# Acetaldehyde Metabolism by Wine Lactic Acid Bacteria and Its Oenological Implications

A Thesis Presented in Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

at

Massey University
Palmerston North
New Zealand

James Osborne
March 2000

# Abstract

Acetaldehyde is one of the most important sensory carbonyl compounds formed during vinification. Excess acetaldehyde can adversely affect the flavour of wine and acetaldehyde plays a role in the colour development of red wines. Excess acetaldehyde is usually masked by the addition of sulphur dioxide ($SO_2$) to the wine ($SO_2$ is also used as an antimicrobial and antioxidant agent in wine and acetaldehyde bound $SO_2$ is less effective in these roles). To date there has been no definitive study of the impact of wine LAB on free and bound acetaldehyde. Therefore, this study investigated the metabolism of free and bound acetaldehyde and its oenological implications.

A survey of 11 commercial malolactic starter cultures (mostly *Oenococcus oeni* strains) showed that 9 out of 11 were able to metabolise acetaldehyde (in a resting state) with the corresponding formation of ethanol and acetic acid as products. $SO_2$ bound acetaldehyde was also metabolised by the two strains tested (*Lactobacillus buchneri* CUC-3 and *Oenococcus oeni* MCW). This is the first evidence that LAB can indeed catabolise $SO_2$ bound acetaldehyde, therefore releasing free $SO_2$.

During growth *Oenococcus oeni* EQ54 and *Oenococcus oeni* VFO were able to metabolise free acetaldehyde in wine at pH 3.3 and pH 3.6. In wine containing $SO_2$ bound acetaldehyde, *Oenococcus oeni* EQ54 and *Oenococcus oeni* VFO were able to metabolise $SO_2$ bound acetaldehyde at pH 3.6 after a period of sluggish growth. At pH 3.3 there was no metabolism of $SO_2$ bound acetaldehyde by *Oenococcus oeni* EQ54 and *Oenococcus oeni* VFO during the incubation period.

Results from growth experiments showed that in broth there was inhibition of growth at 300 mg/L concentration of acetaldehyde for all strains. In wine, no significant inhibition or stimulation of the cultures examined was found at any acetaldehyde concentrations up to 300 mg/L.

In a simultaneous resting cell incubation of *Saccharomyces bayanus* Première Cuvée and *Oenococcus oeni* Lol11, acetaldehyde produced by the yeast was metabolised by the wine LAB.

The metabolism of acetaldehyde by wine LAB is expected to influence wine flavour as small amounts of ethanol and acetic acid are produced and acetaldehyde is removed. This removal of acetaldehyde by wine LAB suggests that less $SO_2$ will need to be added to the wine to mask excess acetaldehyde when malolactic fermentation is performed. Inhibition of wine LAB growth in broth by high levels of acetaldehyde suggests a role for acetaldehyde in stuck or sluggish MLF. Sluggish growth in wine containing $SO_2$ bound acetaldehyde also suggests a possible role of $SO_2$ bound acetaldehyde in stuck and sluggish MLF. This is due to the release of free $SO_2$ through the metabolism of the acetaldehyde moiety of $SO_2$ bound acetaldehyde.

# Acknowledgements

There have been many people that have helped me through this past year and have enabled me to undertake and complete this little project. Firstly, I'd like to thank my Lord and Saviour Jesus Christ. Without his continual support I never would have made it this far. I'd like to say a big thank you to Mum and Dad for supporting me through all these years of study. Thanks for all the support and for nodding politely and looking interested when I try to explain to you exactly what it is I do.

Thanks to all my friends who have been there for me and a big thank you to Paula, Janet and Sheralee for always sharing their lunch with me.

A big thank you to Ramon, my fellow researcher in the Wine Cellar. You have been a tremendous help to me during my research and it has been great having someone so knowledgeable just across the bench who is willing to help me out with any little problems. I wish you all the best for the completion of your PhD and for the future.

Thanks to the Institute of Molecular BioSciences for providing the facilities, equipment and consumables necessary for this research.

Last, but certainly not least, I'd like to thank my supervisors, Gordon Pilone and Shao-Quan Liu. Thanks Gordon for always being supportive and friendly and for the guidance which has kept me on track with my research. Enjoy your retirement next year! (although your new tractor-mowing venture is sure to keep you busy). Thanks Shao for your valuable input into this research and for your guidance in determining the direction of this research. Also, thanks for all the time you put in reviewing and editing the drafts of my thesis (I hope you didn't find it too boring!)

# Table of Contents

# List of Figures

# List of Tables

# Chapter 1 Introduction to the Thesis

## 1.1. Winemaking: Meaning Behind the Madness

For many thousands of years, wine has been part of human diet. It represents a safe and healthy beverage providing calories and vitamins and, more importantly, offering relaxation and relief from the stresses of every day life (Amerine *et al*, 1980). Species of the grapevine *Vitis* are grown all over the world, and the most important species for winemaking is *Vitis vinifera*. For several thousands of years, wine fermentations have spontaneously occurred, producing wines of varying quality. This still occurs in some wineries today, but pure cultures of microorganisms are now available to induce fermentations in grape juice so that a more uniformed quality of wine can be produced.

Since Pasteur's studies on wine, scientists have been striving to unravel the physical, chemical and biological complexities of wine and the winemaking process. Although much is now known about this subject, there is still much that remains unexplained. It is to this end that we, as researchers, continue to work towards.

### 1.1.1. From the Vine to the Bottle

The winemaking process begins at the vineyard. Here, grapes of certain variety are grown and harvested at an appropriate sugar level. The grapes are then destemmed and crushed. In white wines, the rapid separation of the juice from the skins and seeds is important as extended contact with the skins allows materials such as tannins to be extracted and remain in the finished wine (Ough, 1992). Sulphur dioxide may be added during or after the crush at levels of 50-100 mg/L to prevent oxidation of the grape must and growth of wild yeast and bacteria that may be present on the grapes. After clarification of the white wine juice, yeast is added to begin the alcoholic fermentation. In modern wineries today, starter cultures of *Saccharomyces cerevisiae* or *Saccharomyces bayanus* are usually used which are selected for their ability to ferment vigorously, their contribution to flavour/style and their tolerance to ethanol and sulphur dioxide.

In red winemaking, the juice remains in contact with the skins during fermentation. This is to help extract colours and flavours from the skins. At the completion of alcoholic fermentation, the wine is racked off into another vessel, leaving behind the yeast and solids.

Some wines undergo a secondary fermentation called malolactic fermentation (MLF). This fermentation is carried out by malolactic bacteria and may occur naturally or is induced by the use of pure starter cultures. This causes deacidification of high acid wines (such as those produced from cool climate regions, e.g. New Zealand) and adds flavour complexity.

The wine may be filtered or fined, then aged and blended depending on the type of wine that is being produced. Finally, the wine can be bottled and packaged and sold for the consumer's enjoyment.

## 1.2. Wine Microorganisms

Wine is a harsh environment for microorganisms to grow in. It has a low pH, can have high ethanol concentrations and has low levels of some nutrients. Therefore, it is only a rather select group of microorganisms that can survive and grow under these conditions. However, it is the cumulative effect of these microorganisms, acting both independently and in conjunction with one another, that determines the final chemical and physical nature of the finished wine.

### 1.2.1. Wine Yeast

Yeasts are significant in wine because they carry out the alcoholic fermentation. They can cause spoilage of the wine and can affect wine flavours and overall quality through autolysis. The principal yeasts used in winemaking are *S. cerevisiae* and *S. bayanus*. This is because they conduct a vigorous fermentation to dryness; they possesses good ethanol and sulphur dioxide tolerance and produce reproducible fermentation characteristics (Boulton *et al*, 1996).

