CHARACTERISATION OF WINE MALOLACTIC BACTERIA AND ACETIC ACID FROM FRUCTOSE METABOLISM

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ABSTRACT

Twenty-four strains of wine Lactic Acid Bacteria from the genera *Leuconostoc, Lactobacillus* and *Pediococcus* were characterised with respect to their growth responses to ethanol, temperature, pH, ability to degrade wine organic acids and utilisation of carbon sources. A novel single broth culture (HFA) was developed for the determination of heterofermentation, mannitol formation and ammonia production. Some strains of *Leuconostoc oenos* were found to produce ammonia from arginine. The implications of this are discussed. The production of mannitol from fructose by heterofermentative strains indicated potential acetic acid (volatile acid) spoilage risk for wines.

To investigate this risk, semi-synthetic media were devised to simulate "stuck" yeast alcoholic fermentation and the spoilage potential was evaluated under conditions of pH, substrate availability and ethanol concentration. Acetic acid production was analysed in the media by HPLC and found to occur at high levels from growth in the presence of fructose, but not glucose. The production was not affected by low pH or ethanol concentrations, or their combined effect. This indicated that acetic acid spoilage could occur under wine conditions. Other mechanisms of acetic acid production relative to this experiment are discussed. Erythritol and glycerol were detected in fermentation media but not quantified by HPLC. Their presence supported evidence of the activity of a novel glucose fermentation pathway in *Lc. oenos*.

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1.0 INTRODUCTION

Grapes grown in cool climate regions around the world yield musts with high acid content. Alcoholic fermentations by yeasts such as *Saccharomyces cerevisiae* do little to remove acidity and consequently the wines produced from these musts are tart and unbalanced. Unless this acidity problem is addressed, most consumers will find these wines unpalatable and therefore they would be considered commercially unacceptable.

Certain strains of lactic acid bacteria are capable of degrading one of the predominant acids found in grapes, malic acid. This degradation, commonly known as malolactic fermentation (MLF), involves the biological conversion of L-malic acid to L-lactic acid and carbon dioxide. The formation of the weaker acid, lactic acid, and the release of carbon dioxide result in a more palatable wine of lower acidity and higher pH. The growth of the bacteria also modifies the composition of the wine by producing minor products from metabolism such as diacetyl which are recognised factors in determining flavour complexity.

In the past, winemakers have depended on MLF occurring spontaneously by the natural flora present in the musts and winery equipment. Although usually successful, such practices often incurred lengthy delays of many months before MLF was completed. Today, it is more common in the modern wineries around the world to add malolactic bacteria as starter cultures, as is practised in the dairy industry with other lactic acid bacteria. Such starters are introduced as actively growing bulk cultures prepared from lyophilised or frozen cultures. This process offers more control over MLF as to its initiation, length of time to completion and selection of the strain of bacteria. This also allows for control over the subtle complexity factors produced by growth of the selected bacterial strain.

Major endproducts from metabolism of grape sugars (glucose and fructose) such as lactic acid and carbon dioxide are expected from heterofermentative lactic acid bacteria, while lactic acid alone is produced by homofermentative strains. The amounts and nature of minor metabolic byproducts during growth of malolactic bacteria are difficult to quantify and will vary with the composition of the wine and the strain of starter culture used. For example, some heterofermentative strains may utilise fructose present in the must to gain additional energy for growth, but produce acetic acid in the process (Pilone *et al.*, 1991; Tracey and van Rooyen, 1988).

Acetic acid formation can be deleterious to wines if concentrations become too great. The formation of acetic acid by heterofermenters may occur when fructose is used as a hydrogen acceptor during grape sugar catabolism. The reduction of some fructose to mannitol by mannitol dehydrogenase (Martinez *et al.*, 1963) may be used to reoxidise reduced coenzymes formed in the heterolactic fermentation of these sugars. As shown in Figure 1.1, this allows acetyl phosphate formed from the phosphoketolase reaction to be hydrolysed, instead of having to be reduced to ethanol for this coenzyme reoxidation (Keenan, 1968). Hydrolysis of acetyl phosphate by acetate kinase, then, may result in extra beneficial energy (ATP) formation for the bacteria. In order for this to occur, fructose must be present in excess of requirements for fermentation. This would happen in situations of early inoculations of starter bacteria in musts where the grape sugars are in abundance, or during "stuck" or "sluggish" fermentations where yeast alcoholic fermentation is incomplete or very slow.

1.1 Objectives

Because of the deleterious effects of high acetic acid content in wine, it would be useful for the winemaker to know which strains of malolactic bacteria are capable of mannitol formation and consequently are potential acetic acid producers. In addition, it would be helpful to understand under what conditions this might occur and to minimise

the risk by proper strain selection. This study, therefore, looks at the characteristics of the bacteria and investigates some of the endproducts and parameters under which they are formed. Most of the malolactic bacteria used in this investigation are commercial strains used world-wide and therefore the results will be of international interest.

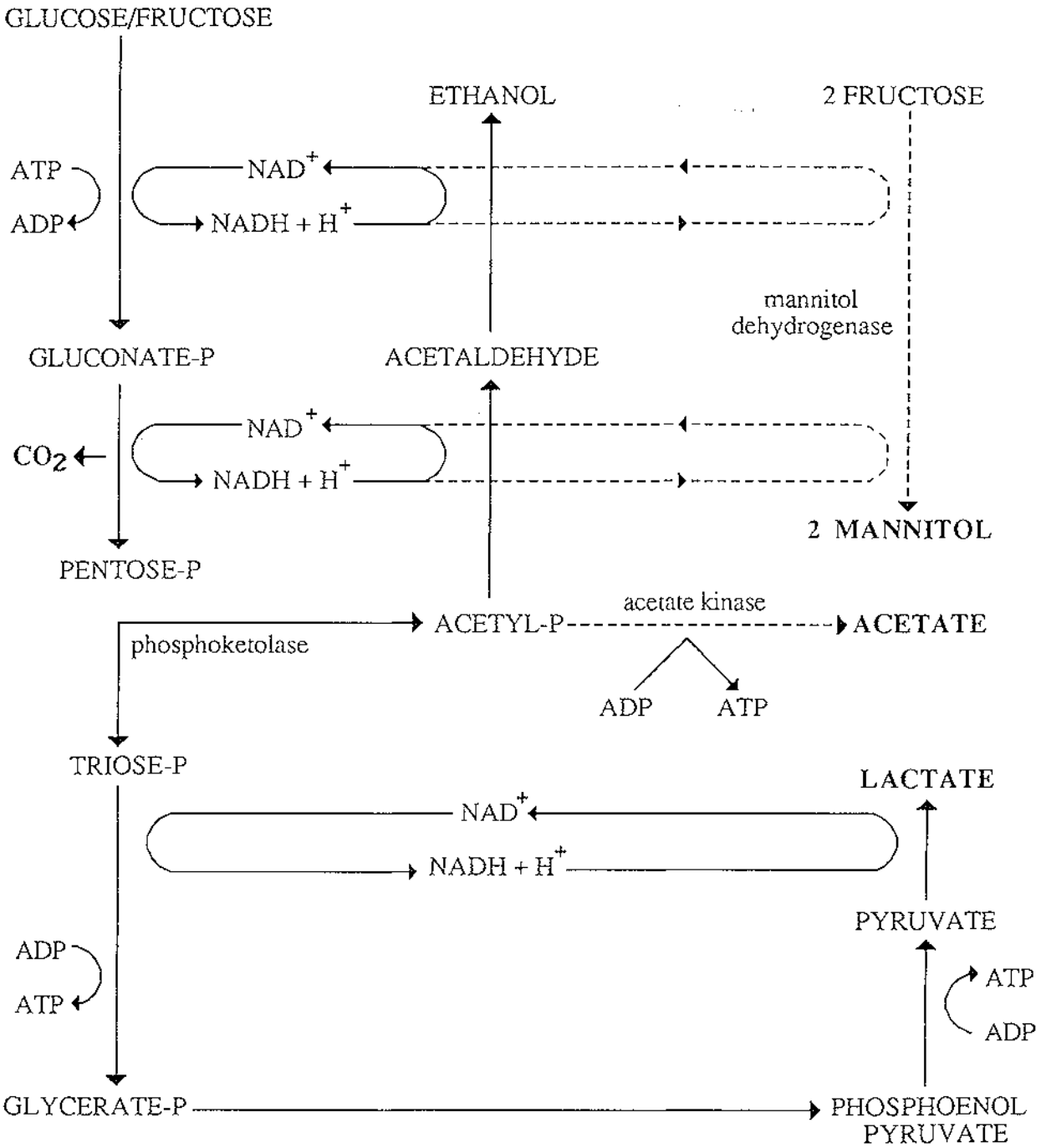

Fig. 1.1 Fructose reduction to mannitol with formation of acetic acid and ATP in heterofermentatative lactic acid bacteria. After Pilone, 1988.

2.0 LITERATURE REVIEW

2.1 Winemaking

Winemaking is an ancient art, dating back to thousands of years B.C. The production of good wines starts with good grapes and harvesting at the right point of maturity. The composition, quality, and in some cases, the type of wine may be determined by the composition of grapes at maturity (Rankine, 1989).

Harvesting may be by hand or by mechanical means. Grapes are received at the winery, and are crushed and destemmed, and the free run juice collected. Sulphur dioxide, at levels of 50-100 mg/L total may be added now or after crushing to resist oxidation, browning and wild yeast and bacterial growth. Juices destined for white wines are treated differently from those intended for reds. For white wine, the skins are removed after gentle pressing and the juice may be clarified by settling for 24 hours or by centrifugation. Juice for red wine remains in contact with the skins to impart colour and flavour components. Care is taken not to crush the seeds, which release excessive tannins.

After clarification, white wine juice is ready for fermentation by yeast. The sulphur dioxide added initially has held wild yeasts in check and the winemaker usually adds selected starter cultures of *Saccharomyces cerevisiae* which ferment strongly and are resistant to sulphur dioxide. Musts undergoing fermentation must be temperature controlled to maintain an even fermentation. Generally, white wines are fermented at lower temperatures than red wines. The skins and pulp in a red wine fermentation are buoyant due to carbon dioxide, and form a cap on the surface. Fermentation proceeds faster in the cap and so mixing or "pumping over" is necessary to provide an even fermentation and to aid in the extraction of colour and tannins (Amerine *et al.*, 1980).

Once fermentation is complete, the wine is racked off into another vessel without disturbing the settled yeast and solids (lees). Extended lees contact has been found to encourage malolactic fermentation (MLF), and is practised if MLF is desired at a later stage. White wines are again clarified either by further racking, filtration or fining, especially if MLF is deemed inappropriate for the wine in question. For red wines, and some whites from cool climate areas, it is considered necessary for malolactic fermentation to occur.

Aging and blending are the next steps in the process of both red and white wines, the timing and ratio of which reflect the skill of the winemaker.

Finally, wines are ready for bottling and are usually stabilised by centrifugation or further racking. Before bottling, wine is usually sterile filtered and dispensed into pre-sterilised bottles. Properly clarified and filtered wine should remain clear up to the time of consumption.

2.2 Malolactic Fermentation

The biological deacidification of wines due to the growth of lactic acid bacteria is considered an integral part of winemaking in cool climate regions, and a spoilage problem in warmer regions. Malolactic fermentation (MLF) generally occurs after yeast alcoholic fermentation, but it is often difficult to initiate or control the timing of this fermentation. In the last 25 years many workers have focussed on MLF, and comprehensive reviews exist (Kunkee, 1991; Edwards and Beelman, 1989; Davis *et al.*, 1985; Wibowo *et al.*, 1985).

Despite the biological deacidification of wines being referred to as a fermentation, the conversion of malic acid to lactic acid is not a true fermentation, but an enzymatic reaction conducted by the bacteria after they have grown in the wine (Wibowo *et al.*,

1985). Tartaric acid and malic acid are the main acids in wines, forming more than 90% of the total acids in grape juice. The acid content of wines from cool climate regions is higher than of those from warm viticultural regions. The tartaric acid content may only be metabolised by some bacteria, and if the pH is above 4.0 (Rankine, 1989). MLF occurs only in table wines, reds and some whites, but not in dessert (fortified) wines as the ethanol content of these wines protects them from most bacteria, with the notable exception of the spoilage organism *Lactobacillus fructivorans* (*trichodes*) (Fornachon *et al.*, 1949). Wine yeasts generally have limited capacity to degrade malic acid and thereby reduce wine acidity, with some selected strains using up to 30% of the malic acid in the must (Gockowiak *et al.*, 1991).

Musts soon after crushing generally contain lactic acid bacteria (LAB) populations of 10^3 to 10^4 cells/mL. The major species of LAB associated with wine are *Leuconostoc oenos*, *Pediococcus damnosus*, *Pd. pentosaceous*, *Pd. parvulus* and several species of *Lactobacillus* (Wibowo *et al.*, 1985). Broadly speaking, the pH of the must is the main factor determining which species develop. Wines below pH 3.5 do not support the growth of pediococci or lactobacilli, and invariably *Leuconostoc oenos* predominates in these wines. The genus *Pediococcus* has not been isolated from French wines but occurs regularly in Australian and other warm climate winemaking regions (Wibowo *et al.*, 1985).

2.2.1 Reasons for MLF

Historically, MLF is encouraged by winemakers for three reasons: 1) the deacidification of high acid musts; 2) the microbial stabilisation resulting from MLF; and 3) the enhancement of flavour and complexity of the wine. Each of these will be discussed in turn.

Deacidification

MLF may reduce the titratable acidity by 1/3 and raise the pH by 0.1 to 0.3 unit (Davis *et al.*, 1985; Amerine *et al.*, 1980). Warm climate wines of high pH may become too low in acid after MLF and become insipid or flat. Due to the difficulty in preventing MLF from occurring in high pH wines, winemakers in these regions encourage MLF to occur and then correct the acidity afterwards with tartaric acid additions. High pH wines are also susceptible to spoilage by other LAB even after MLF (Rankine, 1977).

Microbial stability

MLF involves the growth of high numbers of bacteria and the release of carbon dioxide. Bottled wines which have not already undergone MLF may do so in the bottle, resulting in turbidity and gasiness, rendering the wine spoiled. Historically, this growth of MLF bacteria was presumed to deplete nutrients thus rendering the wine incapable of supporting further LAB growth (Davis *et al.*, 1985). Microbial stability after MLF, however, is not absolute. Wine still contains many residual sugars capable of supporting the growth of spoilage organisms. Wines of higher pH, above 3.5, are especially susceptible to growth of pediococci and lactobacilli, even after MLF. The increase in pH associated with MLF may even encourage spoilage species. Costello *et al.* (1983) found that wines at pH 3.65 showed a succession of bacterial growth with *Lc. oenos* conducting MLF, and species of *Pediococcus* and *Lactobacillus* growing afterward. Wibowo *et al.* (1988) found that a wine in which bacterial growth and MLF had occurred could be filter sterilised and later inoculated with different strains of *Lc. oenos* and growth observed. Davis *et al.* (1986) also showed that wines still contained sufficient substrates for bacterial growth after MLF. They concluded that microbial stability of wine is better controlled through the management of pH, addition of sulphur dioxide and sterile filtration at bottling.

Changes in flavour complexity

The growth of bacteria to high numbers in wine (10^6-10^8 cells/mL) uses wine constituents and produces byproducts of growth, as well as changing the pH of the wine. French producers of Bordeaux and Burgundy wines believe that the high quality of their red wines is in part due to MLF (Amerine *et al.*, 1980). MLF is generally encouraged in all low pH red wines, but is discouraged in most white table wines of delicate varietal character and fruity aroma (Rankine, 1977). Several studies have investigated the influence of MLF on the sensory characteristics of wine. Pilone and Kunkee (1965) reported the sensory evaluation of a wine that had undergone MLF with six different strains of bacteria. No striking differences could be distinguished by the panel of judges. Rodriguez *et al.* (1990) using a panel of experts in the field of oenology found that extended lees contact and fermentation in wood may be as effective as MLF in imparting complexity to a wine. van Wyk (1976) attempted to correlate the occurrence of MLF with a wine's ability to win awards, but found that only 59% of award winning samples had undergone MLF. Rankine (1977) noted that opinion was divided on the desirability of MLF amongst Australian winemakers. He commented that while wines of high organoleptic quality may benefit from MLF by developing more complexity in aroma and flavour, they lost fruit and varietal characteristics and developed transient off-flavours during MLF. Iso-amyl acetate, a fruity ester in varietal wines, has been reported to decrease by up to 80% after MLF (Rankine, 1989). This may be important as there is a market trend towards young varietal wines in this part of the world. Pilone *et al.* (1966) attempted to show chemical differences in wines fermented with different strains of malolactic bacteria, but found only a few differences in composition. These were in volatile acidity, acetoin, diacetyl and diethyl succinate. Malolactic bacteria produce lactic acid as the major end-product, but also produce flavour compounds such as acetaldehyde, acetic acid, ethanol, diacetyl, acetoin and 2,3-butanediol in small amounts. Diacetyl in amounts of 1 to 4 mg/L is regarded as adding complexity to a wine, but if in concentrations above 5 to 7 mg/L it becomes

dominant, imparting a butter-like taste to a wine (Davis *et al.*, 1985; Rankine, 1977). Volatile acidity generally increases during MLF in young wines by about 0.1 g/L (Edwards and Beelman, 1989). Most wines designated as dry in fact contain 1 to 3 g/L residual hexose and pentoses. These sugars are available to LAB as substrates for growth, with the potential for flavour changes from their metabolism. Chromatographic studies by Esau and Amerine (1964) showed dry wines to contain a variety of disaccharides, trisaccharides, pentoses and polyols. The glucose and fructose levels in wines before and after MLF may in fact rise initially and fall later. *Leuconostoc oenos* has been seen to follow a diauxic growth pattern with a preference for pentose sugars, using glucose only after MLF is completed (Wibowo *et al.*, 1985).

2.2.2 Factors influencing the induction of MLF

pH

The pH of wine is generally between 3.0 and 4.0 depending on the concentration of malic and tartaric acids and other buffering systems. Wine pH is one of the most important parameters that affect the behaviour of wine LAB. pH influences the lag phase and the rate of growth of LAB, and therefore influences the duration of MLF. pH also influences the species that can grow and the metabolic behaviour of these species (Wibowo *et al.*, 1985; Bousbouras and Kunkee, 1971). Generally, the higher the pH is above 3.0, the more likely that MLF will occur and the quicker the onset. *Leuconostoc oenos* strain ML 34 required 23 weeks to complete MLF in a wine at pH 3.15, 9 weeks at pH 3.3 and 2 weeks at pH 3.8 (Bousbouras and Kunkee, 1971). Costello *et al.* (1983) and Davis *et al.* (1986) found similar results. The pH of a wine exerts a selective pressure on the species that develop. *Leuconostoc oenos* predominates in wines below pH 3.5 which do not seem to support the growth of pediococci or lactobacilli. These latter species become more prevalent in wines as the pH nears 4.0. In high pH wines *Pediococcus* and *Lactobacillus* species are capable of

conducting MLF, or growing as spoilage species after MLF (Davis *et al.*, 1988; Wibowo *et al.*, 1985; Rankine, 1977). When inoculating wines with cultures of LAB, an initial die-off is often noted, and this appears with increased severity at lower pH (Edwards and Beelman, 1989; Wibowo *et al.*, 1988). The metabolic activity of LAB appears to be influenced by pH, both with respect to carbohydrate metabolism and secondary metabolite production. Davis *et al.* (1988) noted a different fermentation profile for strains of *Lc. oenos*, *Pediococcus* and *Lactobacillus* at pH 6.0 and 4.0 in commercial diagnostic carbohydrate galleries (API 50 CH). They noted a reduced range of carbohydrates fermented at pH 4.0 compared with that at pH 6.0, and propose that similar changes in fermentation profiles might occur in wine. This suggests that wines of higher pH risk increased formation of metabolic by-products such as acetic acid, due to more sugar fermentation at the higher pH. However, Bousbouras and Kunkee (1971) found that increased amounts of volatile acidity were formed by MLF at lower pH, but in amounts considered too small to be of organoleptic significance. The production of diacetyl from citric acid is also favoured by low pH (Wibowo *et al.*, 1985).

Sulphur dioxide

Sulphur dioxide (SO_2) is widely used in winemaking as an antioxidant and as an inhibitor of yeast and bacteria. Sulphur dioxide added to a wine or juice enters a pH dependent equilibrium of reactive forms. These include bound SO_2 and the free SO_2 species of molecular SO_2, bisulphite and sulphite ions. The sum of these different forms is referred to as total SO_2. The most toxic free SO_2 species is molecular SO_2. The amount of free SO_2 is pH dependent – the lower the pH the more is formed. Carbonyl compounds such as acetaldehyde in wine bind SO_2 to form bound SO_2, and this removes free SO_2 from solution. Wibowo *et al.* (1985) suggest that 100-150 mg/L total, 50-100 mg/L bound, or 1 to 10 mg/L free SO_2 is sufficient to affect LAB in wines. These values will vary with the pH, the species involved, and the insoluble

solids present in the wine. *Leuconostoc oenos* appears to be less tolerant to SO_2 than the pediococci or lactobacilli at similar pH levels (Davis *et al.*, 1988; Wibowo *et al.*, 1988; Tracey and Britz, 1987). Winemakers seeking to encourage MLF in wine should limit the use of SO_2 at crushing, and should not exceed 50 mg/L total SO_2 or 10 mg/L free SO_2 (Britz and Tracey, 1990).

Temperature

Not unexpectedly, temperature affects the rate of growth of LAB, and as MLF is not seen to occur until cell density reaches about 10^6 cells/mL, it also affects the rate of MLF (Wibowo *et al.*, 1988). Kelly *et al.* (1989) studied specific growth rates of *Lc. oenos* strains. The maximum specific growth rate for most strains was at 30-33°C. It is generally recognised by the wine industry that below 15°C MLF is difficult to promote, and higher temperatures of 18 to 25°C are recommended (Rankine, 1987). Below 15°C, MLF can be delayed for long periods (Lafon-Lafourcade, 1975). Storing young wines under conditions favourable to MLF (about 25°C) presents conditions favourable to spoilage yeast species such as *Brettanomyces* and *Dekkera* as well. Refrigeration of wine to delay or avoid MLF is a useful tool available to the winemaker, especially in combination with SO_2 additions.

Ethanol

The concentration of alcohol limits the species which can grow in wine. Alcohol tolerance is affected by temperature and pH. Higher temperature and lower pH decrease tolerance. Generally, wine LAB survival and growth decrease as the ethanol concentration rises above 10%. Lafon-Lafourcade (1975) notes that most *Leuconostoc* and *Pediococcus* species have a maximum tolerance of 12-14%, while most *Lactobacillus* species tolerate 15% ethanol. The spoilage organism *Lb. fructivorans* (*trichodes*) has been isolated from dessert wines of 20% ethanol (Wibowo *et al.*, 1985;

Fornachon *et al.*, 1949). Lafon-Lafourcade *et al.* (1983) noted that the survival of *Lc. oenos* in wines after MLF was less in wines with a higher ethanol content (12.5%) than at 10%. There is evidence that ethanol may affect the activity of the enzyme(s) responsible for MLF. The ability of resting cells to degrade malic acid was unaffected at 11% ethanol, but was reduced to 56% at 12% ethanol and to 16% activity at 13% ethanol (Lafon-Lafourcade, 1975).

