable for vinification. Volschenk *et al.* (2001) constructed a *S. cerevisiae* strain containing the *S. pombe mae1* and *mae2* genes integrated in the genome, degrading 5 g/L of L-malate in synthetic and Chenin Blanc grape must. Recombinant malo-alcoholic strains of *S. cerevisiae,* however, produced, higher levels of ethanol during fermentation.

PREVENTION OF MLF

Although MLF is occasionally difficult to induce, prevention of the development of LAB is likewise difficult. Several methods have been implemented with varying degrees of success. Fumaric acid inhibits malolactic fermentation, but is metabolised by yeast and lactic acid bacteria, rendering it unstable (Ough & Kunkee, 1974). Dimethyldicarbonate (DMDC) is lethal against yeast and bacteria, and can be used to sterilise wine (Terrell *et al.,* 1993). DMDC is hydrolysed to CO_2 and a toxic compound, methanol. A

further concern is that no activity is left to protect the bottled product. The winemaking process relies on the use of SO_2 to inhibit microbial growth. However, its use is strictly regulated due to associated health risks and organoleptic changes. Mounting consumer demands for safe alternatives to chemical preservatives has led researchers to focus on natural antimicrobial compounds from plants, e.g. phenolics; animals, e.g. enzymes such as lysozyme; and microorganisms, e.g. bacteriocins (Abee *et al.,* 1995). Lysozyme and bacteriocins, such as pediocin PD-1 and nisin, are interesting candidates for wine preservation. Lysozyme is bacteriolytic against most gram-positive bacteria (Gould, 1996), but has no effect on yeast (Fugelsang, 1997). The activity of lysozyme is not affected by alcohol and it is active in the pH range of wine (Fugelsang, 1997). The Office International de la Vigne et du Vin (OIV) has recently approved the application of lysozyme in winemaking, but the high cost of using lysozyme is still a limiting factor.

BACTERIOCINS AND THEIR ROLE IN MLF

Bacteriocins are ribosomally synthesised antimicrobial peptides or proteins. LAB isolated from wine are capable of producing bacteriocins and they may be responsible for some of the antagonistic effects observed amongst LAB during vinification (Lonvaud-Funel & Joyeux, 1993; Strasser de Saad & Manca de Nadra 1993). Bacteriocin production in grape must or wine may have a significant impact on the completion of MLF. Bauer *et al.* (2003), however, have shown that grape must does not contain the required growth factors needed for production of pediocin PD-1, a bacteriocin produced by *P. damnosous* NCFB 1832. Whether this is true for other bacteriocins, has to be assessed on an individual basis. To our knowledge, no papers have been published on the presence of bacteriocins in finished wines.

Bacteriocins are odourless, colourless, and non-toxic (Hansen, 1994). Contrary to lysozyme and antibiotics, bacteriocins are very specific and only affect a small group of microorganisms. Bacteriocins are introduced into foods by either direct addition of the peptide (usually in a purified form), adding of the culture supernatant (a crude extract of the peptide), or by using a bacteriocin-producing starter culture in fermented foods. Although several bacteriocins with novel applications in the food industry have been developed (Ross *et al.,* 1999), nisin is the only purified bacteriocin currently allowed in food (Van Kraaij *et al.,*1999). Nisin is a bacteriocin isolated from *Lactococcus lactis* of non-oenological origin. The effectiveness of nisin (Radler 1990a) and pediocin PD-1 (Bauer *et al.,* 2003) in preventing the growth of LAB in wine has been demonstrated. The peptides have a bactericidal mode of action against a number of LAB, including malolactic strains of *Lactobacillus, Leuconostoc* and *Oenococcus* spp. (Bauer, 2003). Unlike pediocin PD-1, nisin is also inhibitory towards pediococci. These peptides are stable under winemaking conditions and do not affect yeast growth (Radler, 1990b; Bauer *et al.,* 2003). Nel *et al.* (2002) have shown that pediocin PD-1, when compared with nisin and plantaricin 423, is the most effective in removal of an established biofilm of *O. oeni* from stainless steel surfaces in Chardonnay must. Adherence of malolactic bacteria to surfaces may have a pronounced affect on the ability of malolactic bacteria to survive during alcoholic fermentation and conduct spontaneous MLF. On the other hand, biofilms may be the source of bacterial contamination in wine or even lead to the development of bacteriophages which may cause stuck or sluggish MLF. The addition of nisin and pediocin PD-1 into wine is, however, not yet authorised and cost considerations will play a major role in the acceptance of peptide-based wine preservation strategies. An additional threat to the future application of antimicrobial agents lies in the development of resistance, which has already been reported for nisin in a variety of Gram-positive bacteria (Verheul *et al.,* 1997).