There are two general classes of problems for a winemaker that can arise during the alcoholic fermentation. These are sluggish or stuck fermenations and production of off-flavour compounds. Stuck or sluggish fermentations may be caused by a lack of

fermentation temperature, presence of inhibitory substances such as agricultural spray residue or fatty acids, and the influences of acetic acid produced by bacteria (Fleet and Heard, 1993; Rasmussen *et al*, 1995; Huang *et al*, 1996; Boulton *et al*, 1996; Bisson, 1999).

The production of off-flavours can markedly affect the overall quality of the wine. Acetic acid, higher alcohols and volatile sulphur compounds can effect the sensory properties of a wine (Boulton *et al*, 1996).

### 1.2.2. Wine Lactic Acid Bacteria

Lactic acid bacteria (LAB) isolated from wine are found in two families and three genera (with several species and strains). The *Lactobacillaceae*, represented by the genus *Lactobacillus*, includes rod-shaped Gram-positive species; while the *Streptococcaceae,* represented by the genera *Pediococcus* and *Oenococcus,* includes Gram-positive coccoid or coccobacilloid-shaped isolates (Dicks *et al*, 1995; Fugelsang, 1997). LAB can be described as being either homofermentative or heterofermentative microorganisms. Homofermentative LAB convert glucose mainly to lactic acid without formation of appreciable amounts of carbon dioxide via the Embden-Meyerhof Parnas (EMP) pathway. Heterofermentative LAB lack the enzyme fructose-diphosphate aldolase and must divert the flow of carbon through the 6-phosphogluconate pathway (phosphoketolase pathway). This produces lactic acid as well as ethanol, acetic acid and carbon dioxide (Cogan & Jordan, 1993; Fugelsang, 1997). LAB are important in wine as they metabolise malic acid forming the weaker lactic acid and carbon dioxide. This process is called malolactic fermentation and will be discussed in detail in a later section.

The major species present in the grape juice after crushing include *Lb. plantarum, O. oeni* and *Pd. damnosus*. The predominant species is generally *O. oeni*. This is due to its greater tolerance of low pH. *O. oeni* predominates in wines with pH values below 3.5 while in wines with a pH above 3.5, *Lb. spp.* and *Pd. spp.* may predominate (Wibowo *et al*, 1985; Henick-Kling, 1993).

The microbiology of wine also involves complex ecological interactions. LAB can interact with yeast, acetic acid bacteria, bacteriophage, and there may be interactions between the different species of LAB present within the wine environment (Wibowo

*et al*, 1985). These interactions between the yeast and the wine LAB are of particular importance. These interactions are not always neutral and can affect the final quality of the wine. Ethanol concentration, competition for nutrients and production of sulphur dioxide and other antagonistic products such as octanoic and decanoic acids by yeast, may inhibit the growth of LAB as well as their ability to degrade malic acid (Fornachon, 1968; Wibowo *et al*, 1985; King and Beelman, 1986; Cannon and Pilone, 1993; Fugelsang, 1997). Yeast may also stimulate the growth of LAB in wines. During yeast growth, amino acids and vitamins are excreted into the wine, which may stimulate growth of LAB. Yeast autolysis also releases these substances and prolonged contact of wine with lees encourages autolysis and may encourage MLF (Wibowo *et al*, 1985; Cannon and Pilone, 1993).

Bacteriophages can cause lysis of LAB during growth in wine which can result in partial or complete inhibition of MLF. However, certain strains of LAB are resistant to a broad range of phage (Fugelsang, 1997).

## 1.3. Malolactic Fermentation

As previously mentioned (section 1.2.2), during growth in wine LAB metabolise malic acid to lactic acid and carbon dioxide. This is an enzyme-mediated decarboxylation of L (-) malic acid to L (+) lactic acid and is unique amongst microorganisms (Fuglesang, 1997; Boulton *et al*, 1996). There is no substrate level phosphorylation directly linked to the decarboxylation of malic acid to lactic acid. The malolactic conversion may be linked to the protonmotive force for the production of ATP during growth at low pH. LAB are able to utilise sugars as a carbon source as MLF does not provide carbon for growth (Cox & Henick-Kling, 1989; Henick-Kling, 1993; Liu *et al,* 1995; Fuglesang, 1997).

MLF is important in wine for three major reasons: (i) for the deacidification of high acid wines, (ii) for the microbial stability of the wine and (iii) for flavour modification. MLF usually occurs naturally or spontaneously, after the alcoholic fermentation but it may occur during (Henick-Kling, 1993). In North and South America, Australia, New Zealand, South Africa and Europe, starter cultures of pure bacteria are being used to induce MLF. This is because the use of natural MLFs are often unpredictable and difficult to control. By using starter cultures, the winemaker can have control over the strains responsible for the fermentation and their

contribution to the wine characteristics (Krieger, 1993; Bartowsky & Henschke, 1995; Pilone, 1995; Fugelsang, 1997). The majority of malolactic starter cultures today consist of one or more strains of *O. oeni*. This is due to its tolerance of low pH and high alcohol and because of the flavours and mouth feel this strain produces (Henick-Kling, 1993). Malolactic bacteria carry out the following processes in wine:

### 1.3.1. Deacidification of Wine

The decarboxylation of malic acid to the weaker lactic acid and the production of carbon dioxide decreases the acidity of the wine and increases the pH. This process is particularly important in wines produced from grapes grown in cool climates (such as New Zealand) because these wines often have high acid content. This reduction in acidity improves the palatability of these high acid wines. While acid reduction is beneficial in high acid wines, it is not desirable in wines with lower acidity (grown in warmer climates) as it will leave a wine with too little acid and too high a pH making it susceptible to spoilage by other microorganisms.

### 1.3.2. Microbial Stability

The use of MLF in warmer wine regions is still common. One reason for this is that the MLF also has a stabilising effect on the microbial population of wine. Once the MLF is completed, the winemaker can consider the wine to be bacteriologically stable and can safely store it for further aging (Amerine *et al,* 1980). LAB are nutritionally fastidious. During growth they deplete the amount of micronutrients in the wine, making the medium less capable of supporting further growth of other fastidious microorganisms such as spoilage bacteria and yeasts (Boulton *et al*, 1996). The removal of malate and some sugars also increases microbial stability, as does the production of antimicrobial agents such as lactic acid and bacteriocins (Henick-Kling, 1993).

### 1.3.3. Flavour Modification

MLF not only affects the taste of wine through deacidification but can also contribute other flavour characteristics. The influence of malolactic bacteria on the flavour and therefore the commercial value of wine can be significant. These contributions to wine flavour can be both negative and positive in nature. The levels of sensory active compounds produced and the style of the wine determine whether a flavour modification is desirable or not (Henick-Kling, 1993; Bartowsky & Henschke, 1995).

Any enhancement in wine flavour resulting from MLF is, however, hard to evaluate and there is even some dispute as to whether MLF contributes recognizable flavours to wine (Pilone & Kunkee, 1965; Amerine *et al*,1980; Henick-Kling, 1993). The flavours produced by MLF have been described as 'nutty', 'oaky', 'lactic', 'buttery', 'yeasty' and sweaty. Some winemakers also believe that MLF may enhance the fruity character of a wine (Henick-Kling, 1993).

Body and length of taste, two important characteristics in wine, are recognized as being increased in white and red wines that have undergone MLF. Strain variation plays a role in increasing or decreasing these characteristics. In general, flavour attributes should be regarded as the result of a combination of bacterial strain, wine and the conditions of fermentation (Bartowsky & Henschke, 1995).