Oxygen tension

Lactic acid bacteria are stimulated by anaerobic to microaerophilic conditions. Any stimulation of growth rate will affect the rate of MLF. Some dissolved oxygen is reported as being necessary for growth. However, Kelly *et al.* (1989) found that *Lc. oenos* was not stimulated by CO_2 but by the absence of inhibitory oxygen, and they describe *Lc. oenos* as an aerotolerant anaerobe. Conversely, Condon (1987) reported faster growth rates for other leuconostoc species in the presence of oxygen. The wine leuconostoc, *Lc. oenos* is, however, distinct from other species in the genus, with little homology with the other species (Kelly *et al.*, 1989).

Yeast interactions

Interactions between wine yeasts used to conduct yeast alcoholic fermentation (YAF) and lactic acid bacteria have been reported by many authors (Lonvaud-Funel *et al.*, 1988; Lemaresquier, 1987; Fleet *et al.*, 1984; Fornachon, 1968). During YAF, some strains of yeast produce SO_2, even to high levels, in addition to ethanol, which may affect the induction of MLF. Yeast growth necessitates the depletion of nutrients such as arginine and other amino acids from the wine. This, however, is balanced by the release of compounds from yeast autolysis towards the end of YAF. Antagonistic compounds produced by yeast have been seen to inhibit LAB, and therefore MLF. Medium chain fatty acids are reported to inhibit LAB. Decanoic, octanoic, hexanoic

and dodecanoic acids have been implicated in yeast-LAB antagonism (Edwards and Beelman, 1989).

Winemaking practices

Winery practice may affect the initial presence, survival and growth of LAB. Clarification, racking, centrifugation and filtration remove LAB from suspension, while refrigeration, pasteurisation and preservative addition inhibit LAB growth (Rankine, 1989; Wibowo *et al.*, 1985). Winemakers are able to vary the factors so far discussed – pH, temperature, ethanol concentration etc. – to encourage or discourage MLF. The level of SO_2 required to guarantee that no MLF takes place is organoleptically unacceptable, so this preservative is used in combination with other practices to prevent MLF, particularly in high pH wines. Other preservatives have been suggested. For example, fumaric acid at 1.5 to 2.0 g/L inhibits LAB growth, with its effect being inversely proportional to pH (Rankine, 1977; Pilone *et al.*, 1974).

2.2.3 Strategies for conducting MLF

A simple cost effective way of stimulating MLF is to select conditions which encourage the growth of natural flora present in the wine. The winemaker has little control over the timing of the fermentation, or the organisms responsible for it. MLF may occur during, or immediately after YAF, or several months later. Established wineries may find their wines undergoing MLF spontaneously due to resident lactic acid bacteria on grapes or in winery equipment (Gockowiak *et al.*, 1991; Davis *et al.*, 1985). Spontaneous fermentation involves some degree of risk from spoilage organisms, and many elements of its regulation are beyond the control of the winemaker, hence its unpredictability. Another method employed is to seed a wine with one already undergoing MLF. If the two wines present similar conditions, then the bacteria should be well adapted to growth in the new wine. One drawback to this

method is the inoculum size, with recommendations ranging from 5% to 50% blends of the two wines (Davis *et al.*, 1985). This method also allows for cross-infection with less desirable strains of LAB and for bacteriophage transfer from vat to vat (Gockowiak *et al.*, 1991; Beelman, 1982). The concept that some strains are less desirable than others from an organoleptic point of view has been suggested but not proven by definitive experimentation. Some wine tasting trials have shown that wines inoculated with starter cultures were preferred over those deacidified spontaneously by the natural flora (Zeeman *et al.*, 1982).

The concept of pure culture inoculation of wines has been investigated since the 1960s. Inoculation with a bacterial starter culture has three advantages: 1) less time is required for the bacteria to grow to a high enough density to carry out MLF; 2) the possibility to select bacteria with desirable characteristics to carry out MLF; and 3) inoculation with a mixed strain starter to give protection against phage attack (Henick-Kling, 1988). The effects of different strains of bacteria used to conduct MLF have been studied by many researchers (Edwards *et al.*, 1991; Henick-Kling *et al.*, 1989; Wibowo *et al.*, 1988; Davis *et al.*, 1985; Zeeman *et al.*, 1982; Beelman, 1982; Pilone and Kunkee, 1965). Since wines differ greatly in composition and pH, matching of particular strains of bacteria to wine conditions should allow for faster growth, and therefore faster MLF. Strains have been isolated from low pH wines in cool climate regions where other strains native to warmer regions would not grow (Beelman *et al.*, 1977). The modern winemaker has at his/her disposal a growing list of strains to induce MLF by inoculation. Surveys in New Zealand on the use of bacterial starter cultures in 1985 and 1988 showed an increasing number of wineries (up to 50%) using prepared commercial starters to induce MLF (Pilone, 1988). The winemaker wishes to complete MLF as soon as possible, and so inoculation should be timed to promote the growth of the bacteria and therefore MLF. Opinion is divided as to when to inoculate. The timing of inoculation with such cultures is divided into three broad periods in the vinification process: 1) simultaneously with yeast inoculation; 2)

during YAF; and 3) after the completion of YAF (Davis *et al.*, 1985). Early inoculation is advocated by some winemakers because, apart from SO_2 and pH, a major inhibitor of bacterial growth is the ethanol content of the wine. Inoculation concurrently with the yeast or shortly after the onset of YAF presents the bacterial starter with low concentrations of both ethanol and any other yeast metabolic products which may be inhibitory to their growth. Also, grape juice nutrients normally removed by yeast during fermentation are then available to the bacteria (Gockowiak *et al.*, 1991). However, studies have shown conflicting results. This faster MLF due to inoculation before YAF was not seen by Gallander (1979), though stimulation of MLF by early inoculation has been reported by Beelman (1982) and Kunkee (1991). Concurrent inoculation is practised successfully in California, but some European workers consider it risky due to the possibility of a sluggish or incomplete yeast fermentation which exposes the wine to lactic souring. Here, the bacteria thrive on residual grape sugars and produce large amounts of lactic acid and, in some cases, acetic acid. In a vigorous YAF these sugars are removed quickly (Davis *et al.*, 1985). The inoculation of bacteria during the alcoholic fermentation is advocated by some oenologists. Here, the ethanol concentration is still moderate, and SO_2-binding compounds produced by yeast have reduced the initial free SO_2 so toxic to LAB.

The possibility of yeast and bacterial antagonistic interactions, as reported by Lonvaud-Funel *et al.* (1988), further complicates inoculation at the start of or during YAF. Lafon-Lafourcade *et al.* (1983) confirmed that the strain of yeast used to conduct YAF affected bacterial growth. Wines fermented with *Saccharomyces cerevisiae* were more favourable to malolactic bacteria than those fermented with *S. bayanus*. They acknowledged that deciding the best time to add starters was a complex problem involving many factors, some of which are not yet understood. In their opinion, early inoculation of bacterial culture could lead to the following: 1) significant inhibition of bacterial growth; 2) a partial inhibition of bacterial growth with delayed MLF; and 3) partial inhibition of the yeast by a high inoculum of bacteria. The inhibition of yeast by

bacteria was greatest if inoculation took place early, less of a problem if bacteria were inoculated towards yeast decline phase, and no inhibition was found if inoculation took place after YAF. Inoculating LAB at the end of alcoholic fermentation provides bacteria with nutrients released from yeast autolysis. The bacteria are, however, subject to high ethanol concentration and this may delay MLF (Davis *et al.*, 1985). Failure of post-fermentation inoculation of LAB has been attributed to poor acclimatisation of bacteria to the wine environment, and a low inoculum of viable cells in the culture (Gockowiak *et al.*, 1991; Lafon-Lafourcade *et al.*, 1983). More recently, bacteriophage have been implicated also (Sozzi and Gnaegi, 1986).

Traditionally, research has focussed on the three practised inoculation times: concurrent, late YAF, and after YAF. Recently, however, a fourth possibility has been investigated. This is the addition of LAB starters to juice before yeast inoculation, which would expose the bacteria to conditions conducive to growth, without competition from yeast. This might allow the slow growing bacteria to attain high cell density, and possibly to conduct MLF before yeast growth occurs. The advantages of this would be a quickly finished wine which could be stabilised immediately after YAF, thereby reducing spoilage risk and heating costs in cool climates. Complications arise, however, with heterofermentative organisms growing unimpeded in grape juice. These organisms may use a variation of the phosphoketolase pathway to produce acetic acid instead of ethanol by reducing the abundant hexose present as well as fermenting it (see section 2.6.3). Homofermentative organisms, however, do not possess this ability and so can be considered for use. Some musts containing high levels of growing bacteria have been seen to inhibit YAF by promoting early decline of the yeast before fermentation is complete (stuck fermentation). Lafon-Lafourcade *et al.* (1983) noted that large bacterial inoculations (10^7 cells/mL) at yeasting were seen to conduct MLF without actually growing. This gave rise to the concept of resting cell fermentation, where bacteria are directly inoculated in high enough numbers to conduct MLF, thereby

removing the need for bacteria to grow in the wine – the primary cause of delay in the completion of MLF.

Problems exist in attaining high enough cell numbers of *Lc. oenos* to inoculate commercial scale juices at 10^7 cells/mL without extensive preculturing. Prahl (1989) circumvented this problem and that presented by heterofermentative metabolism by studying other strains of malolactic bacteria. Here, a strain of the homofermentative bacterium *Lb. plantarum* that could be highly concentrated by lyophilisation and that retained a high specific malolactic activity was used. Prahl advocated a massive inoculum in order to conduct MLF before yeast growth, after which the ethanol produced quickly inhibits the growth of the bacteria at lower pHs. In a commercial trial, 10^7 cells/mL of *Lb. plantarum* were inoculated prior to yeast addition and MLF was completed before the ethanol content reached 8%, the toxic level at low pH for this strain (Prahl *et al.*, 1988). This method for conducting a resting cell fermentation has been trialled in New Zealand. Trials used Sauvignon Blanc, Chardonnay, and Cabernet Sauvignon juices which were inoculated with *Lb. plantarum* strain VINIFLORA LP at yeasting or before. No inhibition of yeast growth was recorded, and reductions of malic acid content ranged from 100% to 69% before the end of YAF. This indicated that the method of early inoculation to conduct resting cell malolactic fermentation with *Lb. plantarum* is feasible in New Zealand (Pilone and Prahl, 1990).

2.3 *Leuconostoc oenos*

Garvie (1986a) describes the genus *Leuconostoc* as spherical to lenticular, Gram positive, catalase negative, non-sporing cells in pairs or chains. They are fastidious, requiring complex growth factors and supplied amino acids. Growth is dependent on the presence of a fermentable carbohydrate. From glucose, ethanol, D(-)lactic acid and carbon dioxide are formed. The species *Lc. oenos* morphologically resembles other leuconostocs, but differs in its slow growth rate and its requirement for the "Tomato

Juice Factor", glucopantothenic acid (Garvie, 1986a, 1984; Amachi, 1975). *Lc. oenos* also has a greater acid tolerance, and will grow in ethanol as well. Garvie (1967) proposed to make the acidophilic leuconostocs a separate species based on the growth in broth cultures of initial pH 4.2. Growth in broth cultures of 10% ethanol also separates *Lc. oenos* from the other leuconostocs. In addition, the lactate dehydrogenases and glucose-6-phosphate dehydrogenases of these acidophilic strains have been shown by polyacrylamide gel electrophoresis to be different from other leuconostoc species. Further studies by Garvie and Farrow (1980) reveal that in *Lc. oenos*, glucose-6-phosphate dehydrogenase is NADP linked, whereas this enzyme requires NAD or NADP as the cofactor in other species, with a preference for NAD. Garvie (1986a) divides the genus into four species. The first, *Lc. mesenteroides*, contains three subspecies – *Lc. mesenteroides* subsp. *mesenteroides*, *Lc. mesenteroides* subsp. *dextranicum* and *Lc. mesenteroides* subsp. *cremoris*. The other species in the genus are *Lc. paramesenteroides*, *Lc. lactis* and *Lc. oenos*.

Concerning sugar fermentations, Garvie (1986a, 1967) indicated that *Lc. oenos* does not ferment sucrose or maltose, unlike other *Leuconostoc* species. Furthermore, *Lc. oenos* does not ferment lactose and so can be differentiated from *Lc. mesenteroides* subsp. *cremoris*. French literature suggests that *Lc. oenos* should be divided further into two species on the basis of pentose fermentation (Dicks *et al.*, 1990). Garvie and Farrow (1980) note that *Lc. oenos* is not a homogeneous group and some strains are considerably easier to grow than others, and that it is inadvisable to split the species further on the basis of a few sugar fermentations alone. As a genus, *Leuconostoc* can be morphologically similar to very short heterofermentative lactobacilli. Classically, the test for ammonia production from L-arginine has been used to differentiate between the lactobacilli, of which most heterofermentative strains are positive, and the leuconostocs which are not. This test is now considered doubtful. There are reports of ammonia-producing strains of *Lc. oenos* (Garvie and Farrow, 1980; Kuensch *et al.*, 1974). Recently, Pilone *et al.* (1991) discussed the test and its limitations. Ammonia

production from L-arginine is a product of the arginine dihydrolase system, which in a three step reaction degrades L-arginine to ornithine, 2 moles of ammonia, ATP and CO_2. Classically, 0.3% arginine was added to broth cultures and the test conducted on the broth after growth. In this research (section 4.1.3), tests with Nessler's reagent showed that this amount of arginine could not always produce enough ammonia to be detected, and so 0.6% was used in broth cultures. Using the more sensitive test, it was found that 75% of leuconostocs tested were positive for ammonia production, casting doubt on the usefulness of this test to distinguish lactobacilli from leuconostocs.

Leuconostocs contain the enzyme phosphoketolase and produce gas (CO_2) from carbohydrates, and so are considered to have a heterolactic (heterofermentative) metabolism. Most species ferment glucose by a combination of the hexose-monophosphate and phosphoketolase pathways, but the pathway for *Lc. oenos* has not been fully confirmed (Garvie, 1986a). Growth in the genus is never rapid, with *Lc. mesenteroides* subsp. *mesenteroides* the fastest and *Lc. oenos* the slowest. Even within the species *Lc. oenos*, large differences in growth rates between strains are reported (Kelly *et al.*, 1989; Champagne *et al.*, 1989; Garvie, 1967). *Lc. oenos* may require seven to ten days incubation before reasonable turbidity is produced in broth culture (Garvie, 1984).

2.4 *Lactobacillus* species

Lactobacillus is a large genus containing about fifty species. All are Gram positive, non-sporing, catalase negative rods. These vary in morphology from long and slender rods to coccobacilli. The genus has complex nutritional requirements for amino acids, nucleic acid derivatives, salts, fatty acids and fermentable carbohydrates. Nutritional requirements are generally characteristic for each species, often for particular strains only (Kandler and Weiss, 1986). Lactobacilli are found in dairy products, grain products, meat, fish, water, sewage, beer, wine, fruits and juices, silage and mash.

They are also part of the normal flora associated with man and animals. Hexose fermentation pathways include the Embden-Meyerhof pathway converting hexose to two moles of lactic acid (homolactic fermentation), and the 6-phosphogluconate pathway (heterofermentation) resulting in CO_2, ethanol and lactic acid in equal amounts from hexose. Depending on the strain, D-, L- or a mixture of D- and L-lactic acid is formed. Enzymatically, homofermentative strains contain aldolase but no phosphoketolase, and the converse is true for heterofermentative strains. Pentoses are degraded by heterofermenters (via phosphoketolase) to equimolar amounts of lactate and acetate. Homofermentative strains, lacking phosphoketolase, are unable to ferment pentoses. One group of homofermentative lactobacilli, however, have an inducible phosphoketolase. These strains ferment hexoses homofermentatively, but pentoses heterofermentatively, and are referred to as facultative heterofermenters (Kandler and Weiss, 1986). In accordance with this, the fifty species of lactobacilli are arranged into three groups. Group I contains obligately homofermentative strains fermenting hexose almost exclusively to lactic acid. Pentoses and gluconate are not fermented. This group includes *Lb. delbrueckii* from wine environments. Group II contains the facultatively heterofermentative organisms which ferment hexoses almost exclusively to lactic acid, but are also able to ferment pentoses by an inducible phosphoketolase to lactic acid and acetic acid. Members of Group II which are found in wine environments include *Lb. sake* and *Lb. plantarum*. The third group, Group III, are obligate heterofermenters which ferment hexose to lactate, acetic acid or ethanol and CO_2. The production of CO_2 is characteristic of Group III, which includes the wine species *Lb. brevis, Lb. hilgardii, Lb. buchneri* and *Lb. fructivorans* (Scheifer, 1987; Kandler and Weiss, 1986).

As a genus, lactobacilli generally have a lower tolerance to low pH than do species of *Leuconostoc*, but a higher tolerance to ethanol. Ethanol tolerance can be as high as 15% for most species, but one species, *Lb. fructivorans*, can colerate 20% ethanol concentrations (Wibowo *et al.*, 1985). The wine lactobacilli occur as spoilage

organisms in wines above pH 3.5, and may be responsible for malolactic fermentation in high pH wines.

2.5 *Pediococcus* species

The pediococci are morphologically distinct from other lactic acid bacteria because of cell division in two planes, which forms pairs or tetrads. Chains are not formed, although short four cell groups of linearised tetrads sometimes can be seen. The pediococci are described as Gram positive, catalase negative, non-sporing facultative anaerobes, with species-specific oxygen tolerance (Garvie, 1986b). Growth is dependent on the presence of a fermentable carbon source. Fermentation of glucose is probably by the homolactic Embden-Meyerhof pathway to form D- and L-, or L-lactic acid alone. Cell size is variable from 0.6 μm to 1 μm, but is constant within a strain (Garvie, 1986b). Species reported as growing in wine related environments include *Pd. pentosaceus, Pd. damnosus* and *Pd. parvulus* (Davis *et al.*, 1986; Costello *et al.*, 1983). The pediococci are less acid tolerant than *Lc. oenos,* and are found only in wines above pH 3.5. In broth cultures strains have grown in pH 3.2, and most strains were unaffected by 12.5% ethanol. The pediococci were found to be similar to the lactobacilli in their tolerance of SO_2, which is greater than that of *Leuconostoc oenos* (Davis *et al.*, 1988).

2.6 Aspects Of Bacterial Metabolism

2.6.1 Arginine hydrolysis

Some bacteria are capable of metabolising the amino acid arginine. This has been reported in species of *Streptococcus, Clostridium, Halobacterium, Pseudomonas* and the acetic acid bacteria (Manca de Nadra *et al.*, 1988). Evidence of arginine utilisation in the lactic acid bacteria is scant. Arginine metabolism may be an important energy

yielding pathway, especially in the genus *Lactobacillus* in which some enzymes of the Krebs cycle appear to be absent (Manca de Nadra *et al.*, 1988). *Lb. plantarum* from fish has been shown to degrade arginine to ornithine (Josson *et al.*, 1983), while *Lb. buchneri* and *Lb. sake* have been shown to degrade arginine completely (Montel and Champomier, 1987). *Pediococcus pentosaceus* of dairy origin is also reported to hydrolyse arginine (Tzanetakis and Litopoulou-Tzanetaki, 1989). Complete arginine metabolism involves three separate enzymatic reactions, collectively referred to as the arginine dihydrolase system (Manca de Nadra *et al.*, 1988). These reactions are:

1) arginine + H_2O ——> citrulline + NH_3

2) citrulline + Pi ——> ornithine + carbamylphosphate

3) carbamylphosphate + ADP ——> ATP + CO_2 + NH_3

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arginine + H_2O + Pi ADP ——> ornithine + ATP + CO_2 + $2NH_3$

Enzymes catalysing the arginine dihydrolase pathway reactions are 1) arginine deiminase, 2) ornithine transcarbamylase and 3) carbamate kinase, respectively. Arginine hydrolysis is active in most of the obligately heterofermentative lactobacilli (group III), but is generally absent from the group I (obligately homofermentative) lactobacilli (Kandler and Weiss, 1986). Some reports of ammonia positive *Leuconostoc* species have occurred (Garvie and Farrow, 1980; Weiller and Radler, 1978; Kuensch *et al.*, 1974).

The classical test for arginine hydrolysis uses media incorporating 3 g/L arginine. Pilone *et al.* (1991) discussed the sensitivity of the test on broths initially containing 3 g/L arginine after finding strains of *Lc. oenos* positive for ammonia production in media containing 6 g/L arginine. According to Garvie (1986a), ammonia production is characteristically negative for this species, and this inability has classically been used to differentiate them from some morphologically similar heterofermentative lactobacilli.

Ingram (1975) also commented on the test, suggesting that the test is only useful if set criteria are stated such as ammonia production in the presence of 2% glucose. At 2% glucose only heterofermentative lactobacilli are positive, but at low concentrations facultatively heterofermentative (group II) species may produce ammonia, and the test loses its significance. Pilone *et al.* (1991), as part of this research (section 4.1.3), found that in broth cultures containing 0.5% glucose, 2% fructose and 6 g/L arginine, homofermentative strains tested negative while heterofermentative strains of lactobacilli and a surprising 75% of the heterofermentative *Leuconostoc oenos* tested positive for ammonia. It was suggested that the increased detection of ammonia producers was due to more arginine in the broth cultures than was used previously.

The arginine dihydrolase system liberates two moles of ammonia – one in reaction 1 and the other in reaction 3 (see above). Not all bacteria have a full complement of the enzymes necessary (Montel and Champomier, 1987), and therefore may produce only one mole of ammonia per mole of arginine. This would happen if reaction 1 or reactions 1 and 2 are functioning only. If the organism lacks carbamate kinase of reaction 3, the second mole of ammonia will not be formed, nor will energy be produced. This may be the case with some *Leuconostoc* species. By using 6 g/L in test broths, even bacteria with only enzymes for the first and possibly the second reaction could produce up to 0.6 g/L of ammonia. The detectable limit of ammonia in the HFA broth used is just greater than 0.3 g/L, so 0.6 g/L is reliably detectable with Nessler's reagent, as found in section 4.1.3 of this research (Pilone *et al.*, 1991). Because of this poor sensitivity, carbamate kinase-deficient bacteria may be undetectable in conventional media (3 g/L arginine). Work presently under way in our laboratory includes screening *Lc. oenos* strains for the presence of the enzymes responsible for the dihydrolase system (Pilone and Liu, 1992).

A positive test for ammonia production indicates arginine hydrolysis, but does not necessarily indicate the formation of energy from the reaction. This energy is only

available to strains with the full complement of enzymes. As grapes and wines can contain relatively high concentrations of arginine, heterofermentative strains capable of complete arginine hydrolysis may be more competitive as starter cultures. Flavour modification due to arginine hydrolysis and ammonia production has not been studied.

2.6.2 Flavour compounds

The aroma and flavour of wine are composed of many factors, those derived from the fruit, from the growth of microorganisms, and from processing and aging. The term 'complexity' is often used to describe the contributions of those factors other than the fruit or varietal characteristics of the wine. Complexity is difficult to define chemically or in sensory terms. For example, Rodriguez *et al.* (1990) found that fermentation in oak, extended lees contact, and aging may be as effective as MLF for imparting complexity to wines. Despite many reports of chemical composition changes in wines after MLF, detectable sensory changes are few (Pilone and Kunkee, 1965).