Since bacteriocins are encoded by genes, a variety of structural analogs of the natural peptide may be constructed through genetic engineering. This opens new possibilities to engineer *S. cerevisiae* wine yeast strains to produce these peptides and to control bacterial populations in wine. The feasibility of this concept has already been demonstrated (Schoeman *et al.,* 1999; Van Reenen *et al.,* 2002), but production efficiency will have to be optimised. Schoeman *et al.* (1999) have cloned pediocin PA-1, a bacteriocin produced by *Pediociccus acidilactici* into *S. cerevisiae*. The bacteriocin is active against most wine spoilage LAB, with the exception of *O. oeni*, and would therefore be ideal as a preservative in wine where MLF, conducted by *O. oeni*, is wanted. Van Reenen *et al.* (2002) cloned a homologous bacteriocin, plantaricin 423, produced by *L. plantarum* into *S. cerevisiae*. Although pediocin PA-1 and plantaricin 423 belong to the same subclass of bacteriocins, the pediocin-like peptides, and the mature peptides are approx. 40% identical, the spectrum of inhibition differs. Plantaricin 423, contrarily to pediocin AcH, is very active against *O. oeni* (Nel *et al.,* 2002). Pediocin PD-1 and nisin, on the other hand, belong to the lantibiotic family of bacteriocins (Bauer, 2003). Unlike pediocin-like peptides and other Class II bacteriocins, lantibiotics are post-translationally modified. The engineering of lantibiotics is less straightforward than that of unmodified proteins, since expression systems have to be developed not only for the structural genes, but also for genes encoding the biosynthetic enzymes and regulatory proteins. The cloning of lantibiotic genes in *S. cerevisiae* and its expression is an exciting challenge. Yeast strains expressing pediocin PD-1, nisin or plantaricin 423, would be applicable in wines where MLF is unwanted. The construction of recombinant wine yeast strains expressing bacteriocin genes together with a malolactic gene would be useful in wines where concurrent alcoholic and malolactic fermentation is required, without the effects associated with bacterial metabolism.

CONCLUSIONS

Sensory and chemical analysis have shown that LAB influence wine quality not only through MLF, but also through other metabolic pathways. Bacterial growth, survival and metabolism in wine depends on a multitude of wine components, environmental conditions, strain-specific enzymatic activities and the availability of fermentable substrate. Although our knowledge has increased considerably over the past 10 years, many questions remain unanswered, of which the most evident concerns the natural adaptation of wine LAB to such a harsh medium. Three cellular mechanisms play a key role in survival of *O. oeni* in wine: MLF, the plasma membrane-bound ATPase systems, and synthesis of specific stress proteins.

The control of MLF may be governed in several ways. It can be promoted through (a) strain selection; (b) starter culture development and improved reactivation; (c) development of malolactic

reactors with free or immobilised bacteria, or enzymes; or (d) the construction of recombinant wine yeast strains conducting concurrent alcoholic fermentation and MLF. MLF can be prevented by (a) employing antimicrobial compounds as wine preservatives and through (b) genetic modification of yeast strains to produce antimicrobial agents such as bacteriocins. Considering the advantages of biological systems over classical chemical control methods, there is little doubt that once such systems have been developed, they will offer a healthier and more ecologically friendly alternative. Systematic studies on natural antimicrobials, such as lysozyme and bacteriocin, in synergistic combination with classical preservation agents will also have an increasing role to play in the future. While "naturalness" alone is not a sufficient objective for these developments, the use of natural inhibitors that will improve preservation strategies, with advantages in product quality and safety, merits further research (Gould *et al.,* 1996).

LITERATURE CITED

Abee, T., Krockel, L. & Hill, C., 1995. Bacteriocins: modes of action and potentials in food preservation and control of food poisoning. Int. J. Food Microbiol. 28, 169-185.

Alexandre, H., Costello, P.J., Remize, F., Guzzo, J. Guilloux-Benatier, M., 2004. *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: current knowledge and perspectives. Int. J. Food Microbiol. 93, 141-154.

Amerine, M.A. & Kunkee, R.E., 1968. Microbiology of wine-making. Ann. Rev. Microbiol. 22, 323-358.

Ansanay, V., Dequin, S., Camarasa, C., Schaeffer, V., Grivet, J. P., Blondin, B., Salmon, J.M. & Barre, P., 1996. Malolactic fermentation by engineered *Saccharomyces cerevisiae* as compared with engineered *Schizosaccharomyces pombe*. Yeast 12, 215-225.

Bastianini, A., Granchi, L., Guerrini, S.L. & Vincenzini, M., 2000. Fatty acid composition of malolactic *Oenococcus oeni* strains exposed to pH and ethanol stress. Ital. J. Food Sci. 12, 333-342.

Bauer, R., 2003. Strategies for the control of malolactic fermentation: Characterization of pediocin PD-1 and the gene for the malic enzyme from Pediococcus damnosus NCFB 1832. Dissertation, Stellenbosch University, Private Bag X1, 7602 Matieland, (Stellenbosch) South Africa.

Bauer, R., Nel, H.A. & Dicks, L.M.T., 2003. Pediocin PD-1 as a method to control growth of *Oenococcus oeni* in wine. Am. J. Enol. Vitic. 54, 86-91.

Beelman, R.B. & Gallander, J.F. 1979. Wine deacidification. Adv. Food Res. 25, 1-53.

Bony, M., Bidart, F., Camarasa, C., Ansanay, V., Dulau, L., Barre, P. & Dequin, S., 1997. Metabolic analysis of *S. cerevisiae* strains engineered for malolactic fermentation. FEBS Lett. 410, 452-456.

Britz, T.J. & Tracey, R.P., 1990. The combination effect of pH, SO_2, ethanol and temperature on the growth of *Leuconostoc oenos*. J. Appl. Bacteriol. 68, 23-31.

Cabras, P., Meloni, M., Melis, M., Farris, G.A., Budroni, M. & Satta, T., 1994. Interactions between lactic acid bacteria and fungicides during lactic fermentation. J. Wine Res. 5, 53-59.

Capucho, I. & San Romão, M.V., 1994. Effect of ethanol and fatty acids on malolactic activity of *Leuconostoc oenos*. Appl. Microbiol. Biotechnol. 42, 391-395.