Although much is known about the production of flavour active compounds during MLF, the contribution of individual compounds to the flavour of the wine is not yet fully known (Henick-Kling, 1993). Without this information and without knowledge of the chemical basis of important precursor compounds, it will not be possible to predict the sensory outcome of MLF (Laurent *et al*, 1994). The most important flavour compound produced by malolactic bacteria is diacetyl (2,3 butanedione). In pure form, diacetyl is described as having various odors including those of butter, rancid butter or butterscotch (Boulton *et al*, 1996). The desirability of this compound is dependent on the concentration and the style of wine being produced. Generally, only small amounts (1-4 mg/L) are formed and are considered to enhance the flavour complexity of the wine (Davis *et al*, 1985). At higher levels (> 5mg/L), it has a characteristic buttery aroma which is regarded by some as a defect (Davis *et al*, 1985; Henick-Kling, 1993). MLF can reduce strong vegetative aromas and can enhance the fruity characteristics of a wine (Henick-Kling, 1993).

Malolactic bacteria can also impact the levels of an important aroma compound in wine, acetaldehyde. Acetaldehyde is a potent volatile flavour compound that can be found in many foods and beverages. It is normally present in recently fermented and aging wines and can also be found in cider, cheese, and yoghurt (Fugelsang, 1997). Acetaldehyde is one of the most important sensory compounds found in wine and makes up more then 90% of the total aldehyde content in wine. Levels typically range from 10-200 mg/L (Henschke & Jiranek, 1993). In wine, the average values for white

wines are about 80 mg/L and for red wines, 30 mg/L, but in sherry levels can reach as high as 500 mg/L (McClosky& Mahaney, 1981). In wine, the sensory threshold value of acetaldehyde is 100 to 125mg/L. Acetaldehyde is characterized by a sour, green apple aroma. It is extremely reactive and can react with amino acids to create various flavour compounds (Griffith and Hammond, 1989). In red wine, acetaldehyde can cause turbidity and may play a role in haze formation. It also plays a role in colour formation during the maturation of red wines (Ribereau-Gayon *et al*, 1983; Somers & Wescombe, 1987; Bakker *et al*, 1993; Boulton *et al*, 1996). While high concentrations of acetaldehyde are considered a unique feature of sherry-type wines (Ough, 1992), high levels are generally undesirable in table wines. Port wine also owes its essential colour and flavour features to excess acetaldehyde (Bakker & Timberlake, 1986).

## 1.4. Research Aims

Despite the importance of acetaldehyde in wine aroma and colour, the metabolism of acetaldehyde by wine LAB and its potential impact on wine aroma and quality has to date not been systematically studied. Therefore, this project investigated, definitively, acetaldehyde metabolism by wine LAB from the following perspectives: (a) metabolism of free and bound acetaldehyde by resting cells and growing cells (Chapter 4 and Chapter 5); (b) the effect of acetaldehyde on the growth characteristics of wine LAB in broth and wine (Chapter 6) and (c); the implications for simultaneous fermentation (Chapter 7).

# Chapter 2 Literature Review

## 2.1. Acetaldehyde Production in Wine

### 2.1.1. Acetaldehyde Formation by Yeast

Acetaldehyde is considered to be a leakage product of the alcoholic fermentation by yeast (Margalith, 1981). It is the product of the decarboxylation of pyruvate during the alcoholic fermentation (Ciani, 1997). The amount of acetaldehyde produced by yeast varies greatly amongst different species and strains. For example, within the species *S. cerevisiae* acetaldehyde production ranges from 0.5 to 286 mg/L (Fleet & Heard, 1993). Sugar is the primary substrate for acetaldehyde production, although acetaldehyde may also be formed through the metabolism of amino acids such as alanine (Henschke & Jiranek, 1993; Boulton *et al*, 1996). Acetaldehyde is excreted into the environment mainly during the first stages of fermentation (growth phase) (Ciani, 1997) and can also be recatabolised by the yeast (Farris *et al*, 1983).

Acetaldehyde can also be formed through the metabolism of ethanol by yeast. Ethanol can be oxidised to acetaldehyde by film yeast which are commonly used in sherry-type wine production and levels in these wines may be as high as 500 mg/L (Zoecklein *et al*, 1995; Fugelsang, 1997). In yeast, the enzyme alcohol dehydrogenase (ADH) is responsible for converting ethanol to acetaldehyde. *S. cerevisiae* contains three ADH isoenzymes. ADH I is the fermentative enzyme, which converts acetaldehyde into ethanol under fermentative conditions. It is associated with the glycolytic pathway and also causes reoxidation of NADH. However, under aerobic conditions this enzyme can be partly or completely repressed (Millan & Ortega, 1988). Under oxidative conditions, ADH II is active and can oxidise ethanol to acetaldehyde. It is catabolically repressed and is usually absent or present in small amounts when *S.cerevisiae* is growing on a fermentable carbon source. Both these enzymes are present in the cytoplasm while ADH III is present in the mitochondria. The biological role of ADH III is not yet fully understood, although it is thought that it may play a role in the respiratory metabolism of yeasts (Millan & Ortega, 1988).

External actors such as temperature, oxygen and $SO_2$ can affect the production of acetaldehyde by yeast. Romano *et al* (1994) showed that increased temperature

promotes acetaldehyde production. In contrast, Cabranes *et al* (1998) reported that the production of acetaldehyde was higher at 12°C than it was at 18°C. It has also been shown that $SO_2$ levels can affect the rate of acetaldehyde production by yeast (Pilkington & Rose, 1988). Wines fermented with $SO_2$ have considerably higher acetaldehyde levels than those fermented without $SO_2$ (Herraiz *et al*, 1989). There appears to be a link between sulphite resistance in yeast and acetaldehyde production with the more sulphite resistant yeast producing larger amounts of acetaldehyde than the more sulphite sensitive yeast (Pilkington & Rose, 1988). This may be due to the sulphite binding properties of acetaldehyde (discussed in a later section).

### 2.1.2. Acetaldehyde Production by Acetic Acid Bacteria

Acetic acid bacteria are able to produce acetaldehyde in wine. These bacteria may be present on the grapes or winery equipment and can produce acetic acid through the oxidation of ethanol (Drysdale & Fleet, 1989). Ethanol is first oxidised to the intermediate, acetaldehyde, which is then further oxidised to acetic acid. The ethanol and aldehyde dehydrogenases responsible for these reactions have been characterised (Adachi *et al*, 1980; Ameyama & Adachi, 1982). Under low oxygen conditions and/or ethanol concentrations higher then 10%, acetaldehyde tends to accumulate rather then being oxidised to acetic acid (Zoecklein *et al*, 1995). Ethanol concentrations above 10% become increasingly inhibitory to the growth of acetic acid bacteria and their ability to oxidise ethanol diminishes. Also, aldehyde dehydrogenase is less stable than ethanol dehyrogenase at these concentrations and this could lead to an accumulation of acetaldehyde (Drysdale & Fleet, 1988; Zoecklein *et al*, 1995; Fugelsang, 1997). Acetaldehyde levels of up to 250 mg/L can be formed by acetic acid bacteria (Drysdale & Fleet, 1989; Fuglesang, 1997).

### 2.1.3. Acetaldehyde Production via Auto-oxidation of Ethanol and Phenolic Compounds

The oxidation of ethanol to acetaldehyde occurs at an appreciable rate in wine only via a coupled auto-oxidation of certain phenolic compounds. A strong oxidant ($H_2O_2$) produced by phenolic oxidation oxidises ethanol to acetaldehyde (Wildenradt & Singleton, 1974). Oxygen, ethanol and a readily oxidisable phenol are required (Ribereau-Gayon *et al*, 1983).

### 2.1.4. Acetaldehyde Production by LAB

Many dairy LAB are able to produce acetaldehyde. These include lactococci ( Keenan *et al*, 1966b; Liu *et al* ., 1997), lactobacilli (Keenan & Lindsay, 1967), leuconostocs (Walsh & Cogan, 1973) and pediococci (Keenan *et al*, 1968). Acetaldehyde is formed from glucose as an intermediate in the formation of ethanol (Lees & Jago, 1976a). Currently, it is not clear whether wine LAB are able to produce acetaldehyde in a similar manner and there is a need for further research in this area.