Apart from lactic acid production, malolactic bacteria are capable of producing acetaldehyde, acetic acid, ethanol, diacetyl, acetoin and 2-3 butanediol. Diacetyl and acetaldehyde are the most flavourful compounds, with the more reduced compounds (acetoin, 2-3 butanediol and ethanol) contributing less. The ratios of production depend on the oxidation-reduction balance of the environment (Kandler, 1983). A diacetyl concentration of between 1 and 4 mg/L is regarded as adding complexity to the wine. However, concentrations of 5 to 7 mg/L or higher can be overpowering and impart a butter-like aroma which is undesirable (Davis *et al.*, 1985). Citric acid is the substrate for acetoin and diacetyl formation. Citrate occurs in milk at 1.5 g/L, but in wine levels are generally less than 0.5 g/L, although they may be higher in *Botrytis* infected grapes (Henick-Kling, 1988). Citrate is degraded according to the overall reaction:

3 citrate ——————> lactate + 3 acetate + 5 CO_2 + diacetyl

The accompanying production of three acetate molecules for each diacetyl corroborates the higher acetate levels found in wines containing diacetyl (Zeeman *et al.*, 1982).

Malolactic fermentation causes increases in ethyl lactate and diethyl succinate concentrations which increase with low pH wines (Zeeman *et al.*, 1982; Pilone *et al.*, 1966). These increases in esters are probably too small to alter wine bouquet as the odour threshold value of ethyl lactate in wine is low. This is also true for diethyl succinate.

The formation of pyruvate by citric acid reduction or by hexose fermentation can be used to form lactate by lactate dehydrogenase (LDH). In most LAB, LDH is fructose 1-6 diphosphate-dependent (Tseng and Montville, 1990). In the absence of fructose 1-6 diphosphate (hexose limitation), other products are formed from pyruvate. Pyruvate may be split into formate and acetate by pyruvate formate lyase. Those bacteria with oxidases, peroxidases or NAD-independent dehydrogenases can oxidise pyruvate to acetate and yield ATP. Aerobically they can use pyruvate oxidase, or anaerobically pyruvate dehydrogenase. These reactions all release acetate into wine, the level of which is important to the winemaker as spoilage occurs if levels are high.

Sorbate has been used as a preservative in wine, but it can be utilised by some LAB. It is reduced to its corresponding alcohol which reacts with ethanol at low pH to form a volatile ester (2 ethoxy-3-5-diene) responsible for 'geranium' off-flavours (Henick-Kling, 1988). Some species of wine LAB may metabolise glycerol to the bitter substance acrolein, but this has received little attention in the literature (Wibowo *et al.*, 1985).

2.6.3 Mannitol and acetic acid formation

The ability to form mannitol from fructose is reportedly positive for heterofermentative lactic acid bacteria (Dicks and van Vuuren, 1988), although exceptions have been reported. Chalfan *et al.* (1975) found the heterofermentative organisms that they identified as *Lc. oenos* and *Lb. brevis* were negative by thin layer chromatography. Tracey and Britz (1987) found that mannitol formation was variable amongst *Lc. oenos*.

The reduction of fructose to mannitol by mannitol dehydrogenase is of physiological as well as oenological importance. Heterolactic fermentation of hexoses produces two moles of reduced coenzyme NADH (nicotinamide adenine dinucleotide) up to the phosphoketolase reaction (see Fig. 1.1.) The usual fate of acetylphosphate is its conversion to acetaldehyde and subsequently to ethanol. This is necessary for the reoxidation of the reduced coenzymes formed earlier. However, in the presence of mannitol dehydrogenase these reduced coenzymes can be reoxidised by the reduction of fructose to mannitol, instead. This negates the need for acetyl phosphate conversion to ethanol. The acetyl phosphate produced from the phosphoketolase reaction is then available for conversion to acetic acid by acetate kinase. The acetate kinase reaction liberates ATP. This results in two net ATPs being formed from each hexose fermented in the presence of mannitol dehydrogenase and excess fructose. This is of physiological importance, as heterofermentation would now be as efficient as homofermentation, producing two moles of ATP from one mole of hexose fermented instead of the usual one mole. Although fructose is the most common substrate available for reduction, other compounds in media or wine may be acted on by specific dehydrogenases. For example, free acetaldehyde may be used for coenzyme reoxidation and result in a similar build-up of acetyl phosphate and its conversion to acetic acid (Eltz and Vandemark, 1960).

From an oenological point of view, the production of acetic acid is detrimental, and even excessive mannitol formation may cause spoilage by increasing the sugar-free extract and the viscosity of the wine (Tracey and van Vuuren, 1988). Mannite spoilage (mannitol formation) is accompanied by excessive acetic acid formation (Dicks and van Vuuren, 1988). In media containing 5% and 10% ethanol, Tracey and van Vuuren (1988) found increased mannitol formation and increased volatile acidity in the lower ethanol medium, but only a slight increase in the 10% ethanol medium. They noted a significant positive correlation between the fructose consumed and the mannitol produced in the 5% ethanol medium, but no correlation at 10% ethanol. They did not relate this, however, to consumption of the glucose that was also present in the medium, or to acetate produced.

Methods for detecting mannitol have included crystallisation following washing with alcohol (Fornachon, 1964), thin layer chromatography (Chalfan *et al.*, 1975), GLC (Tracey and van Vuuren, 1988) and plate pouring and drying of broth cultures (Pilone *et al.*, 1991). Yamamaka and Sakai (1968) and Martinez *et al.* (1963) studied the enzyme mannitol dehydrogenase and its properties. The enzymes purified from *Lb. brevis* and *Lc. mesenteroides* exhibited a high degree of substrate specificity, uncommon to other hexitol dehydrogenases with NADH as the oxidising species. Fructose was essential to induce the enzyme in *Lb. brevis* – growth on glucose or sucrose did not. However, in *Lc. mesenteroides*, similar amounts of enzyme were produced from growth on fructose as well as glucose and sucrose as carbon sources. In aerobic cultures, mannitol itself induced mannitol dehydrogenase production in both *Lactobacillus* and *Leuconostoc* species (Yamamaka and Sakai, 1968). The enzyme is inactive for pentitols. NADH is required for fructose reduction, and there is a 20% reduction of activity with NADPH. The enzyme, however, is specific for NADH when oxidising mannitol to fructose. The scavenging of NADH by dehydrogenases causes acetic acid production in anaerobic environments. Some LAB contain NADH oxidases and peroxidases in their cytoplasm. These enzymes are responsible for interactions

between LAB and oxygen and their end-products are H_2O_2 and H_2O. In the presence of oxygen, these enzymes reoxidise NADH to NAD^+ and allow production of acetic acid and energy in a similar fashion to mannitol dehydrogenase activity (Condon, 1987). Other oxidases that may be present include pyruvate oxidase in some homofermentative species, and α-glycerophosphate oxidase in those LAB which can use glycerol as an oxygen dependent growth substrate. The accumulation of H_2O_2 and other toxic metabolites due to the action of oxidase enzymes may halt the growth of some aerobic cultures. Most LAB, however, possess NADH peroxidases to break down H_2O_2, but do not possess a true catalase.

Under both anaerobic or aerobic conditions, cultures of LAB have enzyme systems to increase ATP production from hexose fermentation by switching from ethanol to acetate production. It has been shown experimentally that the molar growth yield of *Lc. mesenteroides* growing aerobically on glucose is twice that of anaerobic cultures, and growth rates are also increased (Lucey and Condon, 1986). However, in strains of *Leuconostoc oenos* isolated from wine, Kelly *et al.* (1989) did not find growth rate stimulation under aerobic conditions. Growth rate, in fact, was stimulated in an anaerobic environment.

2.6.4 Carbohydrate utilisation

Studies on carbohydrate utilisation have been conducted in either a minimal medium with an added carbohydrate source, or in commercially available test kits. Garvie (1967) used a basal medium with added pH indicator and carbohydrate. The broths were incubated at 22°C for 21 days at initial pH 5.2. The study for this thesis project, however, uses a minimal medium of pH 5.5 and incubation at 30°C for 21 days. Growth was detected using optical density measurements.

There exist conflicting reports in the literature regarding the use of commercial carbohydrate test kits. The API 50 CH system has been used by Millière *et al.* (1989) for phenotypic characterisation of *Leuconostoc* spp. and by Davis *et al.* (1988) in a study of some 166 strains of wine LAB. Lafon-Lafourcade reported that the API 50 L system was quick, easy and reliable for classifying isolates from wine. On this basis, Tracey and Britz (1987) used the API 50 CH system in their study of 70 malolactic bacteria, but noted that they could not distinguish between the genera *Pediococcus* and *Leuconostoc*. They also could not confirm the results of Lafon-Lafourcade with respect to hexoses, but had good agreement with pentose sugars. Millière *et al.* (1989), using API 50 CH galleries, also conducted control experiments in the API 50 CH medium in test tubes (or plates) with 0.5% carbohydrate added. No comment was given on the variation in results between the two methods, nor was the test tube pH or the length of incubation stated. Pardo *et al.* (1988), in a technical brief, studied forty-two LAB in both API 50 CHL galleries and in test tubes. Tube tests were conducted in duplicate and test kits assayed twice according to manufacturer's instructions. The study was undertaken to determine if the test kit assay was in fact suitable for classification of *Lc. oenos*, after results obtained in preliminary studies were not in accordance with the literature. They reported that *Lc. oenos* showed restricted fermentation patterns in the API 50 CHL galleries. In this study, they included strain ML34, a malolactic strain used in this thesis research study. ML34 fermented only two carbohydrates in test galleries (esculin and fructose), while in test tubes ML34 fermented esculin, fructose, cellobiose, glucose, ribose, and trehalose. The culture medium for the API 50 CHL is similar to the commonly used lactic acid bacterial medium of de Mann, Rogosa and Sharpe (MRS), and is incubated for only 48 hours. The researchers increased the incubation time to 14 days, but did not find an increase in the number of substrates fermented. Sugars such as ribose were fermented in two to three days in test tubes, whereas 14 days in the test galleries showed no reaction. Overall, they concluded that the API 50 CHL system gave less accurate results than test tube methods for fermentation patterns of *Lc. oenos*. A survey of the literature shows

much variation in fermentation patterns, especially for *Lc. oenos*. It is recognised that *Lc. oenos* is not a homogeneous group (Davis *et al.*, 1988; Garvie, 1967). It is possible that fermentation patterns in lower pH media and in wines may bear little resemblance to those tested at higher pH levels, and may be further dependent on the physiochemical properties of the medium. Strains of *Lc. oenos* have been seen to lose the ability to ferment some carbohydrates on repeated subculturing (Beelman *et al.*, 1977). Benno and Mitsuoka (1983) compared the Minitek system with test tube methods and found that with modification, an 86% agreement could be attained between the two methods.

The testing of fermentation patterns of LAB has shown a range of results. The results depend on the system used, the length of incubation, and the pH at which the tests are conducted. It is common, therefore, that the literature contains conflicting conclusions as to the fermentative capabilities of LAB, especially *Lc. oenos* and the pediococci. It is difficult to speculate on the carbohydrate utilisation of LAB in wine because of the organisms' slow growth, the stress placed on the organisms by this harsh environment, and the undefined composition of wine. Thus, fermentation patterns derived from testing under set conditions are of only limited taxonomic and oenological use. They provide an abstract insight into the possible capabilities of the LAB genome.

In an applied approach, Davis *et al.* (1986) studied the behaviour of LAB in wines. The uninoculated wines were analysed for organic acid, hexose, pentose and amino acid content, and re-analysed after MLF had occurred. Surprisingly, they reported that the concentrations of glucose and fructose increased after MLF. They also found that arabinose and mannose concentrations decreased, while galactose, ribose, xylose, rhamnose, glycerol, myo-inositol and glucitol remained constant. Similar results were also recorded in control wines (pH 3.0) which showed no bacterial growth. They concluded that the growth of *Lc. oenos* during MLF was not accompanied by the

utilisation of any specific hexose or pentose. They speculated that residual enzymatic activity from grapes and yeast could be responsible for the observations. For example, residual sucrose in the wines might hydrolyse and liberate both glucose and fructose; trehalose hydrolysis would yield glucose; and the hydrolysis of phenolic glucosides to their monosaccharides might also occur. The utilisation of glucose and fructose by *Lc. oenos* still, however, remains a possibility since hexose generation could be at a faster rate than its utilisation. The decrease in mannose and arabinose was not due to MLF alone. Uninoculated wines also decreased in mannose and arabinose concentrations, after which some wines showed an increase in mannose concentration at later stages. This delayed increase was speculated to arise from the hydrolysis of mannan polysaccharide from yeast cell walls by autolysis. This study indicates that the changes in concentration of carbon sources in wines undergoing MLF may not necessarily be due exclusively to bacterial metabolism. This possibility further complicates any analysis of wines and the activities of LAB in them. These findings provide a new aspect to the debate on the timing of inoculation with LAB into young wines, because the theoretical metabolic activities and end products are unlikely to closely resemble those actually occurring in the wine.

3.0 MATERIALS AND METHODS

3.1 Basic Characteristics

3.1.1 Gram reactions

Gram staining method

The Gram staining method of Paik and Suggs (1974) was used with modification. A heat fixed smear was covered with crystal violet (0.2%) for 1 minute and washed briefly with iodine solution. The slide was then flooded with iodine solution and left to stand for 1 minute, then washed with water. Destaining was done using acetone-iodine solution by tilting the slide and dripping the solution onto the smear until the washings became colourless, and then rinsing with water. Counterstaining was done with safranin (1%) for 1 minute, washed off with water and blotted dry. Gram positive organisms stain blue, Gram negative organisms take up the counterstain and appear red. Iodine solution contains 6 g of ground iodine and 12 g of KI per litre stored in the dark. Acetone-iodine decolouriser consists of 3.5 mL of a strong iodine solution (6 g KI, 10 g ground iodine, 10 mL DI water, made up to 100 mL with 90% ethanol) and 96.5 mL of acetone.

KOH method

The KOH method used was that of Lin (1980). Single colonies were picked off agar using an inoculating loop, and mixed with one drop of freshly prepared 3% KOH on a glass slide. The emulsion was stirred gently for up to one minute and examined for ropiness by withdrawing the loop upwards and noting the viscosity. Gram negative bacteria become viscous, and when lifting the loop off the emulsion a thread of viscous

material (DNA) will be pulled up with it. A Gram negative bacterium, *Escherichia coli*, served a a positive control.

3.1.2. Catalase test

Testing for the presence of catalase enzyme involves placing a drop of hydrogen peroxide (3%) onto a glass slide containing a portion of a colony picked from an agar streak plate. After mixing, a coverslip is placed over the emulsion. This facilitates the observation of gas bubbles from the breakdown of H_2O_2 into oxygen and water in the presence of catalase (Collins and Lyne, 1985).

3.1.3 Colony and cell morphology

Strains were grown on VjG agar (section 3.2.2) for 7 to 10 days at 30°C and single colonies were examined using a stereomicroscope (Olympus, Japan) at low power (2x) for colony form, elevation and margin. Portions of colonies were picked off and wet mounts made and examined for cell morphology using a Zeiss compound microscope (model 4706-9901) under phase contrast at 400x magnification.

3.2 Media

3.2.1 Basal broth (BB)

Basal broth (BB) contains the following ingredients per litre: tryptone, 5 g; peptone, 5 g; yeast extract, 5 g (all Difco, Detroit); mineral solution, 1 mL; and Tween 80 solution (Difco), 1 mL. The ingredients were dissolved in 450 mL Milli-Q treated, deionised water (DI), and pH adjusted to 5.5 with 50% H_3PO_4 or 30% KOH. The solution was then made up to 500 mL only, to give 2-fold strength (2x BB) and stored frozen. Mineral solution contains 20 g $MgSO_4 \cdot 7H_2O$ and 5 g $MnSO_4 \cdot 4H_2O$ in 100 mL

DI water and stored frozen. Tween 80 solution is prepared as a 5 g per 100 mL solution in DI water, and also stored frozen.

3.2.2 Vegetable juice broth (VjG)

Vegetable juice serum was prepared from Campbell's V8 juice™ with no added sugar (Campbell's Soups, Australia). The juice was vacuum filtered through a 1 cm cake of diatomaceous earth (Kenite Diatomite 3000, WitcoCorp, N.Y.) supported by one sheet of filter paper (Whatman No. 1). Approximately 50 mL of diatomaceous earth was also added to 1 L of juice and mixed prior to filtering. Vegetable juice broth (VjG) contains per litre: 2x BB, 500 mL; glucose (BDH), 20 g; vegetable juice serum, 200 mL; and DI water, 250 mL. The ingredients were mixed and adjusted to pH 5.5 with dilute potassium hydroxide. The solution was made up to 1 litre with DI water and sterilised by autoclaving at 121°C for 15 minutes.

3.2.3 Heterofermentation-arginine broth (HFA)

HFA broth is a single broth culture containing both glucose and fructose, as well as L-arginine. It is used for testing biochemical characteristics. HFA contains the following ingredients per litre: tryptone, 5 g; yeast extract, 5 g; peptone, 5 g; glucose, 5 g; fructose (BDH), 20 g; L-arginine (Sigma), 6 g; 5% w/w Tween 80 solution, 1 mL; and vegetable juice serum (section 3.2.2), 200 mL. The pH was adjusted to 5.5, and 9.0 mL dispensed into culture tubes containing Durham tubes. For comparison, the Vaspar method (Pilone *et al.*, 1991) was also used to detect heterofermentation. Here, 1 part petroleum jelly and 6 parts paraffin were poured molten onto inoculated broths and allowed to solidify and seal the tubes.

3.3 Lactic Acid Bacterial Strains

Wine lactic acid bacteria (LAB) used in this study represent members of the genera *Leuconostoc, Pediococcus* and *Lactobacillus*. These belong to the Food and Fermentation laboratory culture collection of the Department of Microbiology and Genetics, Massey University, Palmerston North, New Zealand. Their original sources are listed in Table 3.1.

3.4 Culture Techniques

All strains were routinely cultured in VjG broth at 30°C. Subcultures were made in the same medium every seven days. It was noticeable that differences in growth rates between strains exist, especially in the species *Lc. oenos*. Strains were also stored at 4°C in stab cultures. Stab medium was MRS broth (Oxoid) supplemented with 20% vegetable juice serum (section 3.2.2) and 5 g/L fructose, at pH 5.5. This rich medium supported luxurious growth of all strains.

3.5 Tests For Metabolic Traits

3.5.1 Test for heterofermentation

Using HFA medium, the tests for heterofermentation, mannitol production from fructose, and ammonia production from arginine were made using a single broth culture. 9 mL of HFA broth was dispensed into 16 x 150 mm culture tubes containing inverted Durham tubes (9.5 x 50 mm). Bubbles trapped in the Durham tubes solubilise on autoclaving. Collection of gas in the Durham tubes is an indication of heterofermentation. Tubes were inoculated in duplicate with 100 µL of active culture from VjG broth cultures and incubated at 30°C for up to 3 weeks. Tubes were observed for gas collection in the Durham tubes periodically. Uninoculated HFA broth

Table 3.1 Wine lactic acid bacteria

Strain	Taxonomy	Comments
ML34	*Leuconostoc oenos*	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. Isolated from a Louis Martini Winery, Napa Valley, California red wine. Commercially available in liquid culture. [Ref: Pilone and Kunkee, 1972]
OENO	*Leuconostoc oenos*	Isolated from a bioactivated frozen culture of Microlife Technics, Sarasota, Florida, U.S.A. Commercially available in frozen culture.
PSU-1	*Leuconostoc oenos*	From R. B. Beelman, Pennsylvania State University, U.S.A. Isolated from a laboratory red wine. Commercially available in liquid culture. [Ref: Beelman *et al.*, 1977]
L181	*Leuconostoc oenos*	From J. Fornachon, Australian Wine Research Institute, Glen Osmond, South Australia, Australia from a 1966 experimental red wine. Used as a commercial starter culture in the 1960s. Currently a laboratory culture. Contains a temperate phage. [Ref: Pilone *et al.*, 1974]
MCW	*Leuconostoc oenos*	From M. Bannister, Vinquiry, Healdsburg, California, U.S.A. Isolated from Matanzas Creek Winery, Santa Rosa, California wine. Commercially available in freeze-dried culture.
Er1a	*Leuconostoc oenos*	From M. Daeschel, Dept Food Science and Technology, Oregon State University, Corvallis, Oregon, U.S.A. Isolated from Oregon Chardonnay wine. Commercially available in freeze-dried culture. [Ref: Izugabe *et al.*, 1985; Henick-Kling *et al.*, 1989]
Ey2d	*Leuconostoc oenos*	From M. Daeschel, Dept Food Science and Technology, Oregon State University, Corvallis, Oregon, U.S.A. Isolated from Oregon Chardonnay wine. Commercially available in freeze-dried culture. [Ref: Izugabe *et al.*, 1985; Henick-Kling *et al.*, 1989]
INO	*Leuconostoc oenos*	Isolated from a freeze-dried culture of INOBACTER from Institute Oenologique de Champagne, Epernay, France. Commercially available in freeze-dried culture.

Table 3.1 (cont'd)

Strain	Taxonomy	Comments
MB	*Leuconostoc oenos*	Isolated from a freeze-dried culture of Microenos B from Lacto-Labo, Dange, France. Commercially available in freeze-dried culture.
2001	*Leuconostoc oenos*	From Lallemand Inc., Montreal, Quebec, Canada. Peynaud strain (ATCC 23277), one of four strains comprising Lalvin Malolactic Starter Culture. Commercially available in mixed freeze-dried culture. [Ref: Champagne *et al.*, 1989]
2006	*Leuconostoc oenos*	From Lallemand Inc., Montreal, Quebec, Canada. PSU-1 strain, one of four strains comprising Lalvin Malolactic Starter Culture. Commercially available in mixed freeze-dried culture. [Ref: Champagne *et al.*, 1989]
2008	*Leuconostoc oenos*	From Lallemand Inc., Montreal, Quebec, Canada. Radler-3 strain, one of four strains comprising Lalvin Malolactic Starter Culture. Commercially available in mixed freeze-dried culture.
2035	*Leuconostoc oenos*	From Lallemand Inc., Montreal, Quebec, Canada. Australian strain, one of four strains comprising Lalvin Malolactic Starter Culture. Commercially available in mixed freeze-dried culture. [Ref: Champagne *et al.*, 1989]
2043	*Leuconostoc oenos*	Isolated from a freeze-dried culture from Lallemand Inc., Montreal, Quebec, Canada. Strain C.I.V.C. (Comité Interprofessionnel du Vin de Champagne). Commercially available in mixed freeze-dried culture. [Ref: Champagne *et al.*, 1989]
CUC-1	*Lactobacillus delbrueckii*	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. Isolated from a Cucamonga Vineyard Co., California 1964 red wine. Obligately homofermentative. A laboratory culture. [Ref: Pilone *et al.*, 1966]

Table 3.1 (cont'd)

Strain	Taxonomy	Comments
CH2	*Lactobacillus plantarum*	Isolated from a freeze-dried culture of Viniflora LP from C. Prahl, Chr. Hansen's Laboratorium A/S, Hørsholm, Denmark. Isolated from fermenting wine. Facultatively heterofermentative. Commercially available in freeze-dried culture. [Ref: Prahl, *et al.*, 1988]
CUC-3	*Lactobacillus buchneri*	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. Isolated from a Cucamonga Vineyard Co., California 1964 red wine. Obligately heterofermentative. A laboratory culture. [Ref: Pilone *et al.*, 1966]
ML30	*Lactobacillus brevis*	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. Isolated from a Livermore Valley, California red wine lees. Obligately heterofermentative. A laboratory culture. [Ref: Pilone *et al.*, 1966]
EQ	*Lactobacillus hilgardii*	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. EQUILAIT, a commercial malolactic culture from France. Obligately heterofermentative.
CUC-4	*Pediococcus* sp.	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. Isolated from a Cucamonga Vineyard Co., California, 1964 red wine. A laboratory culture. [Ref: Pilone *et al.*, 1966]
C5	*Pediococcus* sp.	From R. E. Kunkee, Dept Viticulture and Enology, University of California, Davis, U.S.A. Isolated from a U.C. Davis experimental red wine. A laboratory culture. [Ref: Pilone *et al.*, 1966]
44.40	*Pediococcus* sp.	Originally from a Sonoma Valley, California Chardonnay. This isolate is from a lyophilised culture obtained from Biologicals, Berkeley, California, U.S.A. Commercially available in freeze-dried culture. [Ref: Silver and Leighton, 1981]
XMP	*Pediococcus* sp.	Isolated as a contaminant from a freeze-dried mixed culture Microenos P from Lacto-Labo, Dange, France. A laboratory culture.

with Durham tubes served as controls. An alternative method for the detection of gas production was also used. Instead of using Durham tubes, tubes of HFA broth were inoculated and covered with 1 cm of molten Vaspar (section 3.2.3). Pressure from gas formation due to heterofermentation pushes the Vaspar plug up the tube and may expel the plug completely. Uninoculated controls were also sealed and incubated.