Carreté, R., Vidal, M.T., Bordons, A. & Constantí, M., 2002. Inhibitory effect of sulfur dioxide and other stress compounds in wine on the ATPase activity of *Oenococcus oeni*. FEMS Microbiol. Lett. 211, 155-159.

Caspritz, G. & Radler, F., 1983. Malolactic enzyme of *Lactobacillus plantarum*: purification, properties and distribution among bacteria. J. Biol. Chem. 258, 4907-4910.

Champagne, C.P., Gardner, N. & Doyon, G., 1989. Production of *Leuconostoc oenos* biomass under pH control. Appl. Environ. Microbiol. 55, 2488-2492.

Cofran, D.R. & Meyer, J. 1970. The effect of fumaric acid on malo-lactic fermentation. Am. J. Enol. Vitic. 21, 189-192.

Cogan, J.F., Walsh, D. & Condon, S., 1989. Impact of aeration on the metabolic end-products formed from glucose and galactose by *Streptococcus lactis*. J. Appl. Bacteriol. 66, 77-84.

Constantí, M., Reguant, C., Poblet, M., Zamora, F., Mas, A.. & Guillamon, J.M., 1998. Molecular analysis of yeast population dynamics: effect of sulfur dioxide and inoculum on must fermentation. Int. J. Food. Microbiol. 41, 169-175.

Couto, J.A., Rozès, N. & Hogg, T., 1996. Ethanol induced changes in the fatty acid composition of *Lactobacillus hilgardii*, its effects on plasma membrane fluidity and relationships with ethanol tolerance. J. Appl. Bacteriol. 81, 126-132.

Cox, D.J. & Henick-Kling, T., 1989. Chemiosmotic energy from malolactic fermentation. J. Bacteriol. 171, 5750-5752.

Cox, D.J. & Henick-Kling, T., 1990. A comparison of lactic acid bacteria for energy-yielding (ATP) malolactic enzyme systems. Am. J. Enol. Vitic. 41, 215-218.

Craig, E.A., Gambill, D.B. & Nelson, R.J., 1993. Heat shock proteins: molecular chaperones of protein biogenesis. Microbiol. Rev. 57, 402-414.

Davis, C.R., Wibowo, D., Eschenbruch, R., Lee, T.H. & Fleet, G.H. 1985. Practical implications of malolactic fermentation: A review. Am. J. Enol. Vitic. 36, 290-301.

De Beer, D., Joubert, E., Geldenblom, W.C.A. & Manley, M., 2002. Phenolic compounds: A review of their possible role as *in vivo* antioxidants of wine. S. Afr. J. Enol. Vitic. 23, 48-61.

Denayrolles, M., Aigle, M. & Lonvaud-Funel, A., 1994. Cloning and sequence analysis of the gene encoding *Lactococcus lactis* malolactic enzyme: Relationship with malic enzymes. FEMS Microbiol. Lett. 116, 79-86.

Denayrolles, M., Aigle, M. & Lonvaud-Funel, A., 1995. Functional expression in *Saccharomyces cerevisiae* of the *Lactococcus lactis mleS* gene encoding the malolactic enzyme. FEMS Microbiol. Lett. 125, 37-44.

Dicks, L.M.T., Dellaglio, F., & Collins, M.D., 1995. Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni* (corig.) gen.nov., comb. nov. Int. J. System. Bacteriol. 45, 395-397.

Diviès, C., Cavin, J.F. & Prévost, H., 1994. Immobilized cell technology in wine production. Crit. Rev. Biotechnol.14, 135-154.

Dombeck, K.M. & Ingram, L.O., 1984. Effects of ethanol in the *Escherichia coli* plasma membrane. J. Bacteriol. 157, 233-239.

Drici-Cachon, Z., Guzzo, J., Cavin, J-F. & Diviès, C., 1996. Acid tolerance in *Leuconostoc oenos*. Isolation and characterization of an acid-resistant mutant. Appl. Microbiol. Biotechnol. 44, 785-789.

Edwards, C.G., Beelman, R.B., Bartey, C.E. & McConnell, A., 1990. Production of decanoic acid and other volatile compounds and the growth of yeast and malolactic bacteria during vinification. Am. J. Enol. Vitic. 43, 233-238.

Firme, P., Leitão, M.C. & San Romão, M.V., 1994. The metabolism of sugar and malic acid by *Leuconostoc oenos*: effect of malic acid, pH and aeration conditions. J. Appl. Bacteriol. 76, 173-181.

Fleet, G.H. & Heard, G.M., 1993. Yeast. Growth during fermentation. In: Fleet, G. (ed.). Wine Microbiology and Biotechnology, Harwood Academic Publishers, Chur, Switzerland. pp. 27-54.

Formisyn, P., Vaillant, H., Lantreibecq, F. & Bourgois, J., 1997. Development of an enzymatic reactor for initiating malolactic fermentation in wine. Am. J. Enol. Vitic. 48, 345-349.

Fornachon, J.C.M., 1963. Inhibition of certain lactic acid bacteria by free and bound sulphur dioxide. J. Sci. Food Agric. 14, 857-862.

Fornachon, J.C.M. 1968. Influence of different yeasts on growth of lactic acid bacteria in wine. J. Sci. Food Agric. 19, 374-378.

Fourcassie, P., Makaga-Kabinda-Massard, E., Belarbi, A. & Maujean, A., 1992. Growth, D-glucose utilization and malolactic fermentation by *Leucocnostoc oenos* strains in 18 media deficient in one amino acid. J. Appl. Bacteriol. 73, 489-496.