## 2.2. Effect of Acetaldehyde on the Chemical and Physical Properties of Wine

### 2.2.1. Effect of Acetaldehyde on Wine Colour

The colour of a red wine is one of its most important and obvious quality indicators. For young red wines, colour is due mainly to high levels of anthocyanins being present in the new wine. However, during maturation and aging red wine colour changes from bright red to a reddish-brown tint (Bakker *et al*, 1993). This is due to anthocyanins, extracted from the grape skins during fermentation, forming pigments by condensation with other flavonoid compounds (Ribereau-Gayon *et al*, 1983; Somers & Wescombe, 1987). One of these condensation reactions involves acetaldehyde. Condensation reactions between anthocyanins and phenolic compounds such as tannins and catechin occur very slowly when acetaldehyde is not present. These reactions, however, occur rapidly in the presence of acetaldehyde and cause an enhancement in the colour intensity of red wines (Timberlake & Bridle, 1976; Somers & Wescombe, 1987). Timberlake & Bridle (1976) showed that the anthocyanin, malvidin 3-glucoside, was the only anthocyanin that was able to condense with acetaldehyde. In that reaction, a dimer consisting of malvididn 3-glucoside linked to a catechin by a $-CH_3CH-$ bridge was formed. In the absence of anthocyanin, acetaldehyde will produce polymeric compounds with catechin alone. In addition to catechin, acetaldehyde is also capable of reacting with more complex flavan-3-ols such as procyanidin dimers, trimers and even condensed tannins. When these react with malvidin-3-glucoside, a polymeric pigment is formed again (Fulcrand *et al*, 1998). Acetaldehyde may also play a role in forming a range of other pigmented polymers in wine. Acetaldehyde can react with catechin to form vinyl catechin. This

vinyl catechin can then react with anthocyanins to form a pigment. Acetaldehyde may also react with larger flavanols, such as condensed tannins, to form a range of pigments in red wines (Franchia-Aricha *et al*, 1998). Therefore, addition of acetaldehyde to red wine is favourable from a colour enhancement point of view. However, it is illegal to do so, and increases in acetaldehyde levels can be gained through practices such as aerobic treatment of the wine to initiate and promote aging reactions (Ribereau-Gayon *et al*, 1983).

### 2.2.2. Effect of Acetaldehyde on Wine Sensory Characteristics

As previously mentioned (section 1.3.3), acetaldehyde at levels above the sensory threshold in wine of 100 to 125mg/L produces a green, grassy or apple-like aroma which is undesirable in table wines. Acetaldehyde can also affect the astringency and bitterness of a wine. Reduction in astringency in red wines has been attributed as being a consequence of anthocyanin-flavanol derived pigment formation by either direct condensation or through acetaldehyde mediated reactions (Timberlake & Birdle, 1976).

### 2.2.3. Effect of Acetaldehyde on Sulphur Dioxide

Sulphur dioxide ($SO_2$) is an antimicrobial and antioxidant compound that has been used in winemaking for centuries. The physical and chemical reactions of $SO_2$ in winemaking include: the inhibition of unwanted bacteria, moulds and yeast; the interaction with wine phenols in the competitive oxidation; the binding of excess acetaldehyde, pyruvate, keto-glutarate, and anthocyanins; and the delay of brown pigment development (Romano & Suzzi, 1993; Boulton *et al*, 1996). Total $SO_2$ exists in three forms. The molecular gas form, $SO_2$; the sulphite anion, $SO_3^{-2}$; and the bisulphite anion, $HSO_3^{-1}$. The levels of each form of $SO_2$ in solution are pH dependent. In wine with a pH of 3 to 4 the bisulphite ion predominates (Zoecklein *et al*, 1995). Several compounds present in wine are active in binding with $SO_2$. These compounds include acetaldehyde and anthocyanins (present in red wine). Acetaldehyde binds strongly with the bisulphite ion to form hydroxy-sulphonate ethane which, compared to the unbound bisulphite ion, has weak antimicrobial and antioxidant properties (Burroughs & Sparks, 1973; Romano & Suzzi, 1993). This binding of the bisulphite ion by acetaldehyde reduces the amount of free $SO_2$ present in the wine and therefore reduces the effectiveness of $SO_2$ as an antimicrobial agent.

However, this bound form of $SO_2$ can still be inhibitory to LAB. Fornachon (1963) and later Hood (1983) showed that growth of LAB may be inhibited in the presence of low levels of acetaldehyde bound $SO_2$ and that sensitivity to bound $SO_2$ varied amongst species and strains of LAB and was greatest at low pH. This effect may be due to the ability of LAB to metabolise the acetaldehyde moiety of $SO_2$ bound acetaldehyde, thus releasing the $SO_2$. Free $SO_2$, even at very low levels, has a strong inhibitory effect on LAB in wine.

Because $SO_2$ binds strongly with acetaldehyde (the dissociation constant being about $1.4 \times 10^{-6}$ at wine pH (Burroughs & Sparks, 1973)), the addition of $SO_2$ to wine can mask high levels of acetaldehyde, thus protecting or improving wine taste and aroma (Zoecklein *et al*, 1995). However, addition of high levels of $SO_2$ is undesirable from both a public health and wine quality point of view. High levels of $SO_2$ can affect the polymerization reaction between acetaldehyde and wine phenolics and can also have a decolourising effect (Ribereau-Gayon *et al*, 1983).

## 2.3. Effect of Acetaldehyde on Wine Microorganisms

### 2.3.1. Effect of Acetaldehyde on Wine Yeast

In wine, yeasts are the primary producers of acetaldehyde. At certain levels acetaldehyde may affect the yeast and therefore the alcoholic fermentation and this may lead to sluggish or stuck fermentations. Many factors are implicated as playing a role in sluggish and stuck fermentation, (Fleet & Heard, 1993; Huang *et al*, 1996; Edwards *et al*, 1998; Edwards *et al*, 1999) but the role of acetaldehyde to date has not been examined. Acetaldehyde is a known inhibitor of a wide range of metabolic pathways. The toxic effects of this compound are due to the reaction of the aldehyde group with cellular amino groups via the formation of Schiff's bases. More concerning the toxic effects of acetaldehyde can be found in a review by Jones (1989).

It has been suggested that intracellular and extracellular accumulation of acetaldehyde is one of the central mechanisms of ethanol inhibition. Ishikawa *et al* (1990) found that a decline in the metabolic activity of *Zymomonas mobilis* under high oxygen

supply was due to inhibition by accumulated acetaldehyde. The oxidation of ethanol to acetaldehyde consumes NAD and produces NADH. Under oxidative conditions, the equilibrium is shifted toward acetaldehyde and NADH production. The resulting imbalance in the NAD/NADH ratio inhibits the tricarboxyl acid cycle (TCA). The TCA cycle usually consumes acetaldehyde, and therefore, there is a possibility of acetaldehyde accumulation within the cell (Jones, 1989). Stanley & Pamment (1993) showed that acetaldehyde accumulates intracellularly in fermenting yeast to concentrations well above extracellular values. However, it is still not clear whether intracellular acetaldehyde accumulation is a major factor of inhibition in yeast during wine fermentation. Stanley *et al* (1993) showed that exogenously added acetaldehyde at above 300 mg/L inhibited yeast growth. Considering the production of acetaldehyde by wine yeast and the toxicity of acetaldehyde, Liu and Pilone (2000) have suggested that acetaldehyde plays a role in sluggish and stuck alcoholic fermentations. This hypothesis remains to be substantiated.