3.5.2. Test for ammonia production from arginine

After incubation and recording of heterofermentation (section 3.5.1), 1 mL of culture was transferred to a white tile spot plate. One to two drops of Nesslers reagent (BDH) were added. The formation of a brick orange colour immediately upon addition of the reagent is positive for the presence of ammonia. Uninoculated HFA medium served as a negative control.

The sensitivity of the ammonia test was determined in HFA broth and DI water with added ammonium nitrate. The highest possible level of ammonia from 6 g/L L-arginine is 1.17 g/L. HFA broth or DI water with added equivalent of 1.17 g/L ammonia was repeatedly diluted two-fold with HFA broth or DI water until detection was no longer possible.

3.5.3 Test for mannitol formation

After tests for heterofermentation and ammonia production were completed, the remaining HFA broth culture was poured into 90 mm open plastic Petri dishes and placed in a 37°C incubator for 2-3 days to dry. This was followed by a 2-3 day period at room temperature for mannitol crystal formation. Often, crystal formation did not occur until the final drying at room temperature. Mannitol crystals on the dried Petri dishes appear as large rosette crystals. Uninoculated HFA broth was used as control, as well as broth cultures where the amount of fructose was replaced with glucose.

3.6 Growth Studies

3.6.1 Low pH

VjG broth was supplemented with 0.2% L-malic acid (VjGM) as a buffer against further pH drop due to the production of lactic acid during bacterial growth. Decarboxylation of the malic acid by malolactic bacteria causes a counteracting increase in pH. The ability of strains to grow at initial pH levels of 4.8, 4.2, 3.7, 3.5 and 3.3 was tested. The medium was adjusted to the desired pH with dilute phosphoric acid and dispensed prior to autoclaving. The final pH of the medium after autoclaving changed by 0.02 units for pH 3.7 and 4.2. The lowest pH medium (3.3) showed a post-autoclaving pH change of 0.05 units. Cultures in early log to log phase were inoculated (1 loopful) in duplicate into 8 mL VjGM broth and incubated at 30°C for 3 weeks. Results were recorded as change in optical density (O.D.) due to growth, and were read on a Bausch and Lomb Spectronic 20 spectrophotometer at 600 nm wavelength (1 cm light path) against a DI water blank. Good growth often yielded high O.D. readings (>2.0 units). Standard curves were generated with a strain from each of the genera, *Lactobacillus*, *Pediococcus* and *Leuconostoc* to determine the dilutions necessary to obtain O.D. readings within the linear range of the instrument. Representatives of each genus were grown to a high density in VjG broth and diluted with VjG until a linear portion of the standard curve was found. All cultures were then diluted to within the linear portion with uninoculated medium. Results were recorded by the following equation:

$$OD_{growth} = [\text{dilution factor} \times OD_{culture}] - OD_{VjG}$$

3.6.2 Ethanol (10%, v/v)

VjG containing 10% (v/v) ethanol was prepared. VjG broth was made slightly concentrated by making the final volume up to only 900 mL. The broth was adjusted to

pH 5.5 and dispensed into 9 mL lots then autoclaved. When cool, 1.05 mL of 95% ethanol (AnalR, BDH) was added aseptically to each tube and mixed thoroughly by withdrawing and ejecting the broth several times with a sterile 5 mL autopipette. The tubes were inoculated in duplicate (1 loopful) from active subcultures and incubated at 30°C for 4 weeks. Results were recorded as change in optical density readings as described above (section 3.6.1).

3.6.3 Temperature

VjG broth (pH 5.5) was inoculated in duplicate from actively growing subcultures (1 loopful) and incubated for 4 weeks at the temperature designated. Due to browning of the medium at the higher temperatures, uninoculated medium was also incubated for 4 weeks at each temperature for use as diluent when reading results. This darker coloured, uninoculated medium provided for a more accurate estimation of growth from change in O.D. readings (section 3.6.1). Temperatures studied in this experiment were 15°, 30°, 35° and 40°C. At 40°C, growth could not always be determined by O.D. readings and visual turbidity observations were sometimes made, instead.

3.7 Carbon Sources

3.7.1 Media

Basal broth (BB) at pH 5.5 (section 3.2.1) was inoculated in duplicate with each strain. After incubation at 30°C for 2 weeks, growth was recorded as change in optical density (section 3.6.1). The O.D. of the BB culture minus the O.D. of the uninoculated BB medium gave the growth due to the nutrients in BB medium alone, with no added carbon source. To test for any increase in growth due to the test carbon sources, BB medium was prepared at pH 5.5 in 900 mL rather than 1 L, dispensed into 9 mL lots and autoclaved. To these, 1 mL of carbon source (see Table 4.7) was added

aseptically. All monosaccharides, disaccharides, trisaccharides, polyols, pentoses and most other carbon sources were dissolved as 5% solutions in DI water. Monosaccharide solutions were autoclaved, but all other solutions were filtered through sterile 0.2 μm cellulose nitrate filters (MSI, Westboro, MA) and dispensed aseptically. Substituted sugars, salicin and esculin, and gluconic acid were made up as 2% solutions and sterile filtered as above. Once aseptically added to the sterile BB concentrate, the final concentration of carbon sources was 0.5% and 0.2%. The tubes were then held at 25°C for two days as a final check on sterility before inoculating.

3.7.2 Inoculation and growth measurement

The carbon source media were inoculated with a loopful of culture from VjG broth. Growth on the added carbon source was measured as change in O.D. (section 3.6.1) after 3 weeks incubation at 30°C. Sterile BB medium was used as diluent where necessary. Growth due to the carbon source alone was determined by the equation:

$OD_{growth} = (OD_{CS} - OD_{UCS}) - (OD_{BB} - OD_{UBB})$, where

OD_{CS} = growth on carbon source

OD_{UCS} = OD of uninoculated carbon source broth

OD_{BB} = OD of growth on BB

OD_{UBB} = OD of uninoculated BB.

This equation takes into account colour changes due to carbon source addition to the BB broth (usually minimal), and the O.D. or growth attributed to the nutrients in the BB broth alone.

3.8 Degradation Of L-Malic And Citric Acids

3.8.1 Media

VjG broth was supplemented with 0.2% L-malic acid and 0.2% citric acid and was designated VjGMC. This was adjusted to pH 5.5 with 30% KOH and autoclaved.

3.8.2 Thin layer chromatography of acids

After inoculation in duplicate from VjG broth cultures and incubation at 30°C for 14 days, chromatography was used to detect the reduction in both the malate and citrate in the VjGMC broth cultures. The method of Stamer *et al.* (1983) was used with modification. To prepare the chromatography solvent, 100 mL of DI water was added to a separating funnel. To this, 0.25 g bromophenol blue (BDH) was added, instead of the 0.10 g originally used. 100 mL of normal butanol (BDH) was then added, followed by 10 mL of 98% formic acid (BDH). The resulting mixture was vigorously shaken for 2 minutes and allowed to stand undisturbed overnight.

The lower aqueous phase was discarded and the organic phase (yellow colour) was used as the chromatography solvent. TLC plastic sheets, 20 cm x 20 cm (EM Science, Cherry Hill, N.J.) with 0.1 mm layer of cellulose (no fluorescent indicator) were cut to 10 cm x 10 cm, and a line marked 1 cm from the base with a soft lead pencil. Along this baseline marks were made for spotting purposes at 1.5 cm intervals with a 0.5 cm margin on each edge. Care was taken not to touch the cellulose surface with fingers, and both ruler and scissors were wiped with 70% ethanol to remove oils. Samples were spotted using autopipette tips (Gilson) as dispensers. 10 µL of sample was loaded and the tip removed from the autopipette. The sample was dispensed by touching the tip briefly on the target mark, then drying it quickly with a hot air dryer to give a spot of 5-7 mm in diameter. Each sample was touched and dried twice on its

mark from the same pipette load. Once loaded the sheet was placed upright in 1-3 mm of solvent in a sealed jar and allowed to run until the solvent front reached 1 cm from the top (approximately 60 minutes). The sheet was then removed and dried in a fume hood. The acid spots appear yellow on a green background. Uninoculated VjGMC medium served as a control.

3.9 Mannitol And Acetic Acid Production

3.9.1 Media

Media used for fermentation studies were based on vegetable juice glucose broth (VjG) (section 3.2.2) with modifications made to pH, hexose additions and ethanol concentrations as described below:

M1 medium (VjF pH 5.5)

M1 medium was prepared as follows per litre: 2 x BB, 500 mL; vegetable juice serum, 200 mL; DI water, 150 mL. No carbon source was included at this stage. The pH of the solution was adjusted to pH 5.5 and made up to 900 mL, only. Then, 18 mL aliquots were dispensed into 20 x 150 mm test tubes and autoclaved at 121°C for 15 minutes. Fructose solution (20%) was autoclaved separately and 2 mL aliquots dispensed aseptically into the medium and mixed thoroughly by pipette.

M2 medium (VjF pH 3.8)

M2 medium was prepared as M1, but with the pH adjusted to 3.8.

M3 medium (VjF pH 5.5 + ethanol

M3 medium was prepared as follows per litre: 2 x BB, 500 mL; vegetable juice serum, 200 mL; DI water, 90 mL. No carbon source was included at this stage. The pH was adjusted to 5.5 and the solution made up to 795 mL, only. Medium was dispensed in 23.85 mL aliquots into 20 x 150 mm test tubes. Then, 3 mL of separately autoclaved fructose solution (20%) was added aseptically to each tube and mixed thoroughly by pipette. To each tube a further 3,15 mL of ethanol (95%, AnalR, BDH) was added and mixed thoroughly by pipette.

M4 medium (VjF pH 3.8 + ethanol)

M4 medium was prepared as for M3 medium, but the pH was adjusted to 3.8.

M5 medium (VjG pH 5.5)

M5 medium was prepared as described in section 3.2.2.

3.9.2 Inoculation and sample preparation

Media M1-M5 were inoculated in duplicate at 1% with early stationary phase cells from subcultures (VjG). After two weeks or when visible growth had occurred, cultures were vortexed and centrifuged at 5,000 rpm (5,000 x g) and the supernatant poured off and frozen at -20°C for HPLC analysis. Samples were thawed, mixed thoroughly and filtered through 0.2 µm cellulose nitrate filters into clean 5 mL sample vials. The samples were frozen until required, then thawed and mixed thoroughly by inverting several times, then analysed.

3.9.3 HPLC methods

Analysis of media M1-M5 after growth of the bacteria was conducted by High Performance Liquid Chromatography (HPLC) using ion exclusion techniques. Analyses were performed on a Shimadzu LC-4A HPLC and samples were injected using a SIL-2AS autosampler. Columns were maintained at the specified analysis temperatures by a CTO-2AS column oven. Peaks were detected on an RID-2AS Refractive Index (RI) detector and recorded on a C-R3A Chromatopac.

Two columns were used in this study: 1) Biorad HPX-87H (300 mm x 7.8 mm) protected by Biorad Microguard® cation H guard cartridge and used under the following operating conditions: column temperature 65°C, flow rate 0.8 mL/min, solvent 0.013N H_2SO_4, RI detector range 2. 2) Biorad HPX-87C (300 mm x 7.8 mm) protected by a Biorad Microguard carbo-C guard cartridge and operated under the following conditions: column temperature 85°C, flow rate 0.7 mL/min, solvent reagent grade Milli-Q water, RI detector range set at 1.

The Chromatopac was calibrated using high and low external standards to generate a two point calibration. Re-calibration was done at regular intervals throughout analysis between runs.

Calibration standards

Standards were made volumetrically from analytical grade reagents in Milli-Q treated water and filter sterilised (0.2 µm) and frozen until required. Several kinds of standards were prepared.

High standard: Fructose 20 g/L, Glucose 20 g/L,
Lactic acid 5 g/L, Acetic acid 5 g/L

Low standard: Fructose 1 g/L, Glucose 1 g/L
Lactic acid 0.5 g/L, Acetic acid 0.5 g/L

Initially, to establish retention times and to tentatively establish the resolution capabilities of the column, solutions of 10 g/L were made for each of glucose, fructose, mannitol, acetate and lactate. Other standards used for investigational rather than analytical purposes were "inverse" standards, where one component was at high concentration and the other at low concentration. These standards were:

"Inverse" standards:
1) Fructose 20 g/L, Mannitol 5 g/L
2) Fructose 10 g/L, Mannitol 10 g/L
3) Fructose 15 g/L, Mannitol 1 g/L

These standards were made to test fructose and mannitol at varying concentrations intended to simulate the decreasing fructose concentration as the mannitol concentration increased, as would be expected from growing organisms in the media.

"Wild"standards: Glucose 5 g/L, Fructose 5 g/L
Mannitol 5 g/L, Lactic acid 2 g/L
Acetic acid 1 g/L, Ethanol 10 g/L

"Wild" standards simulate a sample of a half grown culture in the presence of ethanol.

FAM standards:
FAM (high) Fructose 20 g/L, Acetic acid 5 g/L, Mannitol 10 g/L
FAM (low) Fructose 1 g/L, Acetic acid 0.5 g/L, Mannitol 0.5 g/L

FAM standards were used in the study of resolution capabilities of the HPX-87C column.

4.0 EXPERIMENTAL RESULTS

4.1 Characterisation

4.1.1 Basic characteristics

All 14 strains used in this project that were reported to be *Lc. oenos* were grown on VjG agar for seven days at 30°C and then colony and cell morphology examined. No easily identifiable morphologies were found to distinguish one strain from any other. The following strains can be described as having colonies that are small (1 mm or less), creamy white opalescent, convex, circular with entire edges; and cells of Gram positive and catalase negative coccobacilli: ML34, OENO, PSU-1, L181, MCW, Er1a, Ey2d, MB, INO, 2001, 2006, 2008, 2035 and 2043.

The strains of *Lactobacillus* used in this study represent five different species from the genus, so each is described separately after seven days at 30°C on VjG agar:

CUC-1: highly convex, opaque off-white colonies, >2mm in diameter, with entire edges, catalase negative, Gram positive bacilli

CH2: white shiny convex colonies, >2mm in diameter, with entire edges, catalase negative (no pseudocatalase observed), Gram positive bacilli

CUC-3: umbonate white colonies, up to 3 mm in diameter, with undulate margins, catalase negative, Gram positive bacilli

ML30: smooth, opaque small colonies, >1mm in diameter, convex, creamy white, catalase negative, Gram positive, short bacilli

EQ: large umbonate white colonies, >5 mm in diameter, with a 'fluffy' appearance and undulate margin, distinctive from other strains, catalase negative, Gram positive bacilli

The *Pediococcus* strains used in this study are from possibly more than one species.

CUC-4:	smooth regular colonies, >1 mm in diameter, off-white, catalase negative, Gram positive cocci forming tetrads
C5:	smooth regular colonies, >1 mm in diameter, off-white, catalase negative, Gram positive cocci forming tetrads
44.40:	smooth regular colonies, >1 mm in diameter, off-white, catalase negative, Gram positive cocci forming tetrads
XMP:	smooth white convex colonies, >2 mm in diameter, entire edges, cells larger than other strains of *Pediococcus*, catalase negative (no pseudocatalase observed), Gram positive cocci forming tetrads

4.1.2 Carbon dioxide production

All strains of *Leuconostoc oenos* produced carbon dioxide (CO_2) from growth on hexose in HFA broth (Table 4.2). Gas was produced by both Durham tube and vaspar detection techniques. Among the *Lactobacillus* strains tested (Table 4.2), strains CUC-1 and CH2 did not produce CO_2 from growth on hexose, while strains CUC-3, ML30 and EQ did evolve CO_2. No gas production was recorded for any of the *Pediococcus* strains tested.

4.1.3 Ammonia production from arginine

Results of tests on the sensitivity of Nessler's reagent in HFA broth and in water are presented in Table 4.1. Ammonia concentrations above 0.3 g/L were required for detection in HFA broth. In water, 0.012 g/L gave a clear positive result. *Leuconostoc oenos* strains were tested for ammonia production from arginine in HFA broth (Table 4.2). Four strains (OENO, Ey2d, 2035, 2043) tested positive and two more (INO, 2001) gave results of variable intensity. The remaining eight strains tested negative. Ammonia production by *Lactobacillus* strains was also tested (Table 4.2). The

Table 4.1 Ammonia detection levels with Nessler's reagent

	Dilution	NH_4^+ (g/L)	Reaction
HFA broth	none	1.172	orange
" "	2-fold [a]	0.586	yellow-orange
" "	4-fold	0.293	faint yellow-orange
" "	8-fold	0.147	faint yellow
" "	16-fold	0.073	faint yellow
" "	32-fold	0.037	faint yellow
" "	64-fold	0.018	faint yellow
" "	none	0.000	faint yellow
H_2O	none	1.1720	dark orange
"	10-fold [b]	0.1172	medium orange
"	100-fold	0.0117	light orange
"	1000-fold	0.0011	faint yellow orange
"	none	0.0000	colourless

[a] Diluted with HFA broth

[b] Diluted with H_2O

Table 4.2 Products from growth in HFA broth

Strain	CO_2	NH_4^+	Mannitol
Leuconostoc oenos			
ML34	+	-	+
OENO	+	+	+
PSU-1	+	-	+
L181	+	-	+
MCW	+	-	+
Er1a	+	-	+
Ey2d	+	+	+
INO	+	var [a]	+
MB	+	-	+
2001	+	var	+
2006	+	-	+
2008	+	-	+
2035	+	+	+
2043	+	+	+
Lactobacillus			
CUC-1	-	-	-
CH2	-	-	-
CUC-3	+	+	+
ML30	+	+	+
EQ	+	+	+
Pediococcus			
CUC-4	-	-	-
C5	-	-	-
44.40	-	-	-
XMP	-	+	-

[a] var, variable

homofermentative strains CUC-1 and CH2 did not produce ammonia, but the heterofermentative strains CUC-3, ML30 and EQ gave clearly positive results. Among the pediococci tested, only strain XMP produced ammonia.

4.1.4 Mannitol production

The formation of mannitol from growth in HFA broth was tested by crystallisation of broth cultures after growth. All 14 strains of *Lc. oenos* produced mannitol crystals upon drying, indicating fructose reduction to mannitol during growth (Table 4.2). *Lactobacillus* strains CUC-3, ML30 and EQ also tested positive for mannitol production. *Lactobacillus* strains CUC-1 and CH2, and all the *Pediococcus* strains tested negative.

4.1.5 Growth in the presence of 10% ethanol

Strains were grown in VjG containing 10% ethanol. Growth after 4 weeks at 30°C and pH 5.5 was often luxuriant, and dilutions were necessary to accurately measure optical density, O.D. (section 3.6.1). The results are shown in Table 4.3 and indicate a large variation among strains of the species *Lc. oenos,* and within the species of *Lactobacillus* and *Pediococcus*. Of the *Lc. oenos* strains, PSU-1 and 2035 grew to the highest O.D., while strain 2043 showed little growth after 4 weeks. Two strains of *Lactobacillus*, CUC-1 and CUC-3, showed considerably more growth than other members of the genus. Strain CH2 was notably affected by the presence of ethanol, as this organism grew luxuriantly in VjG without ethanol. Strain 44.40 gave an O.D. level considerably lower than other members of the genus *Pediococcus*. Strain XMP, which is not a commercial starter culture nor a characterised strain (see Table 3.1), also grew in the presence of 10% ethanol; and, along with C5, grew to the highest O.D. in the genus.

Table 4.3 Growth in the presence of 10% ethanol

Strain	Corrected OD_{600nm} [a]
Leuconostoc oenos	
ML34	0.76
OENO	0.67
PSU-1	1.11
L181	0.71
MCW	0.53
Er1a	0.86
Ey2d	0.56
MB	0.38
INO	0.38
2001	0.88
2006	0.83
2008	0.78
2035	1.35
2043	0.03
Lactobacillus	
CUC-1	2.11
CH2	0.23
CUC-3	1.31
ML30	0.45
EQ	0.33
Pediococcus	
CUC-4	1.08
C5	1.61
44.40	0.26
XMP	1.41

[a] corrected for OD of uninoculated broth (section 3.6.1)

4.1.6 Growth at pH levels in VjGM

Table 4.4 shows growth of strains after 3 weeks of incubation in VjG containing malic acid (VjGM) at initial pH levels of 4.8 to 3.3. Most strains grew equally well at pH 4.8 and 4.2, with a few having a slight preference for the lower pH level. *Leuconostoc oenos* strains never grew to high optical densities, even in high pH broths but appear to be relatively less affected from pH 4.8 to 3.3 than the lactobacilli. Most lactobacilli show about a 30-fold decrease in O.D. between high and low pH. Among strains of *Lc. oenos*, reasonable differences were seen at higher pHs, but at lower pHs more pertinent to wine environments, general differences between strains were less. Two strains, INO and 2043, are notable for low growth at pH 3.7 to 3.3. Although reasonable growth was attained at higher pHs, growth of these two strains was always less than that of most other strains. Strain Ey2d was unusual, as at pH 3.5 and 3.3 growth was comparable to other strains of *Lc. oenos* but at higher pHs growth was less than that of other strains. Strains 2001 and 2008 generally attained the highest O.D. across the range of pH. All strains of *Lactobacillus* grew to high O.D. at high pHs but showed a large drop from pH 4.2 to 3.7. The lactobacilli show reasonable uniformity in their response to low pH. This is surprising given the diversity of species within the genus. At pH 3.3 strains CH2 and CUC-3 showed the most growth, with O.D. levels twice those of ML30 and EQ. Within the *Pediococcus* strains tested, XMP grew to a higher O.D. in high pH media, but the growth of this strain resembled that of the other members of the genus at low pH.