Fuck, E., Stärk, G. & Radler, F., 1973. Äpfelsäureerstoffwechsel bei *Saccharomyces*. II. Anreicherung und Eigenschaften eines Malatenzymes. Arch. Mikrobiol. 89, 223-231.

Fugelsang, K.C., 1997. Wine Microbiology. Chapman and Hall, New York.

Gallander, J. F., 1979. Effect of time of bacterial inoculation on the stimulation of malo-lactic fermentation. Am. J. Enol. Vitic. 30, 157-159.

Gao, C. & Fleet, G.H., 1994. The degradation of malic acid by high density cell suspensions of *Leuconostoc oenos*. J. Appl. Bacteriol. 76, 632-637.

Garbay, S., Rozès, N. & Lonvaud-Funel, A., 1995. Fatty acid composition of *Leucocnostoc oenos*, incidence of growth conditions and relationship with malolactic efficiency. Food. Microbiol. 12, 387-395.

Garcia-Cazorla, J. & Xirau-Vayreda, M., 1994. Persistance of dicarboximidic fungicide residues in grapes, must and wine. Am. J. Enol. Vitic. 45, 338-340.

Garvie, E., 1967. The growth factor and amino acid requirement of the genus *Leuconostoc,* including *Leuconostoc paramesentero* (sp. nov.) and *Leuconostoc oenos*. J. Gen. Microbiol. 48, 439-447.

Gottschalk, G. 1986. Bacterial metabolism. Heidelberg, Springer Verlag.

Gould, G.W., 1996. Industry perspectives on the use of natural antimicrobials and inhibitors for food applications. J. Food Protection, 82-86.

Grobler, J., Bauer, F., Subden, R.E. & Van Vuuren, H.J.J., 1995. The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C_4 dicarboxylic acids. Yeast 11, 1485-1491.

Groisilliers, A. & Lonvaud-Funel, A., 1999. Comparison of partial malolactic enzyme gene sequences for phylogenetic analysis of some lactic acid bacteria species and relationships with the malic enzyme. Int. J. System. Bacteriol. 49, 1417-1428.

Guerrini, S., Bastianini, A., Granchi, L. & Vincenzini, M., 2002. Effect of oleic acid on *Oenococcus oeni* strains and malolactic fermentation in wine. Current Microbiol. 44, 5-9.

Guzzo, J., Cavin, J-F. & Diviès, C., 1994. Induction of stress proteins in *Leuconostoc oenos* to perform direct inoculation of wine. Biotechnol. Lett. 16, 1189-1194.

Guzzo, J., Delmas, F., Pierre, F., Jobin, M-P., Samyn, B., Van Beeuman, B., Cavin, J-F. & Diviès, C., 1997. A small heat shock protein from *Leucocnostoc oenos* induced by multiple stresses and during stationary growth phase. Lett. Appl. Microbiol. 24, 393-396.

Guzzo, J., Jobin, M.-P. & Diviès, C., 1998. Increase of sulfite tolerance in *Oenococcus oeni* by means of acidic adaptation. FEMS Microbiol. Lett. 160, 43-47.

Guzzo, J., Jobin, M-P., Delmas, F., Fortier, L-C., Garmyn, D., Tourdot-Maréchal, R., Lee, B. & Diviès, C., 2000. Regulation of stress response in *Oenococcus oeni* as a function of environmental changes and growth phase. Int. J. Food. Microbiol. 55, 27-31.

Hansen, J.N., 1994. Nisin as a model food preservative. Crit. Rev. Food Sci. Nutr. 34, 69-93.

Henick-Kling, T., 1988. Yeast and bacterial control in winemaking. In: Linskens, H.F. & Jackson, J.F. (eds). Modern Methods of Plant Analysis, New Series, Vol. 6. Springer-Verlag. pp. 276-316.

Henick-Kling, T., 1990. pH and regulation of malolactic activity in *Leuconostoc oenos*. In: Actualités Oenologiques 89. Comptes rendus du 4^e Symposium International d'Oenologie (Bordeaux, 1989). pp. 320-325. Institut d'Oenologie Université de Bordeaux II. Paris: Dunod.

Henick-Kling, T., 1993. Malolactic fermentation. In: Fleet, G.H. (ed). Wine Microbiology and Biotechnology. Taylor & Francis Inc, New York. pp. 289-326.

Henick-Kling, T., 1995. Control of malolactic fermentations in wine: energetics, flavour modification and methods of starter culture preparation. J. Appl. Bacteriol. 79, 29-37.

Hood, A., 1983. Inhibition of growth of wine lactic-acid bacteria by acetaldehyde-bound sulphur dioxide. Aust. Grapegrow. Winemaker 232, 24-43.

Hugenholtz, J., 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. 12, 165-178.

Johnsson, T., Nikkila, P., Toivonen, L., Rosenqvist, H. & Laakso, S., 1995. Cellular fatty acid profiles of *Lactobacillus* and *Lactococcus* strains in relation to the oleic acid content of the cultivation medium. Appl. Environ. Microbiol. 61, 4497-4499.

Jones, R.P., 1989. Biological principles for the effects of ethanol. Enzyme Microb. Technol. 11, 130-153.

Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria. Antonie Leeuwenhoek J. Microbiol. 49, 209-24.

King, S.W. & Beelman, R.B., 1986. Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. Am. J. Enol. Vitic. 37, 53-60.

Kodama, S., Suzuki, T., Fujinawa, S., De la Teja, P. & Yotsuzuka, F., 1994. Urea contribution to ethyl carbamate formation in commercial wines during storage. Am. J. Enol. Vitic. 45, 17-24.