In contrast to the inhibitory effects of acetaldehyde it has also been shown that at low concentrations, acetaldehyde increases the specific growth rate of yeast in the presence of added ethanol and greatly reduced the lag phase (Stanley *et al*, 1993). This stimulatory affect may be due to the role of acetaldehyde in $NAD^+$ regeneration and energy production via glycolysis (Stanley *et al*, 1997).

### 2.3.2. Effect of Acetaldehyde on Wine LAB

Acetaldehyde consumption and/or depletion during MLF has been observed (Somers & Wescombe, 1987), but to date little is known about the effects acetaldehyde may have on wine LAB. It has been shown that wine LAB can metabolise the acetaldehyde moiety of acetaldehyde bound $SO_2$ (Fornachon 1963; Hood, 1983), but the implications of this metabolism are unknown. Much is known however about the effect of acetaldehyde on dairy LAB. Some dairy LAB (in particular *Leuconostoc mesenteroides* ssp *cremoris*) are able to metabolise acetaldehyde (Keenan *et al*, 1966a; Liu *et al*, 1997). Studies have shown that low concentrations of acetaldehyde (<100 mg/L) stimulates the growth of the dairy LAB, *Leuconostoc mesenteroides* ssp. *cremoris*, while high levels (>100 mg/L) inhibits growth (Collins & Speckman, 1974; El-Gendy *et al*,1983; Schmitt & Divies, 1990). It is thought that acetaldehyde acts as a hydrogen acceptor in the oxidation of NADH during heterofermentation, resulting in extra ATP being produced and thus an increase in growth of the bacteria.

If wine LAB can utilise acetaldehyde, then this could have an impact on wine flavour. Winemakers try to minimise the production of excess acetaldehyde because of the adverse sensory effect it produces (section 2.2.1). Therefore, the removal of acetaldehyde by wine LAB is desirable as it will also lessen the need for the addition of large amounts of $SO_2$ to mask this unwanted compound. In the dairy industry, LAB have been used to remove acetaldehyde and thus improve flavour (Keenan & Lindsay, 1966).

In dairy LAB, the metabolism of acetaldehyde produces ethanol and acetic acid (Lees & Jago, 1976a; Collins & Speckman, 1974; Liu *et al*, 1997). If wine LAB are able to metabolise acetaldehyde, than they may produce products which could influence wine flavour. Any inhibition or stimulation of wine LAB could affect MLF. Given that high concentrations of acetaldehyde may cause growth inhibition, as described above, Liu and Pilone (2000) have proposed a possible role of acetaldehyde in stuck and sluggish MLF.

# Chapter 3 General Materials & Methods

## 3.1. Preparation of Wine LAB Media

### 3.1.1. Preparation of Apple MRS (AMRS) Broth/Agar

Commercial MRS Broth..... 52 g
Apple Juice*.....200 mL
DI Water.....800 mL

Heat to dissolve, cool, then adjust pH to ~5.5** with 10% (w/v) KOH or 5 M HCl

Agar..... 20 g

*Use clear apple juice with no preservatives. 250 mL package of 'Fresh-Up Old Fashioned Apple Juice Concentrate' (Frucor Beverages, New Zealand) and dilute with DI water to make 1 L of juice.

**The addition of the acidic apple juice is usually sufficient to lower the pH to the desired pH of 5.5 from the normal pH of 6.2 of the commercial medium.

Sterilise in a pressure cooker at 15 psig for 15 minutes.

### 3.1.2. Preparation of 2X Basal Broth (BB)

Tryptone ..... 5 g
Peptone..... 5 g
Yeast Extract ..... 5 g
*Mineral Solution.....5 mL
**Tween 80 (5% w/w aqueous)..... 1 mL
DI Water..... ca 450 mL

Heat to dissolve, cool, then adjust pH to 5.5 with 10% (w/v) KOH or 5 M HCl

DI Water.....add to 500 mL (to give 2X strength)

Sterilise in a pressure cooker at 15psig for 15 min.

*Mineral solution = 20 g $MgSO_4.7H_2O$ plus 5 g $MnSO_4.4H_2O$ in 100 mL DI water. Store frozen.

**5% Tween 80 is prepared by adding 5 g of this component to 95 g DI water. Warm to dissolve and store frozen.

### 3.1.3. Preparation of Vegetable Juice Glucose (VJG) Broth/Agar

2X BB medium.....500 mL
Glucose..... 5 g
*Vegetable juice serum .....200 mL
DI water.....300 mL

Heat to dissolve, cool, then adjust pH to 5.5 with 10% (w/v) KOH or 5 M HCl

Agar..... 20 g

Sterilise in a pressure cooker at 15 psig for 15 minutes

*Prepare vegetable juice serum by filtering Campell's V8 Juice (Australia) through Whatman No.1 filter paper in a Buchner funnel with diatomaceous earth as a filtering aid (Kenite Diatomite 3000, Witco Corp., N.Y).

## 3.2. Preparation of Wine Yeast Media

### 3.2.1. Preparation of Yeast Media Broth (YM)

Commercial YM Broth (Difco) consists of, per litre:
Yeast Extract ..... 3 g
Malt Extract..... 3 g
Peptone..... 5 g
Dextrose ..... 10 g

For YM agar, add:

Agar.....20 g/L

Dissolve 21 g of commercial YM broth (Difco) in 1 L DI water. Heat to dissolve and then sterilise in a pressure cooker at 15 psig for 15 minutes.

### 3.2.2. Preparation of Grape Juice Broth (GJ)

Mix one volume white grape juice (Grapetise™, New Zealand Apple & Pear Marketing Board, Wellington), with one volume DI water. Per litre, add 5 g yeast extract (YE) and 3 g L-Malic Acid. Adjust pH to 4.5. Heat to dissolve and then sterilise in a pressure cooker at 15 psig for 15 minutes.

## 3.3. Preparation of Buffers

### 3.3.1. Preparation of Tartrate Buffer (50 mM)

Tartaric acid..... 7.5 g
Mineral Solution (section 3.1.2)..... 5 mL
DI Water.....add up to 900 mL

Heat to dissolve, cool, and then adjust pH as required with 10% (w/v) KOH or 5 M HCl. Then dilute with DI water to 1 litre.

### 3.3.2. Preparation of Phosphate Buffer (50 mM)

*di*-Potassium hydrogen orthopohosphate trihydrate .....11.4 g
DI Water..... 900 mL

Heat to dissolve, cool, and then adjust pH to 7.0 with 5 M HCl. Then dilute with DI water to 1 litre.

## 3.4. Chemical Analysis

### 3.4.1. Determination of Free and Total Sulphur Dioxide

Ripper Procedure: modified from Amerine & Ough (1974).
The Rippler determination of sulphur dioxide is based on the oxidation reaction:

$$SO_2 + I_3 + H_2O \longrightarrow SO_3 + 3\,I^- + 2\,H^+$$

REAGENTS

Sodium hydroxide solution (10% (w/v))
Sulphuric acid solution (Four-fold dilution of concentrated (98%) $H_2SO_4$).
Standardised iodine solution (0.01N)
Starch indicator (BDH Prod. No. 200543P)

FREE SULPHUR DIOXIDE DETERMINATION:

1) Place 5 mL wine in an Erlenmyer flask
2) Mix in 1 mL sulphuric acid
3) Add a spatula of starch indicator
4) Rapidly titrate with standardised iodine to a blue endpoint that lasts 30 seconds

DETERMINATION OF TOTAL SULPHUR DIOXIDE:

1) Place 5 mL wine in an Erlenmeyer flask
2) Mix in 1 mL sodium hydroxide solution and let stand for 15 minutes
3) Proceed as with FREE SULPHUR DIOXIDE DETERMINATION adding sulphuric acid and starch indicator before titrating with standardized iodine

CALCULATIONS:

$$\text{Sulphur Dioxide (mg/L)} = \frac{\text{mL Iodine Titrant X Iodine Normality X 32 X1000}}{\text{mL Wine}}$$

### 3.4.2. Bradford Protein Assay

Protein concentration was determined using the method of Bradford, (1976)

Coomassie Brillant Blue..... 100 mg
Ethanol (95%) .....50 mL

Mix and leave overnight to dissolve, then add

$H_3PO_4$ (85%) .....100 mL
DI Water.....850 mL

Procedure

1) Prepare a standard curve using protein standard of known concentration (0 - 300 µg/mL). Use DI water as a diluent when making dilutions.
2) Add 0.1 mL of protein standards (known concentrations, 0 - 300 µg/mL) to 2 mL Commassie Brillant Blue reagent (see above) in a cuvette, mix and wait exactly 10 minutes. Read absorbance at 595 nm in a spectrophotometer. Plot a standard curve.
3) Repeat the above procedure but use the sample (unknown protein concentration) and calculate protein concentrations using the standard curve.