4.1.7 Growth response to temperature

The corrected optical densities for all strains grown in VjG at various temperatures for 4 weeks are presented in Table 4.5. Generally strains of *Leuconostoc oenos* grew to higher optical densities (O.D.) after 4 weeks at 30°C than they did at 35°C or 15°C. A notable exception is strain INO, which preferred 15°C. Four strains of *Lc. oenos*

Table 4.4 Growth at pH levels in VjGM

Strain	Corrected OD_{600nm} [a]				
Initial pH	4.8	4.2	3.7	3.5	3.3
Leuconostoc oenos					
ML34	3.43	3.89	2.02	0.93	0.73
OENO	4.73	4.09	3.07	1.58	0.81
PSU-1	3.83	3.44	1.72	1.28	0.83
L181	4.63	4.89	2.47	1.63	0.60
MCW	3.53	3.44	1.42	0.96	0.49
Er1a	3.43	3.44	2.22	0.85	0.63
Ey2d	1.26	1.44	1.52	0.83	0.69
MB	3.88	3.74	2.02	1.23	0.56
INO	1.93	0.84	0.29	0.28	0.26
2001	5.33	5.34	4.12	1.53	0.86
2006	4.53	4.54	3.12	1.28	0.75
2008	5.33	5.29	4.22	1.63	0.72
2035	3.13	2.94	2.07	0.95	0.48
2043	2.53	1.49	0.46	0.46	0.20
Lactobacillus					
CUC-1	35.50	45.90	5.57	1.58	0.93
CH2	38.93	38.94	5.17	1.88	1.43
CUC-3	34.43	29.94	4.17	1.83	1.43
ML30	32.43	25.94	3.77	1.13	0.74
EQ	27.43	28.44	3.72	1.48	0.89
Pediococcus					
CUC-4	1.38	1.89	1.77	0.33	0.26
C5	1.65	1.84	1.87	0.56	0.25
44.40	2.23	2.09	1.62	0.59	0.24
XMP	6.18	5.54	3.42	0.29	0.27

[a] corrected for OD of uninoculated broth (section 3.6.1)

Table 4.5 Growth response to temperature in VjG

Strain	Corrected OD_{600nm} [a]			
Temperature (°C)	15	30	35	40
Leuconostoc oenos				
ML34	1.17	1.15	0.62	-[b]
OENO	2.29	5.10	0.78	0.37
PSU-1	0.77	1.15	0.63	-
L181	2.15	4.90	0.60	0.30
MCW	0.92	2.90	0.17	-
Er1a	0.89	4.10	0.25	-
Ey2d	0.77	1.05	0.21	-
MB	2.07	4.20	0.37	-
INO	0.57	0.18	0	-
2001	3.87	6.10	1.33	0.37
2006	3.67	4.80	0.31	-
2008	4.20	5.70	0.98	0.09
2035	1.07	2.80	0.26	-
2043	0.08	2.00	0	-
Lactobacillus				
CUC-1	5.27	50.90	67.90	+
CH2	5.27	34.90	56.90	+
CUC-3	3.87	32.90	47.40	+
ML30	2.22	23.90	26.90	-
EQ	3.17	28.90	43.40	-
Pediococcus				
CUC-4	1.79	1.80	2.25	-
C5	1.85	1.40	11.20	-
44.40	1.87	2.00	2.90	-
XMP	5.07	6.70	44.90	+

[a] corrected for OD of uninoculated broth (section 3.6.1)

[b] + or -, visual recording of growth (section 3.6.3)

grew at 40°C, but only to low O.D. levels. *Lactobacillus* strains grew luxuriantly at higher temperatures, but only three strains grew at 40°C – ML30 and EQ did not. A large difference in growth is seen between 15°C and 30°C for all the lactobacilli. Growth at 15°C, 30°C and 35°C among the pediococci was relatively constant compared with that of the leuconostocs and the lactobacilli, with the exception of XMP which grew to high O.D. at 35°C and also grew at 40°C.

4.1.8 Utilisation of malic and citric acids

The ability to dissimilate citric and malic acids in the presence of glucose was examined, and the results are shown in Table 4.6. L-malic acid was degraded by all strains of *Lc. oenos*, *Lactobacillus* and *Pediococcus*, concurrent with the appearance of a large lactic acid spot as observed by thin layer chromatography (section 3.8.2). Citric acid degradation was complete for all strains of *Lc. oenos*, and only in *Lactobacillus* strain CH2. Other lactobacilli gave a reduced citric acid spot over that of the uninoculated medium, indicating that degradation was incomplete. One strain, *Lactobacillus* EQ, did not seem to degrade citric acid, as the acid spot was similar in intensity to that of the uninoculated control. *Pediococcus* strains CUC-4, C5 and 44.40 did not degrade the acid, while strain XMP showed some degradation.

4.1.9 Growth on carbon sources

Growth responses of the wine LAB to carbon sources in basal broth (BB) are given in Tables 4.7 to 4.9.

Leuconostoc oenos

Glucose served as a fermentable carbon source for all strains of *Lc. oenos*. Strains OENO and L181 grew strongly on glucose, but Er1a, Ey2d, MB, INO, 2035 and 2043

Table 4.6 Malic and Citric Acid Degradation

Strain	Malic Acid	Citric Acid
Leuconostoc oenos		
ML34	+	+
Oeno	+	+
PSU-1	+	+
L181	+	+
MCW	+	+
Er1a	+	+
Ey2d	+	+
INO	+	+
MB	+	+
2001	+	+
2006	+	+
2008	+	+
2035	+	+
2043	+	+
Lactobacillus		
CUC-1	+	± [a]
CH2	+	+
CUC-3	+	±
ML30	+	±
EQ	+	-
Pediococcus		
CUC-4	+	-
C5	+	-
44.40	+	-
XMP	+	±

[a] ±, partial utilisation

Table 4.7 Growth of *Leuconostoc oenos* strains on carbon sources

	Corrected OD[a] and interpretation[b]													
Leuconostoc oenos strains	ML34		OENO		PSU-1		L181		MCW		Er1a		Ey2d	
Sugars														
D-Glucose	.180	+	.79	+	.025	+	.435	+	.015	+	.01	sl	.005	sl
D-Fructose	.825	+	.695	+	1.01	+	.435	+	.455	+	.60	+	.505	+
D-Galactose	-.035	-	.038	+	-.03	-	-.015	-	-.005	-	-.005	-	-.01	-
D-Mannose	.025	+	.19	+	.033	+	.29	+	-.01	-	0	-	-.01	-
L-Sorbose	-.005	-	-.01	-	-.025	-	-.035	-	-.005	-	0	-	-.005	-
L-Fucose	-.008	-	-.003	-	-.033	-	-.033	-	-.033	-	-.008	-	-.008	-
L-Rhamnose	.010	sl	.005	sl	0	-	-.025	-	-.005	-	-.003	-	-.005	-
Lactose	-.030	-	-.015	-	-.010	-	-.01	-	.008	sl	.008	sl	0	-
Maltose	0	-	.03	+	.01	sl	-.017	-	-.007	-	-.005	-	-.008	-
Sucrose	-.025	-	.015	+	-.058	-	-.022	-	-.015	-	-.005	-	-.012	-
Cellobiose	-.058	-	.035	+	-.045	-	-.035	-	-.012	-	-.015	-	-.018	-
Melibiose	0	-	.225	+	.01	sl	.095	+	0	-	0	-	-.02	-
Trehalose	.095	+	.635	+	-.035	-	.10	+	-.008	-	-.012	-	-.02	-
Raffinose	-.010	-	.045	+	-.04	-	-.015	-	-.008	-	-.005	-	-.025	-
Melezitose	-.025	-	-.01	-	0	-	-.01	-	-.01	-	-.005	-	-.02	-
Substituted sugars														
Amygdalin	-.040	-	.06	+	-.045	-	-.02	-	-.01	-	.005	sl	-.015	-
Salicin	-.030	-	.52	+	-.065	-	-.04	-	-.025	-	-.02	-	-.04	-
Esculin	.060	+	.295	+	.11	+	.055	+	.055	+	.055	+	.05	+
Pentoses														
D-Arabinose	-.032	-	-.012	-	-.04	-	-.045	-	-.07	-	-.005	-	-.012	-
D-Ribose	.405	+	.605	+	.585	+	.365	+	.34	+	.455	+	.475	+
D-Xylose	.405	+	.010	sl	-.005	-	-.013	-	0	-	.370	+	.005	sl
Sugar alcohols														
Glycerol	-.015	-	-.02	-	-.045	-	-.035	-	-.005	-	-.015	-	-.017	-
D-Mannitol	-.060	-	.01	sl	-.05	-	-.015	-	0	-	-.007	-	-.012	-
D-Sorbitol	-.035	-	.008	sl	-.05	-	.10	+	0	-	.003	sl	-.007	-
Acids														
D-Gluconic acid	.095	+	.09	+	.035	+	.005	sl	0	-	-.005	-	-.01	-

[a] corrected for growth on BB (section 3.7.2)

[b] interpretation: >0.01 = +, >0-0.01 = slight (sl), 0 and <0 = -

Table 4.7 (cont'd)

Leuconostoc oenos strains	Corrected OD[a] and interpretation[b]													
	MB		INO		2001		2006		2008		2035		2043	
Sugars														
D-Glucose	.005	sl	.003	sl	.029	+	.07	+	.06	+	.005	sl	.008	sl
D-Fructose	.61	+	.08	+	.92	+	.815	+	.795	+	.36	+	.39	+
D-Galactose	-.025	-	-.005	-	-.005	-	.05	+	.03	+	-.005	-	-.005	-
D-Mannose	-.015	-	-.015	-	.125	+	.22	+	.17	+	-.005	-	-.01	-
L-Sorbose	-.025	-	-.005	-	.025	+	.035	+	.013	+	-.007	-	-.005	-
L-Fucose	-.033	-	-.003	-	.842	+	.075	+	.052	+	-.003	-	.005	sl
L-Rhamnose	-.01	-	0	-	.025	+	.035	+	.025	+	-.003	-	-.005	-
Lactose	-.02	-	.005	sl	.045	+	.05	+	.03	+	.005	sl	.02	+
Maltose	-.02	-	0	-	.025	+	.055	+	.045	+	.005	sl	0	-
Sucrose	-.028	-	-.005	-	0	-	-.015	-	.03	+	-.002	-	-.005	-
Cellobiose	-.035	-	-.01	-	.065	+	.075	+	.05	+	-.012	-	-.005	-
Melibiose	-.04	-	0	-	.115	+	-.03	-	.035	+	-.01	-	0	-
Trehalose	-.04	-	-.022	-	.055	+	.675	+	.53	+	-.012	-	-.002	-
Raffinose	-.025	-	-.005	-	.055	+	.05	+	.07	+	-.005	-	0	-
Melezitose	-.03	-	-.01	-	-.025	-	.05	+	.035	+	.005	sl	0	-
Substituted sugars														
Amygdalin	-.037	-	-.005	-	.03	+	.01	sl	.035	+	.005	sl	.007	sl
Salicin	-.05	-	-.025	-	.625	+	-.02	-	.24	+	-.02	-	-.02	-
Esculin	.015	+	.075	+	.445	+	.250	+	.235	+	.105	+	.125	+
Pentoses														
D-Arabinose	-.03	-	-.01	-	-.02	-	.01	sl	.01	sl	-.07	-	.005	sl
D-Ribose	.425	+	.065	+	.905	+	.91	+	.87	+	.295	+	.115	+
D-Xylose	-.01	-	0	-	.03	+	.045	+	.05	+	0	-	0	-
Sugar alcohols														
Glycerol	-.03	-	-.01	-	-.015	-	.03	+	.02	+	-.01	-	-.005	-
D-Mannitol	-.035	-	-.005	-	.845	+	-.025	-	-.005	-	-.01	-	-.002	-
D-Sorbitol	-.03	-	-.002	-	.03	+	.013	+	.065	+	-.002	-	-.007	-
Acids														
D-Gluconic acid	.185	+	-.002	-	.05	+	.51	+	.470	+	0	-	-.007	-

[a] corrected for growth on BB (section 3.7.2)

[b] interpretation: >0.01 = +, >0-0.01 = slight (sl), 0 and <0 = -

Table 4.8 Growth of *Lactobacillus* strains on carbon sources

	Corrected OD[a] and interpretation[b]									
Lactobacillus strains	CUC-1		CH2		CUC-3		ML30		EQ	
Sugars										
D-Glucose	-.015	-	.03	+	.31	+	.555	+	.94	+
D-Fructose	>1.5	+	>1.5	+	.825	+	.735	+	.884	+
D-Galactose	1.67	+	1.395	+	-.035	-	.08	+	.450	+
D-Mannose	1.62	+	1.390	+	-.05	-	.28	+	.07	+
L-Sorbose	-.02	-	-.100	-	-.035	-	.045	+	.03	+
L-Fucose	.007	sl	.087	+	-.035	-	.032	+	.007	sl
L-Rhamnose	.013	+	.055	+	-.02	-	.04	+	.03	+
Lactose	-.005	-	1.255	+	-.04	-	.075	+	.37	+
Maltose	.905	+	1.30	+	1.25	+	.08	+	1.23	+
Sucrose	.725	+	1.145	+	-.01	-	.075	+	1.45	+
Cellobiose	1.68	+	1.545	+	-.02	-	.21	+	.215	+
Melibiose	-.025	-	1.19	+	-.05	-	.135	+	.785	+
Trehalose	1.63	+	1.195	+	-.065	-	.645	+	.01	sl
Raffinose	-.015	-	1.245	+	-.04	-	.045	+	-.035	-
Melezitose	1.57	+	1.24	+	-.06	-	.065	+	.570	+
Substituted sugars										
Amygdalin	1.38	+	1.25	+	-.035	-	.055	+	.045	+
Salicin	1.46	+	1.23	+	-.045	-	.310	+	0	-
Esculin	1.35	+	.865	+	.02	+	.285	+	.08	+
Pentoses										
D-Arabinose	-.09	-	.06	+	.003	sl	.10	+	.01	sl
D-Ribose	1.225	+	1.045	+	.945	+	.515	+	.975	+
D-Xylose	0	-	.035	+	1.05	+	.400	+	.780	+
Sugar alcohols										
Glycerol	-.005	-	.20	+	-.055	-	.035	+	.04	+
D-Mannitol	.930	+	.15	+	-.045	-	.07	+	.08	+
D-Sorbitol	.025	+	—		-.02	-	.065	+	.04	+
Acids										
D-Gluconic acid	.44	+	-.23	-	.560	+	.175	+	.38	+

[a] corrected for growth on BB (section 3.7.2)

[b] interpretation: >0.01 = +, >0-0.01 = slight (sl), 0 and <0 = -

Table 4.9 Growth of *Pediococcus* strains on carbon sources

	Corrected OD[a] and interpretation[b]							
Pediococcus strains	CUC-4		C5		44.40		XMP	
Sugars								
D-Glucose	.72	+	.535	+	.755	+	1.59	+
D-Fructose	.52	+	.635	+	.68	+	1.28	+
D-Galactose	-.015	-	-.025	-	-.03	-	1.38	+
D-Mannose	.575	+	.625	+	-.16	-	-.18	-
L-Sorbose	-.03	-	-.03	-	-.06	-	-.08	-
L-Fucose	-.03	-	-.023	-	-.048	-	-.163	-
L-Rhamnose	.165	+	.13	+	.17	+	.93	+
Lactose	-.025	-	-.015	-	-.03	-	1.435	+
Maltose	.49	+	.535	+	.285	+	.18	+
Sucrose	-.015	-	-.005	-	-.04	-	1.525	+
Cellobiose	.465	+	.68	+	-.188	-	1.425	+
Melibiose	-.035	-	-.04	-	-.045	-	.137	+
Trehalose	.475	+	.47	+	.475	+	1.475	+
Raffinose	0	-	-.03	-	-.045	-	.775	+
Melezitose	-.035	-	-.03	-	-.075	-	-.01	-
Substituted sugars								
Amygdalin	.395	+	.560	+	.555	+	1.28	+
Salicin	.610	+	.69	+	.705	+	1.36	+
Esculin	.935	+	.365	+	.515	+	1.145	+
Pentoses								
D-Arabinose	-.03	-	-.025	-	-.045	-	.665	+
D-Ribose	-.005	-	0	-	0	-	1.775	+
D-Xylose	-.02	-	-.015	-	-.025	-	.005	sl
Sugar alcohols								
Glycerol	-.035	-	-.025	-	-.04	-	—	
D-Mannitol	-.025	-	-.025	-	-.045	-	-.01	-
D-Sorbitol	-.015	-	-.02	-	-.03	-	.03	+
Acids								
D-Gluconic acid	-.05	-	-.042	-	-.05	-	—	

[a] corrected for growth on BB (section 3.7.2)

[b] interpretation: >0.01 = +, >0-0.01 = slight (sl), 0 and <0 = -

showed only slight increases in growth over that recorded in BB without added glucose. Fructose was fermented by all *Lc. oenos* strains and gave rise to the highest O.D. recorded for most strains. Only strains 2006, 2008 and OENO fermented galactose, but not to high O.D. levels. Half of all strains utilised mannose to some extent (ML34, OENO, PSU-1, L181, 2001, 2006, 2008), while three strains (2001, 2006, 2008) were able to use sorbose. Of the three strains using fucose (2001, 2006, 2008), 2001 attained a notably high O.D. level, similar to that obtained with fructose and ribose, whereas 2043 grew only slightly. Five strains fermented rhamnose to some extent (ML34, OENO, 2001, 2006, 2008), but of these ML34 and OENO were only stimulated slightly. Three strains grew to reasonable O.D. levels in the presence of lactose (2001, 2006, 2008), while four strains (2035, INO, MCW, Er1a) were slightly stimulated. Maltose encouraged growth for 2001, 2006, 2008 and OENO, and slightly encouraged growth for PSU-1 and 2035, while sucrose was fermented only by 2008. The addition of cellobiose to BB encouraged growth in four strains (2001, 2006, 2009, OENO), while melibiose was also fermented by four strains (OENO, L181, 2001, 2008), one of which (OENO) produced the highest change in O.D. levels for any carbon source tested. PSU-1 was also slightly stimulated by melibiose. Trehalose stimulated five strains strongly (ML34, OENO, L181, 2006, 2008), and 2001 to an intermediate extent. Of the trisaccharides, raffinose had an intermediate effect on four strains (OENO, 2001, 2006, 2008), but melezitose was fermented by 2006 and 2008, and slightly by 2035.

Of the phenolic glycosides (substituted sugars), amygdalin served as a carbon source to some extent for 2001, 2008 and OENO, and slightly for four others (Er1a, 2006, 2008, 2043). Salicin was also stimulatory, again, to 2001, 2008 and OENO, all of which attained reasonable changes in O.D. levels. Esculin was a useful carbon source for all 14 strains of *Lc. oenos* tested.

Pentoses showed a range of results with arabinose slightly stimulating only 3 strains (2043, 2006, 2008). Ribose was a preferred carbon source for all 14 strains, producing changes in O.D. levels second only to fructose. Two strains, 2006 and 2008, preferred ribose to fructose. Xylose slightly stimulated Ey2d and OENO, while 2001, 2006 and 2008 grew well. ML34 equally prefers xylose to ribose, while Er1a gave similar O.D. readings to ribose and fructose. Of the sugar alcohols, glycerol stimulated growth of 2006 and 2008, but not greatly. Mannitol stimulated 2001 and yielded an O.D. level similar to fructose and ribose. Mannitol also slightly stimulated strain OENO. Sorbitol encouraged growth in strains 2001, 2006, 2008 and L181, and slightly in strains Er1a and OENO. Gluconic acid was a carbon source for seven strains (ML34, OENO, PSU-1, MB, 2001, 2006, 2008) and slightly stimulated strain L181.

Lactobacillus species

Glucose was utilised by *Lactobacillus* strains CUC-3, ML30 and EQ with growth to high levels. However, strain CH2 did not grow to high levels, and strain CUC-1 was not stimulated at all by the presence of glucose in the basal broth. Fructose proved to be a good growth substrate for all five strains, and high O.D. levels were recorded. Galactose was fermented by CUC-1, CH2 and EQ, but only weakly by ML30, while CUC-3 was not stimulated at all. Similarly, mannose was not fermented by CUC-3, weakly by EQ, and strongly by the remaining strains. Sorbose was fermented only by ML30 and EQ. Fucose was fermented by CH2 and ML30, slightly by CUC-1 and EQ, but not by CUC-3. Rhamnose was fermented to some extent by all strains except CUC-3. Lactose did not prove stimulatory to CUC-1 or CUC-3, while maltose strongly enhanced the growth of all five strains. Sucrose and cellobiose were strongly fermented by all strains except CUC-3. Melibiose was strongly fermented by EQ, ML30 and CH2. Trehalose proved non-stimulatory to CUC-3, and only slightly so to EQ, but other strains fermented it strongly. Trisaccharides such as raffinose were

fermented by CH2 and ML30, while melezitose was a suitable carbon source for all strains except CUC-3. Esculin served as a carbon source for all strains. Amygdalin and salicin were not fermented by CUC-3, and salicin was not fermented by EQ. The lactobacilli grew poorly on arabinose, with only CH2 and ML30 showing some growth. CUC-1 was not stimulated at all. Ribose was a favoured carbon source for all strains, producing O.D. levels greater than or similar to glucose and fructose. Xylose generally supported good growth except for strain CUC-1. Glycerol served as a carbon source for CH2, ML30 and EQ, while mannitol was strongly fermented by CUC-1, and to a lesser extent by CH2, ML30 and EQ. Sorbitol was fermented weakly by three strains, CUC-1, ML30 and EQ. Gluconic acid was not stimulatory to CH2, but all other strains were able to use it as a carbon source.

Pediococcus species

All strains of *Pediococcus* ferment glucose and fructose, with strain XMP always giving the highest O.D. response. Strain XMP was also the only strain to utilise galactose, lactose, sucrose, melibiose, raffinose, arabinose, ribose and sorbitol. CUC-4 and C5 fermented mannose, while none of the strains fermented sorbose or fucose. Rhamnose was fermented by all strains to varying degrees, as was maltose. CUC-4, C5 and XMP fermented cellobiose, while all strains fermented trehalose, and none melezitose. The substituted sugars amygdalin, salicin and esculin were carbon sources for all strains, and were fermented strongly. XMP was the only strain able to use pentose sugars as carbon sources, with ribose being strongly fermented and xylose only slightly. Gluconic acid was not fermented by CUC-4, C5 or 44.40.

4.2 Acetic Acid Production

Data in Tables 4.10 to 4.13 were drawn only from HPLC analysis runs where the repeatability of external standards was 10% or better.