Krieger, S.A., Hammes, W.P. & Henick-Kling, T., 1992. Effect of medium composition on growth rate, growth yield, and malolactic activity of *Leuconostoc oenos* LoZH1-t7-1. Food Microbiol. 9, 1-11.

Kunkee, R.E., 1967. Malo-lactic fermentation. Adv. Appl. Microbiol. 9, 235-279.

Kunkee, R.E., 1967. Control of malo-lactic fermentation induced by *Leuconostoc citrovorum.* Am. J. Enol. Vitic. 18, 71-77.

Kunkee, R.E., 1991. Some roles of malic acid in the malolactic fermentation in wine making. FEMS Microbiol. Rev. 88, 55-72.

Kunkee, R.E.,1997. A heady concoction of alcoholic and malolactic fermentations. Nature Biotech. 15, 224-225.

Labarre, C., Guzzo, J., Cavin, J.F. & Davies, C., 1996. Cloning and characterization of the genes encoding the malolactic enzyme and malate permease of *Leuconostoc oenos*. Appl. Environ. Microbiol. 62, 1274-1282.

Lafon-Lafourcade, S., 1970. Etude de la degradation de l'acide L-malique par les bactéries lactiques non proliférantes des vins. Ann. Technol. Agric. 19, 141-154.

Lafon-Lafourcade, S., 1975. Factors of the malolactic fermentation of wines. In: Carr, J.G., Cutting, C.V. & Whiting, G.C. (eds). Lactic Acid Bacteria in Beverages and Food. Academic Press, New York. pp 43-53.

Lafon-Lafourcade, S., Carre, E. & Ribéreau-Gayon, P., 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. 46, 874-880.

Lafon-Lafourcade, S., Geneix, C. & Ribéreau-Gayon, P., 1984. Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeasts and their elimination from yeast ghosts. Appl. Environ. Microbiol. 47, 1246-1249.

Lehninger, L.A., Nelson, D.L. & Cox, N.M., 1993. Principles of Biochemistry, Worth, New York.

Lehtonen, P., 1996. Determination of amines and amino acids in wine – a review. Am. J. Enol. Vitic. 47, 127-133.

Liu, S.-Q. & Pilone, G.J., 2000. An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. Int. J. Food Sci. Technol. 35, 49-61.

Liu, S.-Q., Pritchard, G.G., Hardman, M.J. & Pilone, G.J., 1994. Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic acid bactria *Leuconoctoc oenos* and *Lactobacillus buchneri*. Am. J. Enol. Vitic. 45, 235-242.

Liu, S.-Q., Pritchard, G.G., Hardman, M.J. & Pilone, G.J., 1996. Arginine catabolism in wine lactic acid bacteria: is it via the arginine deaminase pathway or the arginase-urease pathway? J. Appl. Bacteriol. 81, 486-492.

Lonvaud-Funel, A., 1999. Lactic acid bacteria in the quality improvement and depreciation of wine. Antonie Leeuwenhoek 76, 317-331.

Lonvaud-Funel, A., 2001. Biogenic amines in wines: role of lactic acid bacteria. FEMS Microbiol. Lett. 199, 9-13.

Lonvaud-Funel, A. & Desens, C., 1990. Constitution en acides gras des membranes des bactéries lactiques du vin: incidences des conditions de culture. Sciences des Aliments 10, 817-829.

Lonvaud-Funel, A. & Joyeux, A., 1993. Antagonistim between lactic acid bacteria of wines: inhibition of *Leuconostoc oenos* by *Lactobacillus plantarum* and *Pediococcus pentosaceus*. Food Microbiol. 10, 411-419.

Lonvaud-Funel, A. & Joyeux, A., 1994. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of *Leuconostoc oenos*. J. Appl. Bacteriol. 77, 401-407.

Lonvaud-Funel, A. & Strasser de Saad, A.M., 1982. Purification and properties from a malolactic enzyme from a strain of *Leucocnostoc mesenteriodes* isolated from grapes. Appl. Environ. Microbiol. 43, 357-361.

Lucey, C.A. & Condon, S., 1986. Active role of oxygen and NADH oxidase in growth and energy metabolism of *Leuconostoc*. J. Gen. Microbiol. 132, 1789-1796.

Maicas, S., 2001. The use of alternative technologies to develop malolactic fermentation in wine. Appl. Microbiol. Biotechnol. 56, 35-39.

Maicas, S., Ferrer, S. & Pardo, I., 2002. NAD(P) regeneration is the key for heterolactic fermentation of hexoses in *Oenococcus oeni*. Microbiology 148, 325-332.

Maicas, S., González-Cabo, P., Ferrer, S. & Pardo, I., 1999a. Production of *Oenococcus oeni* biomass to induce malolactic fermentation in wine by control of pH and substrate addition. Biotechnol. Lett. 21, 349-353.

Maicas, S., Natividad, A., Ferrer, S. & Pardo, I., 2000. Malolactic fermentation in wine with high densities of non-proliferating *Oenococcus oeni*. World J. Microbiol. Biotechnol. 16, 805-810.

Maicas, S., Pardo, I. & Ferrer, S., 1999. Continuous malolactic fermentation in red wine using free *Oenococcus oeni*. World J. Microbiol. Biotechnol. 15, 737-739.

Maicas, S., Pardo, I. & Ferrer, S., 2001. The potential of positively charged cellulose sponge for malolactic fermentation of wine, using *Oenococcus oeni.* Enzyme Microb. Technol. 28, 415-419.

Martineau, B., Acree, T.E. & Henick-Kling, T., 1995. Effect of wine type on threshold for diacetyl. Food Res. 28, 139-143.