Protein concentrations of up to 300 µg/mL in sample can be measured.

## 3.5. Enzymatic Analysis

### 3.5.1. Enzymatic Determination of Acetaldehyde

Acetaldehyde concentration was determined using a modified version of an enzymatic assay using the enzyme aldehyde dehydrogenase (Al-DH) (Bergmeyer, 1974). This is an enzymatic determination that measures the absorbance of NADH formed corresponding to the conversion of acetaldehyde to acetic acid catalysed by the enzyme Al-DH. Acetaldehyde is oxidised by Al-DH to acetic acid and the amount of NADH formed is stoichiometric with the amount of acetaldehyde in the sample. The equilibrium lies to the right, in favour of acetate and NADH. Absorbance of NADH was measured using a visible light spectrophotometer (Nova Tech) at 340 nm.

$$\text{Acetaldehyde} + NAD^+ + H_2O \xleftrightarrow[\text{Al-DH}]{} \text{Acetate} + NADH + H^+$$

Reagents:

| NAD-Buffer mixture with Tris/EDTA/KCl/NAD/BSA (pH 8.0) | |
|---|---|
| Tris ..... 0.214 M | Tris (121.1g/mol) ..... 2.6g/100ml $H_20$ |
| EDTA ..... 1.75 mM | EDTA (370g/mol) ..... 65mg/100ml Buffer |
| KCl ..... 104 mM | KCL (74.5g/mol) ..... 775mg/100ml Buffer |
| NAD ..... 0.27 mM | NAD (663.4g/mol) ..... 18mg/100ml Buffer |
| BSA ..... Pure | BSA (pure) ..... 55.5mg/100ml Buffer |
| Aldehyde dehydrogenase ..... 22.2 U/ml | Ald-DH (20U/mg) ..... 1.11mg/ml |
| Sample (up to)(= 40 mg/L) ..... 0.88 mM | |

Enzymatic assay:

Wavelength: 340nm; Lightpath 10mm
Final volume: 1.11ml

| Add | Concentrations in assay |
|---|---|
| Buffer..... 1 ml | Tris..... 0.193 M<br>EDTA..... 1.58 mM<br>KCL..... 94 mM<br>NAD..... 0.24 mM<br>BSA..... 0.5 g/L |
| Sample..... 0.1 ml<br>Mix, wait for constant Extinction and read $A_1$. | up to 80 µM acetaldehyde |
| Aldehyde-DH..... 0.01 ml<br>Mix, wait for completion of the reaction and read $A_2$ | Aldehyde-DH..... 200 mU/ml |

$\Delta A_{Acetaldehyde} = \Delta A_2 - \Delta A_1$.

Linear to 60 mg/L with 30 min at room temperature

Prepare a standard curve using standard acetaldehyde solutions of known concentration (range 0 - 60 mg/L).

Calculate acetaldehyde concentration of sample using the standard curve.

### 3.5.2. Enzymatic Determination of Ethanol

Ethanol concentration was determined using a modified version of an enzymatic assay using the enzyme alcohol dehydrogenase (ADH) (Bergmeyer, 1974). This is an enzymatic determination that measures the absorbance of NADH formed corresponding to the conversion of ethanol to acetaldehyde catalyzed by ADH. Ethanol is oxidized by ADH to acetaldehyde and the amount of NADH formed is stoichiometric with the amount of ethanol in the sample. The equilibrium can be virtually completely displaced to the right at alkaline pH and by trapping the acetaldehyde formed as the hydrazone. Absorbance of NADH was measured using a visible light spectrophotometer (Nova Tech) set at 340 nm.

$$\text{Ethanol} + NAD^+ + H_2O \xleftrightarrow[\text{ADH}]{} \text{Acetaldehyde} + NADH + H^+$$

Reagents:

| NAD-Buffer mixture with Sodium Pyrophosphate/Semicarbazide/Glycine (pH 8.7) | |
|---|---|
| Sodium Pyrophosphate..... 87.12 mM | Sodium Pyrophosphate (446.1g/mol).... 3.9g/100ml $H_2O$ |
| Semicarbazide..... 87.12 mM | Semicarbazide (111.53g/mol)..... 972mg/100ml Buffer |
| Glycine..... 26.86 mM | Glycine (75.1g/mol)..... 202mg/100ml Buffer |
| BSA..... Pure | BSA (Pure)..... 56mg/100ml Buffer |
| NAD..... 0.88 mM | NAD (663.4g/mol)..... 58mg/100ml Buffer |
| Alcohol dehydrogenase..... 6534 U/ml<br>Sample (up to) ..... 1.7 mM | Alcohol-DH (400U/mg)..... 16.4mg/ml |

Enzymatic assay:

Wavelength: 340nm; Lightpath 10mm
Final volume: 1.11ml

| Add | Concentrations in assay |
|---|---|
| Buffer..... 1 ml | Sodium Pyrophosphate..... 87.12 mM<br>Semicarbazide..... 87.12 mM<br>Glycine..... 24.2 mM<br>NAD..... 0.73 mM<br>BSA..... 0.5 g/L |
| Sample..... 0.1 ml<br>Mix, wait for constant Extinction and read $A_1$. | up to 150 µM Ethanol |
| Alcohol-DH..... 0.01 ml<br>Mix, wait for completion of the reaction and read $A_2$ | Alcohol-DH..... 54 U/ml |

$\Delta A_{Ethanol} = \Delta A_2 - \Delta A_1$.

Prepare a standard curve using standard ethanol solutions of known concentration (range 0 - 60 mg/L).

Linear to 60 mg/L with 30 min at 37°C

Calculate ethanol concentration of sample using the standard curve.

### 3.5.3. Enzymatic Determination of L-Malic acid

L-Malic acid concentration was determined using a modified version of an enzymatic assay using the enzyme malate dehydrogenase (MDH) (Bergmeyer, 1974). This is an enzymatic determination that measures the absorbance of NADH formed corresponding to the conversion of L-malic acid to oxaloacetate catalyzed by MDH. L-malic acid is oxidised by MDH to oxaloacetate and the amount of NADH formed is stoichiometric with the amount of L-malic acid in the sample. Protons are trapped by

an alkaline medium and oxaloacetate by hydrazine. A high concentration of $NAD^+$ displaces the equilibrium further to the right. Absorbance of NADH was measured using a visible light spectrophotometer (Nova Tech) at 340 nm.