Table 4.10 Comparison of growth products in M1 and M2 media[a]

	M1 (VjF, pH 5.5)					M2 (VjF, pH 3.8)				
	Lactic	Acetic	Ratio A/L	Glucose	Fructose	Lactic	Acetic	Ratio A/L	Glucose	Fructose
Uninoculated	0[b]	0		2.52	26.20	0	0		2.64	24.50
Leuconostoc oenos										
PSU-1	5.28	2.69	.51			4.57	2.99	.65	0	
	5.29	2.74	.52	0	0.72	3.29	2.06	.63	0	7.80
OENO	6.75	3.14	.47	0		1.79	1.27	.71	0	
	6.55	3.09	.47	0.04		5.77	3.56	.62	0	
Ey2d	5.07	3.42	.67	0.60		4.38	2.40	.55	0	
						4.64	3.07	.66	0.23	
INO	2.66	1.78	.67	0.90		1.36	1.10	.81	1.96	
	3.93	2.56	.65	0.47						
Lactobacillus spp										
CUC-1	20.50	0.30	.01	0.27		15.08	0.22	.01	0	
	20.17	0.33	.02	0.20		14.23	0.22	.02	0	
CUC-3	6.69	3.57	.53	0		5.34	3.21	.60	3.13	
	4.70	3.41	.73	0.06		5.48	3.27	.60	3.01	
EQ	6.64	2.75	.41	0		5.90	2.33	.39	0	
	7.62	3.11	.41	0.12		6.23	2.42	.39	0	
Pediococcus spp										
CUC-4	3.75	0		0.48		1.50	0		1.76	
	4.10	0		0.58		1.23	0		1.84	
44.40	4.73	0		0.35		1.97	0		1.79	
	8.48	0.15	.02	0.33		1.35	0		1.79	
C5	3.80	0		0.46		2.30	0		0.29	
	2.69	0		0.31		2.30	0		0	
XMP	9.79	0		0		5.17	0		0	
	9.99	0		0.04		5.91	0		0	

[a] products in g/L

[b] 0, none detected

Table 4.11 Comparison of growth products in M1 and M5 media[a]

	M1 (VjF, pH 5.5)					M5 (VjG, pH 5.5)				
	Lactic	Acetic	Ratio A/L	Glucose	Fructose	Lactic	Acetic	Ratio A/L	Glucose	Fructose
Uninoculated	0[b]	0		2.52	26.20	0	0		25.5	3.80
Leuconostoc oenos										
ML34	5.00	2.95	.59	0		7.07	1.07	.15	0	
						7.23	1.10	.15	0	
L181	6.43	3.68	.57	0		6.18	1.11	.18	0	
						6.83	1.23	.18	0	
PSU-1	5.28	2.69	.51			6.62	0.93	.14		
	5.29	2.74	.52	0	0.72	6.76	0.73	.11		
OENO	6.75	3.14	.47	0		6.24	1.30	.21	0	
	6.55	3.09	.47	0.04						
MCW	3.52	2.51	.71	0.38		5.89	1.33	.23	0	
	3.64	2.58	.71	0.39		6.03	1.54	.26	0	
Er1a	5.38	2.72	.51	0.08		6.65	1.56	.23	0	
	0.13	0		2.54						
Ey2d	5.07	3.42	.67	0.60		2.51	2.05	.82	5.90	
INO	2.66	1.78	.67	0.90		1.38	0.81	.59	11.78	
	3.93	2.56	.65	0.47		1.46	0.95	.65	12.43	
Lactobacillus spp										
CUC-1	20.50	0.30	.01	0.27		17.62	0.27	.02	9.54	
	20.17	0.33	.02	0.20						
CH2	0.08	0		2.87		13.89	0.37	.03	1.6	
	0.10	0		2.75		14.14	0.39	.03	1.3	
CUC-3	4.70	3.41	.73	0.06		11.24	2.99	.27	0	
	6.70	3.57	.53	0		9.07	2.14	.24	0.20	
EQ	6.64	2.75	.41	0		10.59	1.69	.16	0.24	
	7.62	3.11	.41	0.12		12.41	1.86	.15	0.22	
Pediococcus spp										
44.40	4.73	0		0.35		9.54	0		23.36	
	8.48	0.15	.02	0.33		9.24	0		20.31	
C5	3.80	0		0.46		7.91	0		7.92	
	2.69	0		0.31		7.94	0		8.58	
XMP	9.79	0		0		11.85	0		3.03	
	9.99	0		0.04		12.61	0		3.30	

[a] products in g/L

[b] 0, none detected

Table 4.12 Comparison of growth products in M1 and M3 media[a]

	M1 (VjF, pH 5.5)					M3 (VjF, pH 5.5+10% ethanol)				
	Lactic	Acetic	Ratio A/L	Glucose	Fructose	Lactic	Acetic	Ratio A/L	Glucose	Fructose
Uninoculated	0[b]	0		2.52	26.20	0.1	0		2.59	28.12
Leuconostoc oenos										
ML34	5.00	2.95	.59	0		1.52	1.10	.72	1.14	
						3.18	2.65	.83	0.92	
PSU-1	5.28	2.69	.51			2.34	1.74	.74	0	
	5.29	2.74	.52	0	0.72					
OENO	6.75	3.14	.47	0		2.42	1.76	.73	0	
	6.55	3.09	.47	0.04		2.00	1.46	.73	0	
MCW	3.52	2.51	.71	0.38		1.57	1.20	.76	0	
	3.64	2.58	.71	0.39		1.73	1.26	.73	0.06	
Er1a	5.38	2.72	.51	0.08		3.17	2.29	.72	0.08	
	0.13	0		2.54		2.05	1.50	.73	0.24	
INO	2.66	1.78	.67	0.90		2.01	1.53	.76	1.91	
	3.93	2.56	.65	0.47		1.97	1.53	.78	2.04	
Lactobacillus spp										
CUC-1	20.50	0.30	.01	0.27		12.65	0.32	.03	0.21	
	20.17	0.33	.02	0.20		13.44	0.30	.02	0.24	
CH2	0.08	0		2.87		0	0		2.65	
	0.10	0		2.75		0.08	0		2.66	
CUC-3	4.70	3.41	.73	0.06		6.53	2.41	.37	0.26	
	6.70	3.57	.53	0		7.21	2.58	.36	0	
EQ	6.64	2.75	.41	0		7.98	2.39	.30		
	7.62	3.11	.41	0.12			2.45		0.24	
Pediococcus spp										
CUC-4	3.75	0		0.48		3.43	0		0.62	
	4.10	0		0.58						
44.40	4.73	0		0.35		0.28	0		2.29	
	8.48	0.15	.02	0.33		6.98	0		0	
C5	3.80	0		0.46		4.97	0		0	
	2.69	0		0.31		5.16	0		0	
XMP	9.79	0		0		3.74	0		0.36	
	9.99	0		0.04		2.83	0		0.59	

[a] products in g/L

[b] 0, none detected

Table 4.13 Comparison of growth products in M3 and M4 media[a]

	M3 (VjF, pH 5.5+10% ethanol)					M4 (VjF, pH 3.8+10% ethanol)				
	Lactic	Acetic	Ratio A/L	Glucose	Fructose	Lactic	Acetic	Ratio A/L	Glucose	Fructose
Uninoculated	0.10	0[b]		2.59	28.12	0.10	0		2.54	25.09
Leuconostoc oenos										
ML34	1.52	1.10	.72	1.14		0.36	0.34	.94	2.67	
	3.17	2.60	.82	1.19						
PSU-1	2.34	1.74	.74	0		2.17	1.68	.77	0	
						1.31	1.20	.92	0.88	
OENO	2.42	1.76	.73	0		1.60	1.27	.79	0	
	2.00	1.46	.73	0		1.23	1.00	.81	0.24	
MCW	1.57	1.20	.76	0		1.16	1.03	.89	0.54	
	1.73	1.26	.73	0.06		1.59	1.32	.83	0	
Er1a	3.17	2.29	.72	0.08		2.10	1.72	.82	0.48	
	2.05	1.50	.73	0.24		2.24	1.75	.78	0.46	
MB	1.75	1.19	.68	1.23		2.34	1.72	.74	0	
INO	2.01	1.53	.76	1.91		0.24	0.25	1.04	2.66	
	1.97	1.54	.78	2.04		0.31	0.42	1.35	2.61	
Lactobacillus spp										
CUC-1	12.65	0.32	.03	0.21		4.54	0		1.29	
	13.44	0.30	.02	0.24		4.91	0.14	.03	1.34	
CH2	0	0		2.65		2.33	0.17	.07	2.34	
	0.08	0		2.66		0.50	0.10	.20	2.39	
CUC-3	6.53	2.41	.37	0.26		4.78	3.56	.74	2.10	
	7.21	2.58	.36	0		4.97	3.51	.71	2.35	
ML30	1.52	1.06	.70	0.31		0.49	0.29	.59	2.38	
EQ	7.98	2.39	.30			4.03	2.41	.63	0.37	
		2.45		0.24		4.95	2.81	.57	0.34	
Pediococcus spp										
CUC-4	3.43	0		0.62		0				
XMP	3.74	0		0.36		0.17	0		2.63	
	2.82	0		0.59		0.37	0		2.49	

[a] products in g/L

[b] 0, none detected

Acetic acid production from growth in fructose broths at pH 3.8 and 5.5 is presented in Table 4.10. Acetic acid production occurred at high levels at both pHs. Generally, acetate levels were half those of lactic acid for *Lc. oenos* in M1 medium (fructose at pH 5.5) and were slightly higher in M2 medium (fructose at pH 3.8). Strain PSU-1 produced an acetate to lactate ratio of .52 in M1, and .64 in M2 medium. Similarly, OENO produced an acetate to lactate ratio of .47 and .67 in M1 and M2 respectively. Heterofermentative lactobacilli (CUC-3 and EQ) also produce acetate at high levels. However, the ratios of acetate to lactate were largely unaffected by the change in pH. Homofermentative lactobacilli and pediococci produced little or no acetate at either pH. Strain CUC-1 produced only small amounts of acetate at both pHs at a ratio of 0.15 acetate to lactate for each pH level. Here, large amounts of lactate were produced (20 g/L), indicating good growth. These results indicate that acetic acid production is not significantly changed by growth at low pH.

The effect of growth substrate on acetic acid production by LAB is demonstrated in Table 4.11. Strains of *Lc. oenos* and heterofermentative lactobacilli still produce acetic acid from growth in glucose based media (M5), although in greatly reduced amounts. The major sugar in M5 broth is glucose, but some fructose was detected by HPLC analysis, apparently from the tomato juice serum ingredient. Most acetate to lactate ratios for these strains in the glucose broths (M5) were low (.15-.23) compared with the ratios found in the fructose broths (.51-.71. Strains Ey2d and INO had similar ratios in both broths. Homofermentative species produced low amounts of acetate in M5 medium, as they did in M1 medium, despite often high levels of growth. No acetate could be detected from the growth of *Pediococcus* strains in the glucose based medium, even with luxuriant growth.

The effect of the presence of 10% ethanol on acetic acid production is examined in Table 4.12. Acetic acid levels appear to increase slightly with the presence of 10% ethanol in the high pH medium (M3). The average ratio of acetate to lactate for *Lc.*

oenos is .58 in M1 medium, whereas in M3 it is .75. Heterofermentative lactobacilli (CUC-3 and EQ) show some decrease in the acetate to lactate ratio when grown in the presence of ethanol. For CUC-3, the ratios are .66 in M1 and .37 in M3. Homofermentative species of *Lactobacillus* and *Pediococcus* still produce only small amounts of acetate, or none at all, despite high levels of growth.

The effect of pH at wine ethanol concentration on acetic acid production is shown in Table 4.13. Medium M4 comes closest to simulating wine environment conditions, with pH 3.8 and 10% ethanol. Levels of acetate produced in relation to lactate were slightly greater in this low pH medium. *Leuconostoc oenos* strain Er1a gave an acetate to lactate ratio of .73 in M3 medium and .80 in M4. Most *Lc. oenos* strains showed a slightly greater production of acetate in the low pH M4 medium, but low growth levels made comparisons of some strains difficult. Growth of lactobacilli and pediococci was reduced, as would be expected in low pH media containing ethanol. The heterofermentative lactobacillus CUC-3 gave a ratio in M3 broth of .37, which increased to .73 in M4 broth, indicating that it, too, produced more acetate in M4 medium.

5.0 DISCUSSION OF RESULTS

5.1 Characterisation

5.1.1 CO_2 production

Carbon dioxide production was detected in 14 strains of *Leuconostoc oenos* and 3 strains of *Lactobacillus* (Table 4.2), indicating heterofermentative metabolism of the hexoses present in HFA broth. Two strains of lactobacilli and all 4 strains of *Pediococcus* tested negative for gas production, suggesting homofermentative metabolism. Both vaspar and Durham tube methods yielded similar results. Vaspar covered broths indicated CO_2 production earlier than Durham tube broths. This is presumably due to the vaspar-plugged tubes collecting evolved gas from the entire volume of medium rather than just the medium within the inverted Durham tube. After 7 to 10 days of incubation, the results from both methods agreed. However, tubes were incubated for a total of 21 days before final reading to confirm negative results. The vaspar method requires pouring molten vaspar on to the inoculated cultures and allowing it to solidify. However, expansion of the test tubes often caused leaks. Therefore, additional vaspar was overlayed to form an airtight seal. Furthermore, the removal of the plug from homofermentative broth cultures for further testing is not always easy. The Durham tube method circumvents these problems and is recommended for use in testing heterofermentation in HFA broth by wine lactic acid bacteria.

5.1.2 Ammonia production from arginine

The results for the production of ammonia from arginine were unexpected (Table 4.2). Four strains of *Leuconostoc oenos* tested strongly positive, and two more gave positive results of intermediate intensity. The remaining eight strains did not produce

detectable ammonia from growth in HFA broth. The *Lactobacillus* strains gave results as expected, with the group III obligately heterofermentative strains producing ammonia from arginine and groups I and II homofermentative strains testing negative. Only one strain of *Pediococcus* produced ammonia in detectable amounts.

The detection of ammonia from *Leuconostoc oenos* strains could possibly be explained by the use of the novel medium (HFA broth) which contains 6 g/L arginine. Previous studies (Izugabe *et al.*, 1986; Pilone and Kunkee, 1972; Garvie, 1967) have used media containing only 3 g/L arginine. The arginine dihydrolase pathway (section 2.6.1) consists of three enzymatic reactions, with ammonia liberated by hydrolysis of arginine in the first reaction catalysed by arginine deiminase and by the action of carbamate kinase in the third reaction. LAB do not always have the complete complement of enzymes for the dihydrolase system (Montel and Champomier, 1987). Thus, the amount of ammonia produced is dependent on both the concentration of arginine available in the broth, and the complement of enzymes possessed by the organism, and presumably, their state of induction. Low levels of arginine available and the possibility that only one ammonia-producing reaction of the dihydrolase system is functioning, may be responsible for negative results in earlier investigations. The test for ammonia by Nessler's reagent requires the formation of a brick-orange complex with the medium. This colour formation is not definite unless high levels of ammonia are present. Decreasing levels of ammonia give decreasing intensities of colour from brick-orange to pale yellow. The amber colour of HFA and other broths used for the test often obscure weak reactions. In water, 0.012 g/L ammonia tested positive (Table 4.1). In HFA broth, however, the minimum amount of ammonia needed to observe a positive result is greater than 0.3 g/L (Table 4.1). A definite positive test (brick-orange) is found at 0.6 g/L. LAB strains possessing only one of the ammonia-producing enzymes of the dihydrolase system would test negative in broths containing only 3 g/L arginine. The maximum amount of ammonia possible from 3 g/L arginine by such a strain is 0.3 g/L, which is just below the level of test sensitivity. Strains

possessing the complete complement of enzymes would, however, produce 0.6 g/L from 3 g/L arginine, and test positive by Nessler's reagent. By incorporating 6 g/L arginine in HFA broth, even those strains possessing or inducing only one ammonia-liberating enzyme would be able to produce 0.6 g/L ammonia if the 6 g/L arginine was completely degraded. 0.6 g/L ammonia is easily detected. By incubating for extended periods (3 weeks), the likelihood of detecting such strains is increased and this is recommended when using HFA broth for testing ammonia production from arginine in wine lactic acid bacteria.

According to Garvie (1986a), ammonia production is characteristically negative for *Lc. oenos* and this inability has classically been used to differentiate between *Lc. oenos* and morphologically similar heterofermentative lactobacilli, which do produce ammonia from arginine (Kandler and Weiss, 1986). However, there are reports of *Leuconostoc* species capable of ammonia production from arginine (Garvie and Farrow, 1980; Kuensch *et al.*, 1974; Weiller and Radler, 1976). Garvie and Farrow went on to confirm their two strains of leuconostocs were *Lc. oenos*. Interestingly, these strains tested positive for ammonia production in broths containing only 3 g/L arginine. Curiously, this information was not incorporated in Garvie's description of the leuconostocs in Bergey's Manual of Systematic Bacteriology, Vol. 2, 1986 (Garvie, 1986a).

Among the lactobacilli used in this study, only obligately heterofermentative strains tested positive for ammonia production. Some obligate heterofermenters reportedly do not produce ammonia (Kandler and Weiss, 1986). The heterofermentative *Lactobacillus* most likely to be confused morphologically with *Leuconostoc oenos* is *Lb. confusus*, which is ammonia positive. The results found in this research indicate that the ability to produce ammonia from arginine using Nessler's reagent test no longer can be used to separate these two groups of LAB. The facultatively heterofermentative (group II) strain of *Lb. plantarum* (strain CH2) tested negative for ammonia production

in HFA broth. However, Josson *et al.* (1983) found that a strain of *Lb. plantarum* isolated from fish was capable of degrading arginine to ornithine. The obligately homofermentative strain of *Lb. delbrueckii* tested negative for ammonia production. Kandler and Weiss (1986), however, reported that this is variable even within the subspecies *delbrueckii*.

Three strains of homofermentative *Pediococcus* spp. tested negative for ammonia production, but one strain (XMP) tested positive. XMP was isolated as a contaminant from a streak plate during purification of a freeze-dried culture of Microenos P from Lacto-Labo (see Table 3.1). Microenos P is a mixed culture of wine LAB strains Microenos B (a *Leuconostoc oenos* strain) and Microenos H (a strain of *Lb. hilgardii*). It is not known if the designated contaminant XMP is, in fact, a wine isolate. It does, however, grow at wine pHs and in 10% ethanol, and can degrade malic acid and citric acid (partially). However, ammonia-producing strains of *Pediococcus* from dairy origins have been reported by Tzanetakis and Litopoulou-Tzanetaki (1989). This test yields valuable information as to the identity of XMP. Only two species of *Pediococcus* hydrolyse arginine. One of them, *Pd. pentosaceus*, is a recognised wine environment species (Costello *et al.*, 1983).

Montel and Champomier (1987) reported that the activity of arginine deiminase in the facultatively homofermentative *Lb. sake* was modulated by glucose concentration. Glucose inhibited arginine deiminase activity at levels of 1 mM (0.018%). Conversely, Manca De Nadra *et al.* (1988) found that enzymes of the dihydrolase system were not repressed by glucose in *Lb. buchneri*, an obligate heterofermenter. Ingram (1975) suggested that the test was only useful if set criteria were adhered to, stating that at low glucose concentrations some facultatively heterofermentative (group II) species may also produce ammonia. Thus, this experiment tests only for ammonia production from arginine in HFA broth under the stated conditions. Ammonia-negative strains may still be capable of arginine degradation in low glucose (hexose) environments such as

vacuum-packed meats, and possibly dry wines. In addition, the Nessler's reagent test is insensitive (see Table 4.1) and strains testing negative may still possibly produce ammonia, but in amounts below the 0.3 g/L sensitivity of the test in HFA broth.

Of the strains testing positive for ammonia, only those strains with the full complement of enzymes for the arginine dihydrolase system will benefit from ATP generated by the action of carbamate kinase (section 2.6.1). As wine contains relatively high concentrations of arginine (up to 3 g/L), this extra ATP may convey a competitive advantage to those strains capable of degrading arginine in wine environments.

5.1.3 Mannitol production

The formation of distinctive mannitol crystals in the dried HFA broth cultures was indicative of mannitol formation from fructose (Table 4.2). All 14 strains of *Lc. oenos* and three strains of heterofermentative lactobacilli (CUC-3, ML30, EQ) showed crystal formation upon drying. No pediococci or homofermentative lactobacilli showed crystal formation. These findings agree with the literature, which shows that heterofermentative LAB are generally capable of fructose reduction (Dicks and van Vuuren, 1988). Controls for the testing included uninoculated HFA broths as well as inoculated and uninoculated HFA broths containing only glucose as a carbohydrate source. None of these broths formed any of the distinctive crystals when dried. A few dried plates showing good crystal formation were resolubilised in a minimum amount of reagent grade water and the presence of high amounts of mannitol was confirmed by HPLC using the standard method described in the HPLC section (section 3.9.3). The reduction of fructose to mannitol produces acetic acid and ATP (section 2.6.3). The ATP produced by acetic acid formation means that heterofermentative strains can yield 2 moles of ATP per mole of hexose fermented by the heterolactic pathway. Enhanced growth would be expected because of this. It may be that the reason *Lc. oenos* appears to be fructophilic (Garvie, 1986a), as noted in this study, is not due to the uptake and

fermentation alone, but the combination of fructose fermentation and reduction. While growth may be enhanced, and thereby the likelihood of MLF increased, the production of acetic acid is of concern to the winemaker, especially where growth has continued in a situation where yeast fermentation was sluggish or "stuck". Here, the high amounts of fructose still present in the must would be available for reduction with the consequent production of high levels of acetate. Mannitol itself may also spoil wines if present in high concentrations. Excessive mannitol formation increases the sugar-free extract and the viscosity of wines, and may lead to mannite spoilage. Mannite spoilage is always accompanied by excessive acetic acid production (Dicks and van Vuuren, 1988). From an oenological viewpoint, the identification of a strain lacking the ability to form mannitol would be beneficial. Such a strain might reduce the spoilage risk posed by "stuck" yeast fermentations containing actively growing LAB. It has long been recognised that the growth of homofermentative LAB poses less of a spoilage threat under such conditions, but winemakers persist in using heterofermentative *Lc. oenos* strains due to their pH tolerance and their perceived beneficial influence on wine complexity.

5.1.4 Growth in the presence of 10% ethanol

Garvie (1986a) uses the ability to grow in 10% ethanol as a means to separate *Lc. oenos* from the other species in the genus. The growth of all 14 strains of leuconostocs used in this research (see Table 3.1) confirms their status as *Lc. oenos*. The notion that lactobacilli are more ethanol tolerant than leuconostocs or pediococci (Davis *et al*, 1988; Wibowo *et al*, 1985) is not immediately borne out by the data in Table 4.3 from growth at 30°C in this medium at pH 5.5. Ethanol tolerance is, however, dependent on pH and temperature, and the combined effects of the two. Variations in resistance among strains of the same species may also occur. Resistance to ethanol decreases with lowering pH and higher temperatures. Incubation at 30°C yields good growth from most strains at pH 5.5 (standard culturing conditions), but the inclusion of 10% ethanol

at this temperature may be responsible for the poor growth of some strains. It is possible that some strains could grow better in the ethanol medium at a lower temperature as noted by Asmundson and Kelly (1990). The temperatures employed by winemakers to encourage MLF in wines, around 20°C, may be below optimum growth temperatures in conventional media, but may be more conducive to growth in wines with high ethanol content. In other studies, Henick-Kling *et al.* (1989) noted that in Chardonnay wine, strains ML34 and Er1a (also used in this study) showed similar sensitivities to high ethanol concentrations. In Table 4.3, these strains also show similar amounts of growth.

5.1.5 Growth at pH levels in VjGM

All strains of *Leuconostoc oenos*, *Lactobacillus* and *Pediococcus* species grew in VjGM at initial pH 3.3 (Table 4.4). This is supportive of other investigations of wine lactic acid bacteria (Davis *et al.*, 1988; Henick-Kling *et al.*, 1989) all of which showed that most lactic acid bacteria isolated from wine environments could grow in semi-synthetic media adjusted to pH 3.3 or lower. An O.D. reading after 3 weeks indicates an organism's ability to survive the initial pH of inoculation. In theory, it is possible that some strains may be unable to actively grow at pH 3.3, but have conducted MLF in a resting state (non-dividing). This could raise the pH beyond some critical limit which would allow them to grow. Reports exist of resting cell MLF conducted at low pH (2.0) by non-growing cells of *Lc. oenos* (Davis *et al.*, 1985). Most strains would, however, be expected to grow at initial pH 3.3 in this medium (VjGM). A possible method to clarify this would be to avoid malic acid in the broths and buffer the media at the desired pH.

Izugabe *et al.* (1985) studied *Lc. oenos* strains ML34, PSU-1, Er1a, and Ey2d and noted that at high pH (3.5 and 4.0) growth responses were similar. The results for strain Ey2d (Table 4.4) show that growth was lower at high pH compared with other

strains. At low pH, the strains responded similarly. Strains INO and 2043 always showed less growth than other strains, especially at lower pHs. The lactobacilli grew to high O.D. levels down to pH 3.7, below which growth rapidly declined. Davis *et al*, (1988) found that 77% of *Lactobacillus* strains tested could grow at pH 3.2 in semi-synthetic media. Only 18% could grow at pH 3.0. The pediococci were affected less by pH than the lactobacilli. With the exception of XMP, their growth is never strong, even at high pH, and they exhibit similar growth to the weakest *Lc. oenos* strains at pH 3.3. Davis *et al.* (1988) reported that all *Pd. parvulus* tested grew at pH 3.4, 43% of strains at pH 3.2, and none at pH 3.0 in semi-synthetic media.