Martineau, B. & Henick-Kling, T., 1995. Formation and degradation of diacetyl in wine during alcoholic fermentation with *Saccharomyces cerevisiae* strain EC1118 and malolactic fermentation with *Leuconostoc oenos* strain MCW. Am. J. Enol. Vitic. 46, 442-448.

Martineau, B. & Henick-Kling, T., 1996. Effect of malic acid on citric acid metabolism in *Leuconostoc oenos*. Abstr. Am. J. Enol. Vitic. 47, 229.

Mascarenhas, M.A., 1984. The occurrence of malolactic fermentation and diacetyl content of dry table wines from north-eastern Portugal. Am. J. Enol. Vitic. 35, 49-51.

Mira de Orduña, R., Patchett, M-L., Liu, S-Q. & Pilone, G.J., 2001. Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. Appl. Environ. Microbiol. 67, 1657-1662.

Miranda, M., Ramos, A., Veiga-da-Cunha, M., Loureiro-Dias, M.C. & Santos, H., 1997. Biochemical basis for glucose-induced inhibition of malolactic fermentation in *Oenococcus oenos*. J. Bacteriol. 179, 5347-5354.

Murphy, M.G. & Condon, S., 1984. Correlation of oxygen utilization and hydrogen peroxide accumulation with oxygen induced enzymes in *Lactobacillus plantarum* cultures. Arch. Microbiol. 138, 44-48.

Naouri, P., Chagnaud, P., Arnaud, A. & Galzy, P., 1990. Purificatiion and properties of a malolactic enzyme from *Leuconostoc oenos* ATCC 23278. J. Basic Microbiol. 30, 577-585.

Nault, I., Gerbaux, V., Larpent, J.P. & Vayssier, Y., 1995. Influence of pre-culture conditions on the ability of *Leuconostoc oenos* to conduct malolactic fermentation in wine. Am. J. Enol. Vitic. 46, 357-362.

Nel, H.A., Bauer, R., Wolfaardt, G.M. & Dicks, L.M.T., 2002. The effect of bacteriocins pediocin PD-1, plantaricin 423 and nisin on biofilms of *Oenococcus oeni* on a stainless steel surface. Am. J. Enol. Vitic. 53, 191-196.

Nielsen, J.C., Prahl, C. & Lonvaud-Funel, A., 1996. Malolactic fermentation in wine by direct inoculation with freeze-dried *Leuconoctoc oenos* cultures. Am. J. Enol. Vitic. 47, 42-47.

Nielsen, J.C. & Riechelieu, M., 1999. Control of flavour development in wine during and after malolactic fermentation by *Oenococcus oeni*. Appl. Environ. Microbiol. 65, 740-745.

Olsen, E.B., Russell, J.B. & Henick-Kling, T., 1991. Electrogenic L-malate transport by *Lactobacillus plantarum*: a basis for energy derivation from malolactic fermentation. J. Bacteriol. 173, 6199-6206.

Osborne, J.P., Mira de Orduña, R., Pilone, G.J. & Liu, S-Q., 2000. Acetaldehyde metabolism by wine lactic acid bacteria. FEMS Microbiol. Lett. 191, 51-55.

Ough, C.S. & Kunkee, R.E., 1974. The effect of fumaric acid on malolactic fermentation in wine from warm areas. Am. J. Enol. Vitic. 25, 188-190.

Pilone, G.J. & Kunkee, R.E., 1976. Stimulatory effect of malolactic fermentation on the growth rate of *Leuconostoc oenos*. Appl. Environ. Microbiol. 32, 405-408.

Pilone, G.J., Rankine, B.C. & Pilone., D.A. 1977. Inhibiting malo-lactic fermentation in Australian dry red wines by adding fumaric acid. Am. J. Enol. Vitic. 25, 99-107.

Plihon, F., Taillandier, P. & Strahaiano, P., 1995. Oxygen effect on batch cultures of *Leuconoctoc mesenteroides*: relationship between oxygen uptake, growth and end-products. Appl. Microbiol. Biotechnol. 43, 117-122.

Poolman, B., 1990. Precursor/product antiport in bateria. Mol. Microbiol. 4, 1629-1636.

Poolman, B., Molenaar, D., Smid, E.J., Ubbink, T., Abee, T., Renault, P. & Konings, W.N., 1991. Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. J. Bacteriol. 173, 6030-6037.

Postel, W. & Meier, B., 1983. Verhalten von 2-Acetolactat, 2-Acetohydroxybutarat, Diacetyl und Acetoin während des bakteriellen Äpfelsäureabbaus in Wein. Zeitschrift für Lebensmittle- Untersuchung und -Forschung 176, 356-359.

Radler, F., 1966. De mikrobiologischen Grundlagen des Säureabbaus im Wein. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 Orig. 120, 237-287.

Radler, F., 1986. Microbial biochemistry. Experientia 42, 884-893.

Radler, F., 1990a. Possible use of nisin in winemaking. II. Experiments to control lactic acid bacteria in the production of wine. Am. J. Enol. Vitic. 41, 7-11.

Radler, F., 1990b. Possible use of nisin in winemaking. I. Action of nisin against lactic acid bacteria and wine yeasts in solid and liquid media. Am. J. Enol. Vitic. 41, 1-6.

Ramos, A. & Santos, H., 1996. Citrate and sugar cofermentation in *Leucocnostoc oenos*, a 13C nuclear magnetic resonance study. Appl. Environ. Microbiol. 62, 2577-2585.