L-malic acid + $NAD^+$ $\xleftrightarrow[\text{L-MDH}]{}$ Oxaloacetate + NADH + $H^+$

Reagents:

| Hydrazine/glycine buffer (pH 9.0) | |
|---|---|
| Glycine..... 0.5 M | Glycine (75.1 g/mol)..... 37.8 g/L $H_20$ |
| Hydrazine..... 0.397 M | Hydrazine (130.1 g/mol)..... 51.7 g/L $H_20$ |
| NAD..... 3 mM | NAD (663.4 g/mol)..... 200 mg/100ml buffer |
| MDH..... 3000 U/ml | MDH (6000 u/ml)..... 1ml/1ml $(NH_4)_2SO_4$ |
| Sample (up to)..... 2.2 mM | |

Alkaline buffer is stable for 3 months

Buffer/NAD is stable for several weeks

Enzyme-solutions are stable at 4°C (> 1 year)

Enzymatic assay:

Wavelength: 340nm; Lightpath 10mm
Final volume: 1.09 ml

| Add | Concentrations in assay |
|---|---|
| Buffer..... 1 ml | Glycine..... 0.46 M<br>Hydrazine..... 0.36 M<br>NAD..... 2.75 mM |
| Sample..... 0.08 ml<br>Mix, wait for constant Extinction and read $A_1$ | up to.....0.15 mM (20 mg/L) |
| MDH-suspension..... 0.01 ml<br>Mix, wait for completion of the reaction and read $A_2$<br>(30 min at 37°C or 60 min at 25°C) | MDH..... 27 U/ml |

$\Delta A_{\text{Malic acid}} = \Delta A_2 - \Delta A_1$

<u>Standard</u>

Malic acid: 3 g/l (0.15g/50 ml)

Dilute 1:10 to 300 mg/l

Prepare a standard curve using standard

Linear within 200 mg/l with 40 min at 37°C

Calculate malic acid concentration of sample using the standard curve.

### 3.5.4. Enzymatic Determination of Acetic Acid

Acetic acid concentration was determined using the acetic acid assay test kit (Boehringer Mannhiem GmbH, Germany). Acetic acid is converted in the presence of the enzyme acetyl-CoA sythetase (ACS[1]) and adenosine-5'-triphosphate (ATP) and coenzyme A to acetyl-CoA

(1) Acetic acid + ATP + CoA $\xrightarrow[ACS^1]{}$ acetyl-CoA + AMP[2] + pyrophosphate

Acetyl-CoA reacts with oxaloacetate to citrate in the presence of citrate synthase (CS)

(2) Acetyl-CoA + oxaloacetate + $H_2O$ $\xrightarrow[CS]{}$ citrate + CoA

The oxaloacetate required for reaction (2) is formed from the L-malate and NAD in the presence of L-malate dehydrogenase (L-MDH)

(3) L-malate + $NAD^+$ $\xrightarrow[L\text{-}MDH]{}$ oxalacetate + NADH + $H^+$

The determination is based on the formation of NADH measured by absorbance using a visible light spectrophotometer (Nova Tech) at 340 nm.
Calculations for the determination of acetic acid can be found in the instruction leaflet that comes with the test kit.

## 3.6. Dry Weight Analysis

The dry weight of cell suspensions was determined by pipetting 1 mL of culture into a preweighed 1.7 mL microcentrifuge tube. The supernatant was removed after centrifugation (10,000 *g* for 5 min) and the tube containing cell pellets was dried overnight at 100°C. The difference in weight after cooling was corrected for by subjecting empty tubes to the same procedure. Dry weight could then be determined.

## 3.7. Vinification

### 3.7.1. Media Preparation

4 L of pure white grape juice (Grapetise$^{Tm}$, New Zealand Apple and Pear Marketing Board, Wellington) was used as the juice for the vinification. The juice had a natural soluble solids content of approximately 13 °Brix (g/100g) and a pH of 3.5. The soluble solids were adjusted to 17 °Brix by the addition of 40 g of sucrose (to give 11% ethanol (v/v) after alcoholic fermentation by yeast).

### 3.7.2. Culture Preparation

The wine yeast used was from the Wine Microbiology Laboratory Culture Collection of the Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand. All yeast cultures were maintained on YM agar slopes at 4°C. The wine yeast *S. bayanus* Première Cuvée was grown on YM agar slope (section 3.2.1) by overnight incubation at 30°C. The yeast culture was then aseptically transferred to YM broth (section 3.2.1) and incubated overnight on a shaker at 30°C (aeration of the culture is beneficial to the yeast as it promotes the formation of strong cell membranes). A subculture was made into 200 mL grape juice broth (section 3.2.2) and incubated overnight on a shaker at 30°C. This culture was used for inoculation of the juice.

### 3.7.3. Experimental Procedure

The juice (section 3.7) was inoculated with 2% (v/v) yeast culture growing in grape juice. The juice was then incubated at 18°C and allowed to ferment to dryness (soluble solids <5 °Brix). During the alcoholic fermentation, optical density measurements were regularly taken. Five mL samples were taken and optical density (OD) was measured in a NovaTech visible light spectrophotometer at 650 nm. Acetaldehyde concentration during the fermentation was also measured from these samples. The sample was centrifuged (10,000 *g* for 5 minutes) and the supernatant was used to determine the concentration of acetaldehyde in the wine (section 3.5.1). Fermentation was terminated by cooling to 4°C and the wine was racked off the yeast lees. L-Malic acid levels were adjusted by the addition of 3 g/L of L-malic acid after alcoholic fermentation so that the malic acid level was approximately 4.2 g/L (section 3.5.3.).

# Chapter 4 Metabolism of Acetaldehyde by Resting Cells of Wine LAB

## 4.1. Metabolism of Free Acetaldehyde by Resting Cells

### 4.1.1. Materials and Methods

#### 4.1.1.1. Stock Solutions

Acetaldehyde stock solution was prepared by adding 1 g of fresh acetaldehyde (stored cold at 4°C) to 100 mL of cold DI water. This solution was then diluted 1:2 to give a final concentration of 5000 mg/L. All dilutions were carried out in a fume hood using cold DI water as acetaldehyde is highly volatile and is an irritant. Ethanol stock solution was prepared by adding 1.05 g of 95% ethanol to 100 mL DI water. This solution was diluted 1:20 to give a final concentration of 500 mg/L. These stock solutions were used for preparing experimental media.

#### 4.1.1.2. Culture Preparation

Wine LAB strains were maintained at 4°C after growth as stabs in AMRS agar. For routine use, subcultures of LAB were prepared in AMRS broth (one loop full in 5 mL broth) and stored at 4°C without prior incubation (growth). When a culture was required, it was removed from the cold room and incubated at 30°C until growth was visible as indicated by turbidity changes. This 'cold subculturing' technique enabled quick recovery of a culture when needed. Cold subcultures remained viable for several weeks because the cells were not under acid stress (low pH).

#### 4.1.1.3. Experimental Procedure

VJG broth (50 mL) was inoculated with 5 mL of culture (section 4.1.1.2) and incubated at 30°C to the late-log/early stationary phase as assessed by the occurrence of sedimentation. This culture was then transferred into 450 mL VJG broth and incubated at 30°C to the late-log/early stationary phase. Bacterial cells were harvested

by centrifugation at 5000 x *g* for 10 minutes at 15°C. The cells were washed twice with washing buffer (tartrate buffer at pH 4.2, see section 3.3.1) and centrifuged at 5000 x *g* for 10 minutes at 15°C. Cell pellets were resuspended in 25 mL tartrate buffer at pH 3.6 and 9 mL of this cell suspension were pipetted into two small 20 mL glass vials. The two vials were placed in a waterbath (30°C) and stirred gently using small submerged magnetic stirrers. A third vial containing 10 mL of acetaldehyde at 50 mg/L was also placed in the waterbath (acetaldehyde positive control). To another vial, 10 mL of ethanol at 50 mg/L was added (ethanol positive control). To start the reaction, 1 mL of acetaldehyde at 500 mg/L was added to one of the cell suspensions (to give a final acetaldehyde concentration of 50 mg/L). To the other cell suspension, 1 mL of DI water was added (negative control). 0.3 mL samples were immediately taken from each vial and centrifuged (10,000 *g* for 5 minutes). These samples were then placed in an ice bath immediately. Immediately samples were either assayed or stored frozen (-18°C) for subsequent analysis. Samples were taken from each vial every 10 minutes. Samples were analysed for acetaldehyde, ethanol and acetic acid as described in section 3.5.