The general notion that decreasing pH decreases the growth of lactic acid bacteria is borne out in these results. However, it is well known that results from growth in semi-synthetic media bear little resemblance to those from growth in wine due to the presence of ethanol, SO_2 and other factors. In general, wines of pH below 3.5 do not support the growth of lactobacilli or pediococci. *Leuconostoc oenos* dominates, or is the only species present in low pH wines. Even in high pH wines (3.5 to 4.0), strains of *Lc. oenos* often grow with *Lactobacillus* and *Pediococcus* developing as spoilage organisms after MLF has been completed (Wibowo *et al.*, 1985). Britz and Tracey (1990) looked at the effect of pH on bacterial growth in combination with SO_2, ethanol and temperature, independently. They also studied 3 factor interactions which included the synergistic effect of pH, SO_2 and ethanol together. Growth was severely affected by the combination, but the extremely negative synergistic effect of pH and SO_2 together was, surprisingly, slightly reduced in the presence of ethanol, depending on the specific combinations of the three. Such studies are beyond the scope of this investigation, which revealed no single strain as being strikingly resistant to low pH environments.

5.1.6 Growth reponse to temperature

Growth response, in terms of optical density after 4 weeks of incubation over a range of temperatures, are presented in Table 4.5. Strains of *Leuconostoc oenos* always grew more at 30°C than at 35°C. The opposite is true for *Lactobacillus* and *Pediococcus* strains, which seem to prefer 35°C. Four strains, OENO, L181, 2001 and 2008, showed growth at 40°C. Kelly *et al.* (1989) also noted that strains OENO and L181 were able to grow at 40°C, while Tracey and Britz (1987) found only a few of 54 strains tested grew at this temperature. Champagne *et al.* (1989) studied strains 2001, 2006 and 2035, and noted similar effects of temperature on their growth rates which were optimal at 30-32°C. In this study, these strains reacted similarly (Table 4.5), with 2001 and 2008 growing up to 40°C. The above authors also studied 2043 and noted that growth rates of this strain were always slower in a variety of media at 28-30°C, but that its growth rate at 15-22°C was similar to other strains tested. This observation is pertinent to winemaking, as temperatures seldom rise over 25°C when wine is held to encourage MLF, and strains which are sluggish growers in laboratory trials could well grow better in wines at these lower temperatures. Kelly *et al.* (1989), however, noted that one strain, OENO, always had a faster growth rate at any temperature, and that its optimum growth rate was at a temperature 4 to 6°C higher than that for other strains. Kelly *et al.* also included strains ML34 and PSU-1 in their study, finding that the two strains behaved similarly and had optimal growth rates at 30-33°C. These two strains behaved similarly in this study (Table 4.5) at 30 and 35°C, but ML34 grew better at 15°C than PSU-1. ML34 grew to a similar O.D. level at 15°C and 30°C, while INO was the only leuconostoc strain clearly preferring 15°C, although growth was never luxuriant even after 4 weeks of incubation at pH 5.5. Kelly *et al.* (1989) and Champagne *et al.* (1989) were studying rates of growth in different media for commercial starter production. The study presented here used an optical density after a fixed time (4 weeks) to give information on strains. While growth rate studies are

probably preferable, the data in Table 4.5 show similar trends in apparent growth to those reported by other authors.

A notable feature of *Lactobacillus* strains is the reduced growth at 15°C, even after 4 weeks. Kandler and Weiss (1986) reported growth of lactobacilli from 2°C to 53°C, with optimum temperatures generally 30-40°C, which would support data obtained in Table 4.5. Two strains, ML30 and EQ, failed to grow at 40°C and always showed less growth than the other three strains at all temperatures tested. That some strains grow better than others is not surprising given the diversity of the species within the genus *Lactobacillus*.

The pediococci showed only small increases in growth from 15°C to 30°C. All strains preferred 35°C, especially XMP which showed a large increase between 30 and 35°C. XMP not only grew to higher O.D. levels, but was the only *Pediococcus* strain to grow at 40°C. Kelly *et al.* (1989) included strain 44.40 in their growth rate studies and found it to grow and have a calculable growth rate from 15 to 39°C. In this study, strain 44.40 failed to grow at 40°C.

5.1.7 Dissimilation of L-malic and citric acids

The dissimilation of L-malic acid during growth in VjG medium containing malic acid and citric acid (VjGMC) by all strains is not unexpected (Table 4.6). Initially, all strains probably were selected for their malolactic fermentative capability. Malic and citric acids, together with tartaric acid, are the major acids in grape juices and wines. Malic acid concentrations may be as high as 6 g/L and citric acid concentrations 0.5 g/L with more in *Botrytis*-infected grapes (Henick-Kling, 1988). The reduction of malic acid from wines raises the pH and helps smooth the wine, while citrate degradation is noted for its flavour modification (section 2.6.2) and an accompanying increase in volatile acidity. Millière *et al.* (1989) cited that citrate utilisation may be plasmid

encoded in the genus *Leuconostoc*, and this, along with vancomycin resistance could be lost on repeated laboratory transfer. Garvie (1986a) also noted that *Leuconostoc* species were mostly capable of citrate degradation, but this ability was lost in cultured strains. *Lactobacillus* strain CH2 clearly degraded citric acid, while CUC-1, CUC-3 and ML30 degraded citric acid to lesser extents. Strain EQ did not degrade the acid. Strain ML30 has previously been described as capable of degrading citrate (Pilone, 1965), while strains CUC-1 and CUC-3 were negative for citrate degradation. Pediococci have been reported as unable to degrade citrate (Davis *et al*, 1986), which is supported by strains CUC-4, C5 and 44.40 in Table 4.6. However, strain XMP degraded some citric acid, and this species may belong to *Pd. pentosaceus* , which is described as a citric acid utilising species by Litopolou-Tzanetaki *et al.* (1989).

5.1.8 Carbon sources

Leuconostoc oenos

Currently, *Leuconostoc oenos* can be classified without the need for carbohydrate utilisation studies (Garvie, 1967), but the fermentative capabilities of the bacteria are of interest to oenologists. Carbohydrate utilisation studies on wine LAB (particularly *Lc. oenos*) seem to be influenced by the system used to study them (basal broths or galleries such as the API 50 galleries), and the pH at which the study is conducted (Davis *et al.*, 1988). The use of API 50 carbohydrate galleries and the results obtained from them are discussed in the literature review (section 2.6.4). Studies conducted in basal broth (BB) media and under similar conditions will be compared, although the composition of BB for many authors is different.

In this study, glucose has been included in all standard culturing broths, along with a host of other fermentable sources from tomato juice. So it was surprising to note that six *Lc. oenos* strains grew only slightly better in BB plus glucose than in BB alone

(Table 4.7). Other strains clearly thrived on the added glucose. Pardo *et al.* (1988) also found strains which grew poorly on glucose.

The fructophilic nature of *Lc. oenos* as reported by Garvie (1986a) and Kreiger *et al.* (1992) is confirmed. Most strains attained high growth levels when grown on fructose. A few strains, however, demonstrate Garvie's (1967) comment that some strains are always difficult to grow, and that *Lc. oenos* is a heterogeneous species. Generally, the greatest growth, even for weakly growing strains, was attained with fructose as the carbon source. The basal broth medium used lacked the tomato juice factor (TJF), but strains such as INO and 2043 always grew slowly and to low final optical densities (O.D.), even in subculturing media which contained tomato serum (personal observation). Kelly *et al.* (1989) reported 9 *Lc. oenos* strains which were unable to ferment fructose. Although glucose and fructose are the most abundant hexoses in wines, and are preferred by most *Lc. oenos*, they are not preferentially fermented during MLF. Davis *et al.* (1986) could not correlate the growth of *Lc. oenos* in Shiraz wines with the utilisation of any specific hexose or pentose. The concentrations of glucose and fructose actually increased, presumably because of residual enzymatic activity hydrolysing polysaccharides and other compounds to their monosaccharide monomers. The use of fructose and glucose by the strain conducting MLF was still, however, a possibility as the hydrolysis reactions may be generating glucose and fructose faster than the bacteria can use them.

Three *Lc. oenos* strains grew on galactose, but not to high O.D. levels. Galactose utilisation has been reported by Izugabe *et al.* (1985), and Jensen and Edwards (1991), and in API 50 CH systems by Davis *et al.* (1988). Galactose has been recorded in dry wines, ranging from trace amounts to 2 g/L (Liu, 1990).

Half of the strains used in this study were able to ferment mannose, which is in agreement with Kelly *et al.* (1989), Garvie (1986a, 1967) and Izugabe *et al.* (1985).

Mannose occurs in dry wines at concentrations up to 300 mg/L (Liu, 1990), and has been seen to increase in concentration in wines undergoing MLF, possibly due to the hydrolysis of mannan polysaccharides in yeast cell walls released by autolysis (Davis *et al.*, 1986).

The utilisation of L-sorbose is the first reported (to our knowledge) by either BB or API 50 methods. The growth of the three strains (2001, 2006, 2008) was, however, not great.

Evidence of L-fucose utilisation in the literature is scant. Davis *et al.* (1988) found that none of 71 strains tested could ferment D- or L-fucose. In this study, strain 2043 showed a slight increase in O.D., and three other strains grew to a reasonable extent on L-fucose. However, strain 2001 grew to a high O.D., second only to that achieved on ribose and fructose. Fucose occurs in wines only in low concentrations, up to 90 mg/L (Liu, 1990).

Rhamnose was utilised by 3 strains, with slight responses from two others one of which, ML34, Pilone and Kunkee (1972) described as negative. Davis *et al.* (1988) found 70% of strains did use rhamnose in API galleries at pH 6.0. Liu (1990) reports that rhamnose concentrations in wines may reach 400 mg/L.

Lactose utilisation has been used to help separate *Lc. cremoris* from *Lc. oenos*, but three positive responses and four more slight responses cast doubt on the usefulness of this particular separating factor. These strains are confirmed *Lc. oenos* due to their growth at low pH and in the presence of 10% ethanol. Lactose utilisation has been reported by Davis *et al.* (1988) in just 4% of *Leuconostoc oenos* strains (API system). Lactose is not found in wine to any great extent (<30 mg/L) but it is an important carbohydrate source in milk fermentations.

Utilisation of the disaccharide maltose is reported as negative for *Lc. oenos* by Garvie (1986a), but Izugabe *et al.* (1985) found some positive strains by test tube methods, as was found in this study. Davis *et al.* (1988) found just 5% of 71 strains positive for maltose. Maltose has only been found in wines at very low levels.

Sucrose is another disaccharide reportedly negative for *Lc. oenos* (Garvie, 1986a). Only strain 2008 showed an increase in O.D. in the presence of sucrose. Kelly *et al.* (1989) described strains L181 and one other as sucrose-positive. In this study, L181 did not grow in the presence of sucrose. Kelly *et al.* conducted their tests under strictly anaerobic conditions (at 30°C for seven days) and at twice the concentration of carbon source. If L181 can utilise sucrose, then in this study, sucrose uptake could somehow be affected, or sucrase expression for some reason be repressed. Sucrose is utilised by other species in the genus *Leuconostoc* to form dextran slimes, but this is not a feature of *Lc. oenos*.

Cellobiose was fermented by just four strains, whereas Garvie (1967) noted most strains were positive or had delayed reaction (Garvie, 1986a). Izugabe *et al.* (1985) reported all strains positive, while Kelly *et al.* (1989) reported one quarter of strains positive. Cellobiose proved a good example of the need to modify the standard API system for use with *Lc. oenos* (Jensen and Edwards, 1991). Strains MCW, OENO, ML34 and PSU-1 all returned positive results upon prolonged incubation, having earlier tested negative by standard API techniques. Davis *et al.* (1988) used standard API techniques and reported 85% of strains positive. Cellobiose occurs in wines, but only at very low levels.

The literature generally suggests that the use of the disaccharide melibiose, which is found only in very low concentrations in wine (<10 mg/L), is variable among *Lc. oenos*. The findings of this study agree with this variability.

Five strains fermented trehalose strongly, and one other showed a slight response. Trehalose is a yeast storage disaccharide which may be released by yeast autolysis and hydrolysed to yield glucose (Davis *et al.*, 1986). Extended lees contact or stirred lees fermentations could increase trehalose availability to LAB. Trehalose has been recorded at concentrations of up to 600 mg/L in dry wines (Liu, 1990). Garvie (1986a) lists trehalose as a delayed reaction, but most other literature suggests strains are usually positive (Kelly *et al.*, 1989; Izugabe *et al.*, 1985; Davis *et al.*, 1988).

Utilisation of the trisaccharide raffinose is reportedly negative for *Lc. oenos* according to Garvie (1986a) and most other authors, but four strains used in this study grew to at least some extent. Izugabe *et al.* (1985) also found a few strains positive. Only traces of raffinose occur in wine.

The trisaccharide melezitose was utilised by 2006 and 2008, and slightly by 2035. Only Davis *et al.* (1989) have previously reported melezitose utilisation, and then in only 3% of strains. Melezitose is not recorded as being found in wines.

The phenolic glycoside amygdalin was utilised by four strains, and slightly by three others. Davis *et al.* (1988) reported 76% of strains positive at pH 6.0, while Milliere *et al.* (1989) found none of ten strains could utilise amygdalin at pH 4.5 (both studies in API systems).

Garvie (1986a) describes *Lc. oenos* reaction toward salicin as delayed, but other literature suggests most strains will grow on this substituted sugar on prolonged incubation (Jensen and Edwards, 1991; Izugabe *et al.*, 1988; Pardo *et al.*, 1988). At pH 6.0, Davis *et al.* (1988) found 88% of strains positive, but Milliere *et al.* (1989) reported all ten strains negative at pH 4.5 (both studies conducted in API systems). In this study only three strains grew on salicin.

The hydrodysis of esculin was conducted by all 14 *Leuconostoc oenos* strains used in this study, in agreement with Garvie (1986a).

Pentoses occur in wine and musts at levels of 0.8-2 g/L, and are not fermented anaerobically by wine yeast (Amerine *et al.*, 1980). This leaves pentoses available for LAB metabolism. Arabinose, ribose and xylose are found in wines in concentrations of up to 1 g/L, 0.6 g/L and 1.4 g/L, respectively (Liu, 1990). Strains capable of pentose fermentation would be capable of utilising this significant carbohydrate source, and may be at an advantage in wines. Only three strains showed a slight response to the addition of arabinose, but strains L181 and OENO have been previously stated as being positive (Kelly *et al*, 1989). Overall, *Lc. oenos* seems variable in its response to arabinose. Strain Er1a was reported as unable to ferment xylose by Izugabe *et al.* (1985), but in this study it fermented xylose strongly, as did four other strains (2001, 2006, 2008 and ML34). ML34 has also previously been reported as negative (Pilone and Kunkee, 1972). Ribose was fermented strongly by all 14 strains of *Lc. oenos*. Growth levels achieved were second only to fructose as the preferred carbon source for most strains except 2006 and 2008, which preferred ribose. Most literature agrees that *Lc. oenos* ferments ribose strongly. Negative results are not uncommon in API testing systems (Davis *et al.*, 1988). Jensen and Edwards (1991) reported negative results with standard API methods, but reversed all results to positive by using a modified API testing system.

Glycerol was fermented to some extent by strains 2006 and 2008. The only reference to glycerol utilisation known to this author is that of Davis *et al.* (1988) who found that just 1% of *Lc. oenos* were positive at pH 6.0. Some LAB can form the bitter substance acrolein from glycerol (Wibowo *et al*, 1985). Acrolein production by strains 2006 and 2008 is unknown. Glycerol occurs in standard wines at concentrations of up to 10 g/L.

Mannitol was a carbon source for strain 2001, and slightly for OENO. Garvie (1986a) reports this as negative for *Lc. oenos*. Authors testing for mannitol utilisation and recording negative results include Pardo *et al.* (1988), Pilone and Kunkee (1972), Jensen and Edwards (1991) and Millière *et al.* (1989). However, Davis *et al.* (1988) did report 27% of strains positive by API testing at pH 6.0. All strains are capable of reducing fructose to mannitol, yielding extra ATP as a result (Table 4.2). Those strains further able to utilise mannitol may succeed mannitol-producing strains during growth in wine, and possibly may reduce excessive mannitol in wines (mannitol spoilage) and thereby increasing quality.

Sorbitol has received little attention by researchers as it occurs only in trace amounts in wines. Pilone and Kunkee (1972) and Jensen and Edwards (1991) have reported negative results. In this study, four strains grew (L181, 2001, 2006, 2008), and two more gave slight responses.

Gluconate was utilised by seven strains, and slightly by one other. Three of these (ML34, OENO, PSU-1) have tested negative for gluconate utilisation in earlier work (Jensen and Edwards, 1991). Davis *et al.* (1988) reported 55% of *Lc. oenos* strains as utilising gluconate at pH 6.0.

Most of the hexoses, pentoses and sugar alcohols tested are found in wine or grape juices (Liu, 1990). The behaviour of carbohydrates in wine undergoing MLF has been studied by Davis *et al.* (1986) in two Shiraz wines, and they suggested that the metabolism of wine components by LAB growing and conducting MLF was complex. They noted that glucose and fructose concentrations rose during MLF while arabinose and mannose decreased, but other fermentable carbohydrates such as galactose, ribose, xylose, rhamnose and glycerol remained unchanged. Similar changes also occurred in low pH wines in which no bacterial growth was detected. They proposed that the changes were due to residual enzymatic activity in the wine. The release of nutrients

from yeast lysis, phenolic glycoside and oligosaccharide hydrolysis was responsible for the increases and decreases of monomer sugars that they detected. They could not attribute the decrease of any particular hexose or pentose to the growth of *Lc. oenos*. In higher pH wines in which species of *Pediococcus* and *Lactobacillus* grew, the changes in carbohydrate concentrations were easier to correlate with the growth of bacteria. This research illustrated that the behaviour of LAB in wines is more complex than previously thought, and probably correlates poorly with the expected fermentative capabilities of the bacteria as determined by the use of BB and API systems. Any conclusions drawn from carbohydrate utilisation tests and applied to wine environments would be highly speculative. This is probably because metabolism is affected by pH, the wine itself, and possibly by as yet unknown factors that occur in wine environments. It is interesting, however, to note the range of carbon sources fermentable by the *Leuconostoc* strains under equal conditions in the basal broths at pH 5.5. Strain 2008 fermented 23 different carbon sources under these conditions, while strains such as MCW, Ey2d, MB and INO fermented only five. However, the relevance of this to growth in wine conditions, again, is unknown. Strains having the capability of utilising numerous carbohydrates may be more appropriate for use as starter cultures to induce malolactic fermentation. It is possible, however, that strains able to use fewer carbon sources might still outgrow other strains if the sources they do use are fermented strongly, and are abundant in wines. Selection of strains for use as starter cultures also involves consideration of the abilities to grow at low pH, in high ethanol concentrations, in the presence of SO_2, and other factors.

Lactobacillus species

Strain CUC-1 is described as *Lb. delbrueckii* by Pilone *et al.* (1966). This species is now considered to contain three subspecies, *Lb. delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii* subsp. *lactis* and *Lb. delbrueckii* subsp. *bulgaricus* (Kandler and Weiss, 1986). Species *Lb. delbrueckii* is obligately homofermentative, producing two moles

ATP per mole hexose fermented via the Embden-Meyerhof pathway, and lactate as the sole end-product. Surprisingly, CUC-1 did not utilise glucose when it was added to BB (Table 4.8). The strain did, however, ferment fructose, galactose, mannose, maltose, sucrose, cellobiose, trehalose and melezitose strongly. Lactose was not fermented, a characteristic of *Lb. delbrueckii* subsp. *delbrueckii* (Kandler and Weiss, 1986). Sorbose, melibiose, raffinose, arabinose, xylose and glycerol were not fermented. Pilone *et al.* (1966) also report CUC-1 as unable to ferment lactose and xylose. The three substituted sugars amygdalin, salicin and esculin were utilised, and mannitol was also fermented strongly. CUC-1, in this study, deviates from the fermentation profile described by Kandler and Weiss (1986) for subspecies *delbrueckii* by strongly fermenting galactose, melezitose, substituted sugars, mannitol, ribose and gluconic acid. Subspecies *lactis* utilises lactose and substituted sugars, but not pentoses. Fermentation of pentoses and gluconate is surprising as this is reportedly characteristically negative for obligate homofermenters. Definitive identification of the subspecies to which CUC-1 belongs is not possible from any characteristics tested here, or from those listed by Kandler and Weiss (1986). They suggest that subspecies *lactis* may be the common ancestor to species *delbrueckii* in keeping with the notion that the nutritional requirements of lactobacilli are the results of numerous minor defects within the genome, and that the subspecies have only evolved by minor changes of the phenotype and the genotype. The three subspecies possess more than 80% DNA/DNA hmomology, and their G+C content is almost identical. The ability of the different subspecies to grow in the presence of high ethanol concentrations is not discussed by Kandler and Weiss (1986).

Lb. plantarum strain CH2 (Prahl *et al.*, 1988) is distinctive in this study for the range of carbon sources utilised. Only gluconate and sorbose were not fermented. Interestingly, after mannitol and glycerol, glucose gave the least growth for any carbon source. Ribose was again a preferred carbon source, and the fermentation patterns of Kandler and Weiss (1986) are different only in rhamnose utilisation. CH2 did grow on

rhamnose, but not to high growth levels. CH2 belongs to the group II facultatively heterofermentative lactobacilli (Kandler and Weiss, 1986) which ferment hexoses exclusively to lactate but possess an inducible phosphoketolase for pentose fermentation.

Pilone *et al.* (1966) describe strain CUC-3 as *Lb. buchneri*, an obligately heterofermentative group III lactobacillus. CUC-3 showed a restricted fermentation pattern from that reported by Kandler and Weiss (1986). Only glucose, fructose, maltose, ribose, xylose and gluconate were fermented strongly, and arabinose and esculin were weakly attacked. Dicks and van Vuuren (1988) report a wider range of carbohydrates fermented by *Lb. buchneri* including lactose, galactose, melibiose, melezitose and the three pentoses.

Strain ML30 is described by Pilone *et al.* (1966) as *Lb. brevis*, which is a group III obligately heterofermentative species. In this study ML30 was able to ferment 24 carbon sources, although growth was never as high as strains CH2 or CUC-1. The extended fermentation pattern differs from those described by Kandler and Weiss (1986), Dicks and van Vuuren (1988) and Pardo *et al.* (1988) by the following compounds: mannose, sorbose, fucose, rhamnose, cellobiose, trehalose, melezitose, amygdalin, salicin, glycerol, mannitol and sorbitol. The differences between *Lb. buchneri* (CUC-3) and *Lb. brevis* (ML30) in this study are striking. However, Kandler and Weiss (1986) comment that *Lb. brevis* is often difficult to distinguish clearly from *Lb. buchneri* and *Lb. hilgardii* by simple physiological tests, especially carbohydrate fermentation reactions. They also comment that *Lb. buchneri* is identical in almost all characteristics with *Lb. brevis* except that *Lb. buchneri* ferments melezitose, although anomalies to this rule have been found. From Table 4.8 it can be seen that these two strains are no longer as closely related with respect to carbon sources. Both strains CUC-3 and ML30 produce ammonia from arginine, are

heterofermentative, and reduce fructose to mannitol, all characteristics of group III lactobacilli.