Ramos, A., Lolkema, J.S., Konings, W.N. & Santos, H., 1995a. An enzyme basis for pH regulation of citrate and pyruvate metabolism by *Leuconostoc oenos*. Appl. Environ. Microbiol. 61, 1303-1310.

Ramos, A., Poolman, B., Santos, H., Lolkema, J.S. & Konings, W.N., 1995b. Uniport of anionic citrate and proton consumption in citrate metabolism generate a proton motive force in *Leuconostoc oenos*. J. Bacteriol. 176, 4899-4905.

Reguant, C., Bordons, A., Arola, L. & Rozès, N., 2000. Influence of phenolic compounds on the physiology of *Oenococcus oeni* from wine. J. Appl. Microbiol. 88, 1065-1071.

Richelieu, M., Houlberg, U. & Nielsen, J.C., 1997. Determination of a-acetolactic acid and volatile compounds by headspace gas chromatography. J. Dairy Sci. 80, 1918-1925.

Rigomier, D., Bohin, J.P. & Lubochinsky, B., 1980. Effects of ethanol and methanol on lipid metabolism in *Bacillus subtilis*. J. Gen. Microbiol. 121, 139-149.

Rodriquez, S.B., Amberg, E., Thornton, R.J. & McLellan, M.R., 1990. Malolactic fermentation in Chardonnay: growth and sensory effects of commercial strains in Leuconostoc oenos. J. Appl. Bacteriol. 68, 139-144.

Ross, R.P., Galvin, M., McAuliffe, O., Morgan, S.M., Ryan, M.P., Twomey, D.P., Meany, W.L. & Hill, C., 1999. Developing applications for lactococcal bacteriocins. Antonie van Leeuwenhoek 76, 337-346.

Sá-Coreia, I., 1986. Synergistic effects of ethanol, octanoic and decanoic acids on the kinetics and activation parameters of thermal death in *Saccharomyces bayanus*. Biotechnol. Bioeng. 28, 761-763.

Saguir, F. & Manca De Nadra, M.C., 1995. Effect of L-aspartic acid on the amino acids requirements in *Leuconostoc oenos*. In: 7th International Symposium on Microbial Ecology, Brazil.

Sakamoto, M. & Komagata, K., 1996. Aerobic growth of and activities of NADH oxidase and NADH peroxidase in lactic acid bacteria. J. Ferment. Bioeng. 82, 210-216.

Salema, M., Capucho, I., Poolman, B., San Ramão, M.V. & Loureiro Dias, M.C., 1996a. *In vitro* reassembly of the malolactic fermentation pathway of *Leuconostoc oenos* (*Oenococcus oeni*). J. Bacteriol. 178, 5537-5539.

Salema, M., Lolkema, J.S., San Romão, M.V. & Loureiro Dias, M.C., 1996b. The proton motive force generated in *Leuconostoc oenos* by L-malate fermentation. J. Bacteriol. 178, 3127-3132.

Salema, M., Poolman, B., Lolkema, J.S., Loureiro Dias, M.C. & Konings, W.N., 1994. Uniport of monoanionic L-malate in membrane vesicles from *Leuconostoc oenos*. Eur. J. Biochem. 225, 289-295.

Salou, P., Loubiere, P. & Pareilleux, A., 1994. Growth and energetics of *Leuconostoc oenos* during cometabolism of glucose with citrate or fructose. Appl. Environ. Microbiol. 60, 1459-1466.

Scalbert, A. ,1991. Antimicrobial properties of tannins. Biochem. 30, 3875-3883.

Schoeman, H., Vivier, M.A., Du Toit, M., Dicks, L.M.T. & Pretorius, I.S., 1999. The development of bacterial yeast strains by expressing the *Pediococcus acidilactici* pediocin gene (*ped*A) in *Saccharomyces cerevisiae*. Yeast 15, 647-656.

Shibata, C., Ehara, T., Tomura, K., Igarashi, K. & Kobayashi, H., 1992. Gene structure of *Enterococcus hirae* (*Streptococcus faecalis*) F_1F_0-ATPase, which functions as a regulator of cytoplasmic pH. J. Bacteriol. 174, 6117-6124.

Sinensky, M., 1974. Homeoviscuous adaptation – a homeostatic process that regulates the viscosity of membrane lipids in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 71, 522-525.

Spettoli, P., Nuti, M.P. & Zamora, A., 1984. Properties of malolactic activity purified from *Leucocnostoc oenos* ML34 by affinity chromatography. Appl. Environ. Microbiol. 48, 900-903.

Splittstoesser, D.F. & Stoyla, B.A., 1989. Effect of various inhibitors on the growth of lactic acid bacteria in a model grape juice system. J. Food Protect. 52, 240-243.

Stead, D., 1993. The effect of hydroxycinnamic acids on the growth of wine-spoilage lactic acid bacteria. J. Appl. Bacteriol. 75, 135-141.

Strasser de Saad, A. M. & Manca de Nadra, M.C., 1993. Characterization of a bacteriocin produced by *Pediococcus pentosaceus* from wine. J. Appl. Bacteriol. 74, 406-410.

Teixeira, H., Gonçalves, M.G., Rozès, N., Ramos, A. & San Romão, M.V., 2002. Lactobacillic acid accumulation in the plasma membrane of *Oenococcus oeni:* a response to ethanol stress? Microbiol. Ecol. 43, 146-153.

Ten Brink, B., Otto, R., Hansen, U.P. & Konings, W.N., 1985. Energy recycling by lactatae efflux in growing and nongrowing cells of *Streptococcus cremoris*. J. Bacteriol. 162, 383-390.