Kunkee describes EQ as *Lb. hilgardii*, a commercial starter culture 'Equilait', from France. *Lb. hilgardii* is a group III obligately heterofermentative lactobacillus. EQ was one of the few strains to grow to a higher O.D. level in the presence of glucose than fructose, but both substrates were fermented strongly. The most preferred carbon sources were maltose and sucrose. Many anomalies were found with the fermentation patterns of Kandler and Weiss (1986), Dicks and van Vuuren (1988) and Pardo *et al.* (1988). These include the ability of EQ to ferment mannose, sorbose, rhamnose, cellobiose, trehalose, amygdalin, salicin and esculin, and its slight responses to glycerol, mannitol and sorbitol. EQ is also an obligately heterofermentative, ammonia-producing, fructose-reducing lactobacillus. Strains EQ and ML30 are able to ferment glycerol, possibly by the method reported by Veiga da Cunha and Foster (1992). Here, some strains of *Lb. brevis* and *Lb. buchneri* can produce 1,3 propanediol from glycerol while metabolising glucose or fructose in a co-fermentation, producing greater amounts of acetate and more ATP, and thereby enhancing growth.

Pediococcus species

Pediococcus strains CUC-4, C5 and 44.40 had similar carbon source profiles. They fermented glucose, fructose, rhamnose, maltose, trehalose, amygdalin, salicin and esculin. CUC-4 and C5 also fermented mannose and cellobiose. Garvie (1986b) reports most strains also ferment galactose, but this is contrary to the findings of this study and to those of Edwards and Jensen (1992). Although *Pd. damnosus* and *Pd. parvulus* share up to 40% DNA/DNA homology, they are difficult to separate by carbohydrate analysis. Only the ability to grow at pH 7.0, and to ferment melezitose and sucrose separate the species. On this basis, the three strains cannot be assigned to one species or the other. It is noteworthy that as a genus, these pediococci grow

slightly better on glucose than on fructose, a trait shared only with the *Lb. hilgardii* strain EQ. Strain XMP was notable for its increased fermentation profile and the high optical densities reached. In addition to the carbon sources utilised by the other strains, XMP was also able to ferment lactose, galactose, melibiose, raffinose, arabinose, ribose, sorbitol and to some extent xylose. The utilisation of lactose separates this strain from *Pd. parvulus* and *Pd. damnosus*, along with its ability to ferment pentoses. Maltose utilisation and strong trehalose fermentation separate XMP from *Pd. acidilacti*, which also produces ammonia from arginine. Only two species of *Pediococcus* hydrolyse arginine. One of them (*Pd. pentosaceus*) is a recognised wine species (Costello *et al.*, 1983). No inofrmation is known by this author on the ethanol tolerance of *Pd. acidilacti*. Based on the above information, strain XMP is best classified as *Pd. pentosaceus*. This strain is notable for growing to higher O.D. than the other three strains in 10% ethanol, at pH 3.5 and above, and temperatures from 15° to 40°C, and generally always grew faster during subculturing. Strain XMP can degrade malic acid, as demonstrated by thin layer chromatography results (Table 4.6). Garvie (1986b) reports species *pentosaceus* as being able to attack malate, but it is not known if XMP conducts a classical malolactic fermentation, or uses malic acid as a carbon source.

5.2 Acetic Acid Production

The production of mannitol from fructose (Fig. 1.1) and the resulting switch from ethanol production to acetate production was thought to depend on the substrate for growth (excess fructose), pH and the ethanol concentration. As wine is an undefined medium and the growth of bacteria in it is slow, these factors were studied in either glucose or fructose broths containing the tomato juice factor to ensure good growth. Many aspects of LAB metabolism are thought to vary with pH (Davis *et al.*, 1988), so acetate production was studied at high and low pH levels. Ideally, this should be studied at pHs similar to cool climate wines (pH 3.5 and below). However, because

Lc. oenos grows only slowly at low pH and strains of *Lactobacillus* and *Pediococcus* may not grow at all, the study was conducted at pH 3.8 to ensure active metabolism and growth of all strains.

Heterofermentative strains behaved as expected in fructose broths (Table 4.10) and produced large amounts of acetate per unit of growth measurement (lactic acid). This occurred both at high pH (pH 5.5) and low pH (pH 3.8). Acetic acid production by heterofermentative strains was found to be unaffected by the change in pH under these conditions. Homofermentative strains which produced little or no acetic acid were similarly unaffected. The results suggest that fructose reduction to mannitol, and the resulting production of acetate from acetyl phosphate, can occur at pHs approximating those of wine.

Under anaerobic conditions, heterofermentative LAB are forced to convert acetyl phosphate to acetyl-CoA and to further reduce acetyl-CoA to ethanol. This is necessary to reoxidise the two reduced NADHs produced by dehydrogenases in the early part of the heterolactic pathway. This forces the bacteria to forego converting acetyl phosphate to acetate and gaining the extra ATP (Fig. 1.1). However, if a reducible substrate is present, and the organism possesses the appropriate dehydrogenase enzyme, then the NADHs from the heterolactic pathway can be reoxidised, allowing the organism to convert excess acetyl phosphate to acetate and benefit from extra ATP.

Reducible compounds that are found in must and fermenting musts include fructose, pyruvate, citric acid, malic acid and acetaldehyde. The most common by far is fructose. Because fructose is one of the two major sugars present in musts, it is expected that acetate will be produced in increased levels from growth in the presence of this reducible substrate. Those organisms possessing mannitol dehydrogenase can reduce fructose to mannitol and thereby free up acetyl phosphate for ATP production. M1 medium contains 2.5% fructose (simulating a "sluggish" yeast fermentation) and

M5 is a glucose based medium containing only 0.3% fructose. The results of growth in both media (Table 4.11) show that the ratios of acetate produced in relation to lactate were low (.15-.23) in M5 broth and high (.51-.71) in M1 broth. This indicated that heterofermentative species produce large amounts of acetic acid from fructose, probably by using fructose as an electron acceptor as well as fermenting it (mannitol was found in fermentation broths, section 4.1.4). As expected, homofermentative species did not produce much acetate from growth in either medium, indicating an inability to reduce fructose.

As wine contains high levels of ethanol, it is necessary to consider its effect on acetate production. Tracey and van Rooyen (1988) noted more volatile acids were produced by strains in media containing 5% ethanol than in 10% ethanol. They also found a significant correlation between the fructose consumed and the mannitol produced in the 5% medium, but not in the medium containing 10% ethanol. If the increase in volatile acidity is due indirectly to mannitol production, then ethanol concentrations may affect its production. M3 broth contains 10% ethanol while M1 does not (Table 4.12). In M3 broth, *Lc. oenos* strains showed high acetate production that was only slightly increased over that found in M1 broth. The heterofermentative lactobacilli also showed a small increase in the high level of acetate produced in relation to lactate in the medium containing ethanol. These results indicate that the presence of ethanol does not markedly increase the ratio of acetate to lactate production of these LAB.

The effect of pH at wine ethanol concentrations was also investigated by comparing acetate to lactate ratios in M3 (high pH) and M4 (low pH) media (Table 4.13). The results show that M4 medium stimulates acetate production in relation to lactate over that occurring in M3. M4 medium simulates wine ethanol concentrations (10%) and pH (3.8), so growth was reduced, as would be expected in this harsh environment.

Overall, acetate production in relation to lactate produced was high in heterofermentative LAB. In wines, acetic acid levels greater than about 1 g/L are considered as spoilage (Amerine *et al.*, 1980). The media (M1-M5) contain greater than 2.5% hexose, a situation that might be encountered in winemaking where a sluggish or "stuck" yeast alcoholic fermentation stops before the must is fermented to dryness. Malolactic bacteria present in the must would be presented with excess fructose and glucose for growth. On the basis of this experiment, the risk of spoilage due to excessive acetic acid production in such a situation would be high. From the results, an ethanol concentration of 10% and a pH of 3.8 would not protect a must from spoilage by acetate production. So, winemakers would be wise to closely monitor "stuck" or sluggish fermentations for acetate production by LAB while they address the problem of encouraging the alcoholic fermentation to go to completion.

The production of acetate is also possible by the interaction of LAB and oxygen. Condon (1987) reports that almost all LAB have NADH oxidase. In aerobic conditions, these NADH oxidases (and NADH peroxidases) provide alternative mechanisms for NAD^+ regeneration. Aerobically, NADH oxidase can reoxidise the NADHs produced in the early part of the heterofermentative pathway. Again, as with fructose metabolism, this relieves the need for acetyl phosphate conversion to ethanol, and so a switch in production from lactate and ethanol to lactate and acetate occurs, and ATP is produced. The possession of NADH oxidase has been reported in the genus *Leuconostoc* by Nuraida *et al.*(1992), so it is possible that strains of *Lc. oenos* contain the enzyme.

The product of NADH oxidase activity is H_2O_2, which may become inhibitory in aerobic cultures, as LAB lack a true catalase. The superoxide anion (O_2^-) is believed to be an intermediate of H_2O_2 formation. H_2O_2 can react with O_2^- to form the hydroxyl radical OH· and this is a stronger inhibitor than H_2O_2 (Condon, 1987). It is interesting

to note that mannitol is a known scavenger of the hydroxyl radical and its production by LAB may help with aerobic growth by removing the inhibitory radical.

The extent of acetate production due to interactions of the bacteria with oxygen in this experiment is unknown. Test tubes were nearly full with media, so a small surface area to volume ratio existed. The relatively high temperature of incubation (30°C) must also help to reduce the amount of dissolved oxygen in the media. Normally, heterofermentative LAB produce quantities of CO_2 which effectively provide a flushing out of O_2. But in harsh conditions, slow growth may allow atmospheric oxygen to enter and dissolve into the media. So, the possibility of some of the acetate production being due to the action of NADH oxidases and peroxidases exists in this experiment. The introduction of oxygen into fermenting musts should not occur. However, some oxygen may be introduced when transferring wines from vat to vat, or during processing. The effect (if any) on bulk wine quality would be small. However, should wine be exposed to aerobic conditions, interactions with LAB could occur, but wine is then at a far greater risk of acetate spoilage from the action of aerobic Acetic Acid Bacteria than any spoilage due to LAB metabolism.

Recently, another mode of acetate production has been shown for *Lc. oenos*. Growing and non-growing cells metabolising glucose were seen by nuclear magnetic resonance spectroscopy to produce erythritol and glycerol. Veiga-da-Cunha *et al.* (1992) determined that, anaerobically, glucose was converted to fructose-6-phosphate and then split into erythritol and acetyl phosphate. The acetyl phosphate was then converted to acetate, yielding ATP. Up to 25% of the glucose fermented was channelled to erythritol production. Aerobically, more glucose was fermented to glycerol and lactate. No erythritol or glycerol formation was noted from growth on fructose or ribose.

It is possible that the acetate produced from growth in M5 (glucose) broth by heterofermentative strains may have been produced by this system. The retention times of pure erythritol and glycerol were checked on both the 87H and 87C columns. Peaks of the same retention times were recorded on many fermentation chromatograms of *Lc. oenos* and heterofermentative *Lactobacillus* strains in all media types (results not shown). On the 87H column, which gave the clearest chromatograms, erythritol eluted just prior to the lactic acid peak, and glycerol eluted just after. Due to time constraints and probable difficulties in accurate integration of peak areas, no attempt was made to quantify erythritol or glycerol production from the chromatograms. However, peak heights obtained after growth in media were smaller than those obtained from standards (Erythritol 1.25 g/L, Glycerol 0.5 g/L). The detection of what are very probably (but not confirmed) erythritol and glycerol from the growth of *Lc. oenos* and heterofermentative lactobacilli supports the novel biosynthesis of erythritol and glycerol recently reported by Veiga-da-Cunha *et al.* (1992). This new mechanism allowing for formation of acetic acid by *Lc. oenos* needs further investigation to determine its relevance to winemaking.

This study of acetic acid and mannitol production was hampered by the inability of the HPLC columns to resolve some compounds and the errors generated by the quantification of compounds that could be resolved. It was originally intended to produce a carbon balance and identify and quantify the products of fermentation to distinguish any strains which might produce less acetate than others.

The HPX-87H column could separate glucose, lactic acid, acetic acid, erythritol, glycerol and ethanol, but quantification (integration of areas below peaks) was seldom within 10% error when checked against internally repeated standard samples. These were usually the high and low standards used for calibration. The 87H column, unfortunately, could not resolve fructose and mannitol, two of the major compounds of interest in this study. After variations of analysis conditions failed to separate fructose

and mannitol, two HPX-87H columns were tried in series. Some improvement in fructose and mannitol separation occurred, but only at high standard concentrations, while other formerly quantifiable peaks were broadened or no longer detected. Only those analysis results with errors 10% or less for acetic acid, lactic acid and glucose from the 87H column were used in Tables 4.10 to 4.13.

The HPX-87C column could resolve fructose, acetate and mannitol contained in standards, but not always in fermentation media, and not to any degree of repeatability. In fermentation samples, the lactic acid peak varied in its retention time depending on the concentration. Lactic acid co-eluted with glucose at the concentrations used in low standards. However, in fermentation samples where lactic acid was present and glucose concentrations were known to be low (determined on HPX-87H), the chromatogram showed no large peak at the retention time expected for lactate. The higher the concentration of lactate, the greater was the interference with later eluting peaks such as fructose, acetate and mannitol. Such behaviour could not be explained, as other compounds retained their retention times within the 5% window for which they were calibrated. Mannitol could be resolved from fructose, but co-eluted with ethanol, which was usually a minor product of growth or a major component of the medium (M3 and M4). Erythritol and glycerol closely preceded mannitol and often caused peak misidentification. Hence, only a few carefully selected results for fructose concentrations where all peaks were positively identified were used from the HPX-87C.

In light of the problems with the 87C column, the methodology of standard preparation is questioned. Traditionally, standards are prepared from pure reagents in Milli-Q reagent grade water. To obtain a complete picture of elution profiles, this author suggests that standards be made in a solvent closely resembling the media and the products of its fermentation. Hence, the use of 'wild' standards for studying the resolution of columns. Here, peaks found in the media and those formed by

fermentation products could be examined for interference. The separation of co-eluting compounds which decrease during fermentations and those that increase during fermentation provide additional problems. Fructose concentrations were initially high and mannitol was formed only after growth, usually on fructose. It was sometimes possible to resolve fructose and mannitol if the concentrations of each were comparable. This was determined by the use of 'inverse' standards, where fructose and mannitol concentrations were varied to investigate if at any specific range, quantitative separation could be achieved. As would be expected, fermentation samples seldom fell within the narrow range over which this was possible.

Overall, a smaller scale experiment with just a few strains, and analysed enzymatically might result in more quantitative data on acetate production rates. No mannitol enzymatic kit is available, so this may have to be detected by GLC methods, as the HPLC columns available to this researcher were unable to accurately measure its concentration due to the presence of ethanol and fructose.

An underlying problem with the study of wine LAB is the need to use rich and undefined media because of their fastidious nature. Ideally, research should be conducted in wine so the results can be directly applicable to winemaking, but the undefined nature of wines and the fickle growth of bacteria in them usually prohibits this. Researchers are forced to approximate wine conditions in richer, more defined media, and tentatively extrapolate their findings to what might occur in wines. In this respect, the findings of this research are in a similar predicament to other studies in this field. The synthetic wine system proposed by Liu (1990) might be a significant step toward addressing this problem.

6.0 CONCLUSION

The biological conversion of L-malic to lactic acid in wines is conducted primarily by lactic acid bacteria (LAB). The growth of LAB and the resulting malolactic fermentation (MLF) is encouraged by winemakers in order to reduce the malic acid content of low pH high acid wines and for flavour modification. The growth of the bacteria in wine is slow and unpredictable. Therefore, much research has been conducted on the growth and metabolism of these bacteria with the aims of understanding the factors which govern their growth, and to make malolactic fermentation a more controllable process in wines.

In this research commercial strains of malolactic starter cultures as well as laboratory strains were studied for their characteristics, with special emphasis on the metabolism of the grape sugar fructose in the formation of wine-spoiling acetic acid.

Basic characterisitcs were studied in a novel broth medium (HFA) that allowed the determination of gas production from hexoses, ammonia from arginine, and mannitol from fructose in one inoculated HFA broth tube. Using this broth, all homofermentative strains did not produce CO_2 or show mannitol crystal formation, while all heterofermentative strains produced large quantities of CO_2 and mannitol crystals in dried broths. This indicated that all strains of *Leuconostoc oenos*, and *Lactobacillus* strains CUC-3, ML30 and EQ possessed mannitol dehydrogenase, and so could reduce fructose to mannitol, and form acetic acid. Therefore, all heterofermentative strains of malolactic bacteria have the potential for forming mannitol and acetic acid in "stuck" wine alcoholic fermentations. This warranted further investigation (see below).

The test for ammonia production from arginine has traditionally (Bergey's Manual of Systematic Bacteriology Vol. 2, 1986) been used to help separate *Lc. oenos*, which

does not produce ammonia, from the morphologically similar heterofermentative lactobacilli which do. The literature search revealed that some wine *Lc. oenos* strains are capable of ammonia production, casting doubt on the usefulness of the test. In this study, four of 14 strains of *Lc. oenos* tested positive for ammonia production in broth containing arginine (HFA), and two more gave variable results. The test for ammonia (Nessler's reagent) was found to be insensitive. In previous tests not enough arginine was included in broths, and therefore conversion to ammonia was not great enough to record positive results for strains which did not possess the full complement of enzymes of the arginine dihydrolase system. These strains only form one mole of ammonia per mole of arginine degraded. Strains possessing the three enzymes of the pathway produce two moles of ammonia per mole of arginine, and this amount of ammonia is easily detectable by Nessler's reagent. In HFA broth, ammonia concentrations above 0.3 g/L could be detected. Because HFA broth contains 6 g/L arginine, twice that of previous tests, strains with incomplete arginine dihydrolase enzymes can produce greater than 0.3 g/L ammonia and, therefore, can be detected. Energy (ATP) is only generated by the last step in the arginine dihydrolase system, and thus only strains with a full complement of enzymes can benefit from ATP production. The metabolism of arginine by such strains may be an important source of energy in wines.

Strains were also characterised by their response to wine environment conditions. All strains were grown in the presence of 10% ethanol. This is useful to distinguish *Leuconostoc oenos* from other *Leuconostoc* species which are not wine LAB and cannot tolerate ethanol. Strains were also grown at a range of pH levels (pH 5.5 to 3.3). All strains grew at initial pH 3.3 in media containing malic acid, indicating their suitability to grow in cool climate wines. *Lc. oenos* proved to be more tolerant of low pH than strains of *Lactobacillus* or *Pediococcus*, and this agrees with the literature. However, no strain was strikingly resistant to low pH. Growth over a range of temperatures from 40°C to 15°C was investigated. Strains of *Lc. oenos* and

Pediococcus species were relatively insensitive to lower temperatures compared with *Lactobacillus* strains which preferred 30 - 35°C. Winemakers sometimes encourage MLF by warming wines, thereby incurring costs and spoilage risks, so strains active at lower temperatures would be preferable. No strain was found especially adapted to low temperature. All strains were able to degrade malic acid during growth in glucose broth. The reduction of malic acid produces a rise in pH, which winemakers depend upon to smooth low pH wines (malolactic fermentation). Citric acid was degraded by *Lc. oenos* strains and most lactobacilli, and one pediococcus. Although citric acid is a minor wine component, its utilisation yields flavour compounds such as diacetyl, which is considered beneficial to wine quality in low amounts but detrimental at high levels.

Pediococcus strain XMP was isolated as a contaminant of a commercial malolactic starter culture. This strain grew in 10% ethanol, produced ammonia from arginine, grew at pH 3.3 and to high levels at temperatures from 15°C to 40°C. It also degrades malic and citric acids. Although not a recognised wine strain, the tests indicate that it is suitable for growth in wine environments and to degrade malic acid (conduct MLF). This strain exhibited vigorous growth at temperatures used to encourage MLF, and might be suitable for conducting MLF in higher pH wines. This organism should be further investigated to determine its suitability as a malolactic starter, as it has unusual characteristics compared with known wine pediococci.

Carbon source utilisation studies were conducted in a basal medium which gave little growth until the added carbon source was introduced. The change in optical density (O.D.) was used as a measure of growth. *Lc. oenos* strains preferred fructose to glucose, and also grew strongly on ribose. Many different fermentation profiles were recorded for *Lc. oenos*, and the range of carbon sources utilised for an individual strain ranged from 23 to just 5. This shows the great heterogeneity of the wine leuconostocs. *Lactobacillus* strains grew to higher O.D. levels than other strains (at pH

5.5). The lactobacilli mostly preferred fructose to glucose, but CUC-1 did not grow on glucose. Ribose was also a favoured carbon source. Obligately and facultatively heterofermentative strains utilised pentoses strongly. The strains vary in the range of carbohydrate utilisation from 7 to 25 compounds. Large variations from the expected fermentation patterns given in the literature meant that strains of *Lactobacillus* could not be positively confirmed as the species they were previously designated. *Pediococcus* strains CUC-4, C5 and 44.40 shared similar fermentation profiles, while strain XMP showed a general increase in the number of carbon sources used, and strongly fermented pentoses. XMP was designated as probably being *Pd. pentosaceus*, while the other strains were clearly either *Pd. damnosus* or *Pd. parvulus*, but could not be positively designated as one or the other. Generally, no particular strain was revealed as being superior in its ability to grow in wine environments. Although carbohydrate utilisation patterns in all the wine lactic acid bacteria tested varied considerably, the relevance of this to growth in wine is unknown since LAB metabolism varies with conditions such as pH.

The formation of mannitol and acetic acid was further investigated after HFA broths indicated the potential for their production. Semi-synthetic media were designed to simulate conditions similar to a "stuck" yeast alcoholic fermentation containing 2.5% hexoses, and in some cases ethanol. Five media were used to study the effects of pH, substrate for growth, ethanol and the effect of pH in the presence of ethanol. Both heterofermentative and homofermentative species were included for comparison. HPLC analysis of media after growth indicated that homofermentative strains produced only small amounts of acetate, or none at all, from growth in the various broths. This confirms their inability to reduce fructose to mannitol as was found in the HFA broths. Heterofermentative strains produced high levels of acetic acid at both high pH and low pH in the presence and absence of ethanol, but not from growth on glucose. This indicates that acetic acid production is due to fructose reduction to mannitol. The results conclude that even at wine pH and ethanol concentrations, which might have

changed the metabolism of the bacteria, the spoilage risk posed by heterofermentative strains still exists if fructose is present in high concentrations. On the basis of this result, it would be wise to closely monitor "stuck" fermentations for acetic acid production by LAB while attempting to restart the yeast alcoholic fermentation.

The acetic acid found in the media may have been contributed to in a minor way by interactions of LAB and oxygen because experiments were not conducted in strictly anaerobic conditions. Moreover, a recently discovered pathway of glucose utilisation in *Lc. oenos* may also have contributed to the acetate detected. The products of this novel pathway, erythritol, glycerol (and acetic acid) were detected by HPLC, but not quantified. This pathway may be responsible for some of the small amount of acetate detected in glucose broths.

The analysis of the media after growth by HPLC could detect but not resolve or quantify mannitol because of the compound's co-elution with fructose on the HPX-87H column, and with ethanol on the HPX-87C column.

LAB present a problem because they cannot be studied easily in wine due to their poor growth and metabolism in this harsh environment. Research on wine lactic acid bacteria is usually conducted in richer media to ensure good growth and the results from the media extrapolated with caution to suggest what might occur in wines.

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8.0 ADDENDUM

The following publication was derived from this research.