Terrell, R.R., Morris, J.R., Johnson, M.G., Gbur, E.E. & Makus, D.J., 1993. Yeast inhibition in grape juice containing sulfur dioxide, sorbic acid and dimethyldicarbonate. J. Food Sci. 58, 1132-1135.

Tonon, T. & Lonvaud-Funel, A., 2000. Metabolism of arginine and its positive effect on growth and revival of *Oenococcus oeni*. J. Appl. Microbiol. 89, 526-531.

Tourdot-Maréchal, R., Fortier, L-C., Guzzo, J., Lee, B. & Diviès, C., 1999. Acid sensitivity of neomycin-resistant mutants of *Oenococcus oeni*: a relationship between reduction of ATPase activity and lack of malolactic activity. FEMS Microbiol. Lett. 178, 319-326.

Tourdot-Maréchal, R., Gaboriau, D., Beney, L. & Diviès, C., 2000. Membrane fluidity of stressed cells of *Oenococcus oeni*. Int. J. Food. Microbiol. 55, 269-273.

Tracey, R.P. & Britz, T.J., 1989a. Cellular fatty acid composition of *Leuconostoc oenos*. J. Appl. Bacteriol. 66, 445-456.

Tracey, R.P. & Britz, T.J., 1989b. The effect of amino acids on malolactic fermentation by *Leuconostoc oenos*. J. Appl. Bacteriol. 67, 589-595.

Tracey, R.P. & Van Rooyen, T.J., 1988. Utilization of glucose, fructose and malic acid by malolactic bacteria: effect of ethanol and formation of mannitol and volatile acids. J. Appl. Bacteriol. 65, 113-118.

Usseglio-Tomasset, L., 1992. Properties and use of sulphur dioxide. Food Additives and Contaminants 9, 399-404.

Van der Westhuizen, L. M., 1980. Isolation, identification and fermentation abilities of malo-lactic bacteria from South African red wines. Thesis, Stellenbosch University, Private Bag X1, 7602 Matieland (Stellenbosch), South Africa.

Van Kraaij C., De Vos, W.M., Siezen, R.J. & Kuipers, O.P., 1999. Lantibiotics: biosynthesis, mode of action and applications. Nat. Prod. Rep. 16, 575-587.

Van Reenen, C.A., Chikindas, M.L., Van Zyl, W.H. & Dicks, L.M.T., 2002. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 81, 29-40.

Van Vuuren, H.J.J. & Dicks, L.M.T., 1993. *Leuconostoc oenos*: A review. Am. J. Enol. Vitic. 44, 99-112

Van Vuuren, H.J.J., Viljoen, M., Grobler M.J., Volschenk, H., Bauer, F. &. Subden, R.E., 1995. Genetic analysis of the *Schizosaccaromyces pombe* malate permease (*mae1*) and malic enzyme (*mae2*) genes and their expression in *Saccharomyces cerevisiae*. In: Gousard P.G.,Archer, E., Saayman, D., Tromp. A & van Wyk, C.J. (eds). Proc. of the first SASEV International Congress, Cape Town, South Africa. pp. 6-8.

Vasserot, Y., Dion, C., Bonnet, E., Maujean, A. & Jeandet, P., 2001. A study into the role of L-aspartic acid on the metabolism of L-malic acid and D-glucose by *Oenococcus oeni*. J. Appl. Microbiol. 90, 380-387.

Veiga-da-Cunha, M., Santos, H. & Van Schaftingen, E., 1993. Pathway and regulation of erythritol formation in *Leuconostoc oenos*. J. Bacteriol. 175, 3941-3948.

Verheul, A., Russell, N.J., Van 'T Hof, R., Rombouts, F.M. & Abee, T., 1997. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol. 63, 3451-3457.

Vidal, M.T., Poblet, M., Constantí, M. & Bordons, A., 2001. Inhibitory effect of copper and dichlofluanid on *Oenococcus oeni* and malolactic fermentation. Am. J. Enol. Vitic. 52, 223-229.

Viljoen, M., Subden, R.E., Krizus, A. & Van Vuuren, H.J.J., 1994. Molecular analysis of the malic enzyme gene (*mae2*) of *Schizosaccharomyces pombe*. Yeast 10, 631-624.

Vivas, N., Lonvaud-Funel, A. & Glories, Y., 1997. Effect of phenolic acids and anthocyanins on growth, viability and malolactic activity of a lactic acid bacterium. Food Microbiol. 14, 291-300.

Volschenk, H., Viljoen, M., Grobler, J., Bauer, F., Lonvaud-Funel, A., Denayrolles, M., Subden, R.E. & Van Vuuren, H.J.J., 1997a. Malolactic fermentation in grape musts by a genetically engineered strain of *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. 48, 193-197.

Volschenk, H., Viljoen, M., Grobler, J., Petzold, B., Bauer, F., Subden, R.E., Young, R.A., Lonvaud, A., Denayrolles, M. & Van Vuuren, H.J.J., 1997b. Engineering pathways for malate degradation in *Saccharomyces cerevisiae*. Nature Biotech. 15, 253-257.

Volschenk, H., Viljoen-Bloom, M., Subden, R.E. & Van Vuuren, H.J.J., 2001. Malo-ethanolic fermentation in grape must by recombinant strains of *Saccharomyces cerevisiae*. Yeast 18, 963-970.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H., 1985. Occurrence and growth of lactic acid bacteria in wine: A review. Am. J. Enol. Vitic. 36, 302-313.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F.S., 1995. Wine analysis and production. Chapman and Hall, New York.