### 4.1.2. Results

Eleven commercial wine LAB were surveyed for their ability to degrade acetaldehyde and produce ethanol. Results are shown in Table 4.1.1. The table shows that all strains, except for *Pd. damnosus* CUC-4 and *Pd. sp.* 44.40, were able to utilise acetaldehyde with the production of ethanol. Figure 4.1.1 to Figure 4.1.11 show these results. Uninoculated positive controls (containing 50 mg/L acetaldehyde) indicated little loss of acetaldehyde through evaporation during the experiment (data not shown). Negative controls, containing cell culture and no added acetaldehyde, showed no increase in acetaldehyde levels during the course of the experiment (data not shown). Figure 4.1.3 and Figure 4.1.11 show the results for *Pd. damnosus* CUC-4 and *Pd. sp.* 44.40. These malolactic bacteria were unable to degrade acetaldehyde under the current conditions and production of ethanol was not observed. On a molar basis, the amount of ethanol produced did not fully account for the amount of acetaldehyde degraded by each strain. Therefore, two strains (*Lb. hilgardii* MHP and *Lb. delbrueckii* CUC-1) were tested for acetic acid production as well as for acetaldehyde degradation and ethanol production. Figure 4.1.1 and Figure 4.1.2 show the results of the analysis of acetic acid production for *Lb. hilgardii* MHP and *Lb. delbrueckii* CUC-

1. Both bacteria produced acetic acid as well as ethanol. The amount of ethanol and acetic acid produced accounted for about 75% of the acetaldehyde degraded in the case of *Lb. hilgardii* MHP and about 60% in the case of *Lb. delbrueckii* CUC-1. Therefore, some utilised acetaldehyde was unaccounted for and it is not known at this stage what the fate of this acetaldehyde was.

**Table 4.1.1** Acetaldehyde utilisation and product formation by resting cells of wine LAB in tartrate buffer (pH 3.6) at 30°C.

| [1]**Organism** | **Strain** | **Acetaldehyde Degradation** | **Ethanol Production** |
|---|---|---|---|
| *O. oeni* | MCW | + | + |
| *O. oeni* | VFO | + | + |
| *O. oeni* | EQ54 | + | + |
| *O. oeni* | ML34 | + | + |
| *O. oeni* | 2001 | + | + |
| *O. oeni* | Lol11 | + | + |
| *Lb. hilgardii* | MHP | + | + |
| *Lb. delbrueckii* | CUC-1 | + | + |
| *Lb. buchneri* | CUC-3 | + | + |
| *Pd. damnosus* | CUC-4 | - | - |
| *Pd. sp.* | 44.40 | - | - |

[1]Dry weights for all strains ranged between 3 and 6 mg.

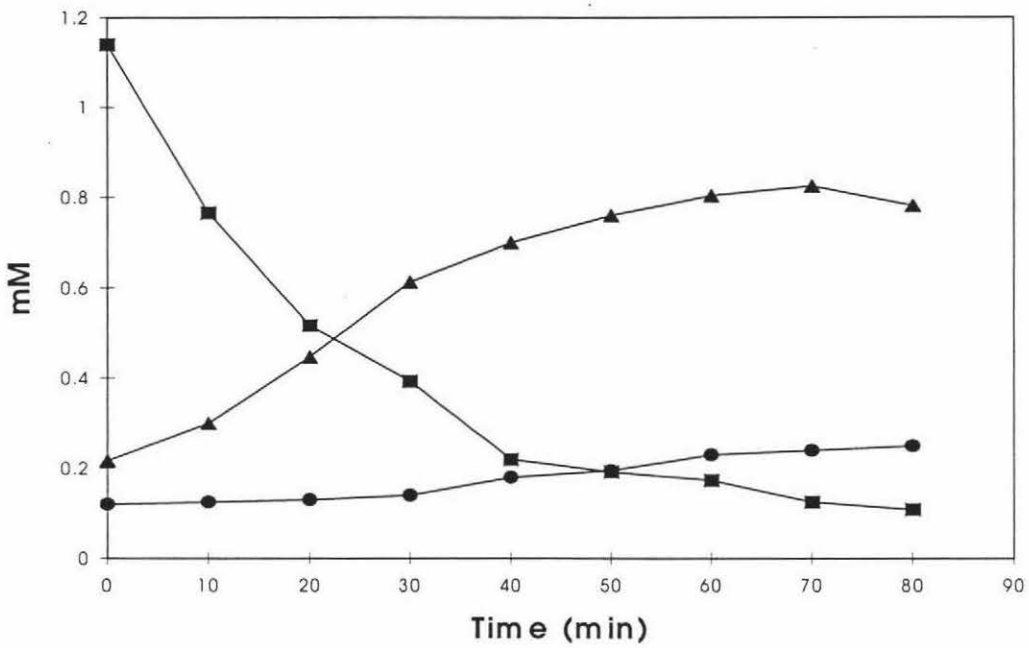


**Figure 4.1.1** Degradation of acetaldehyde and production of ethanol and acetic acid by resting cells of *Lb. hilgardii* MHP in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ● acetic acid; ▲ ethanol.

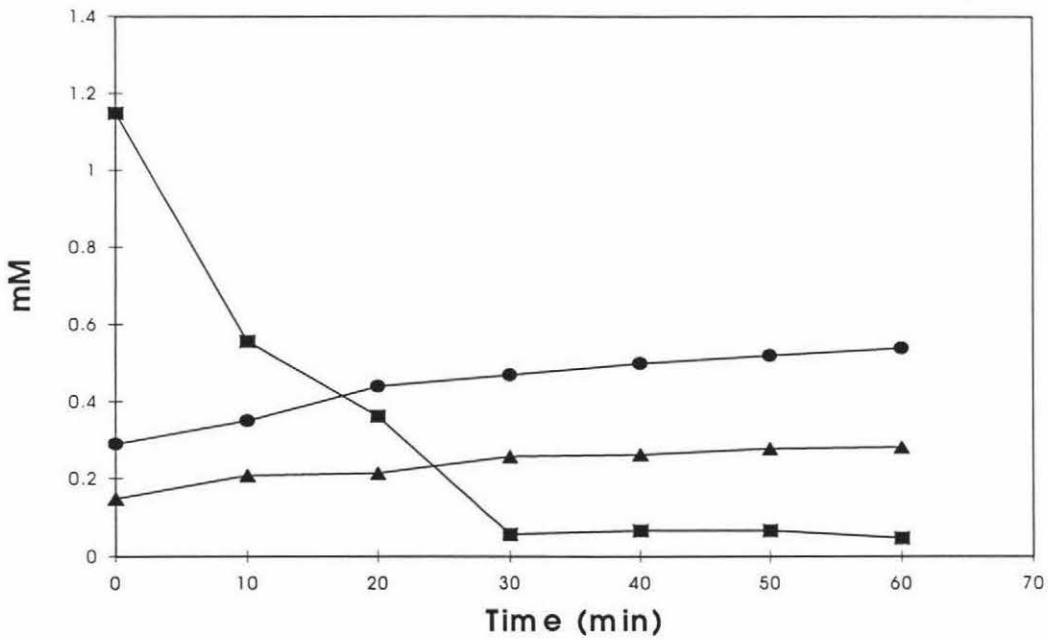


**Figure 4.1.2** Degradation of acetaldehyde and production of ethanol and acetic acid by resting cells of *Lb. delbrueckii* CUC-1 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ● acetic acid; ▲ ethanol.

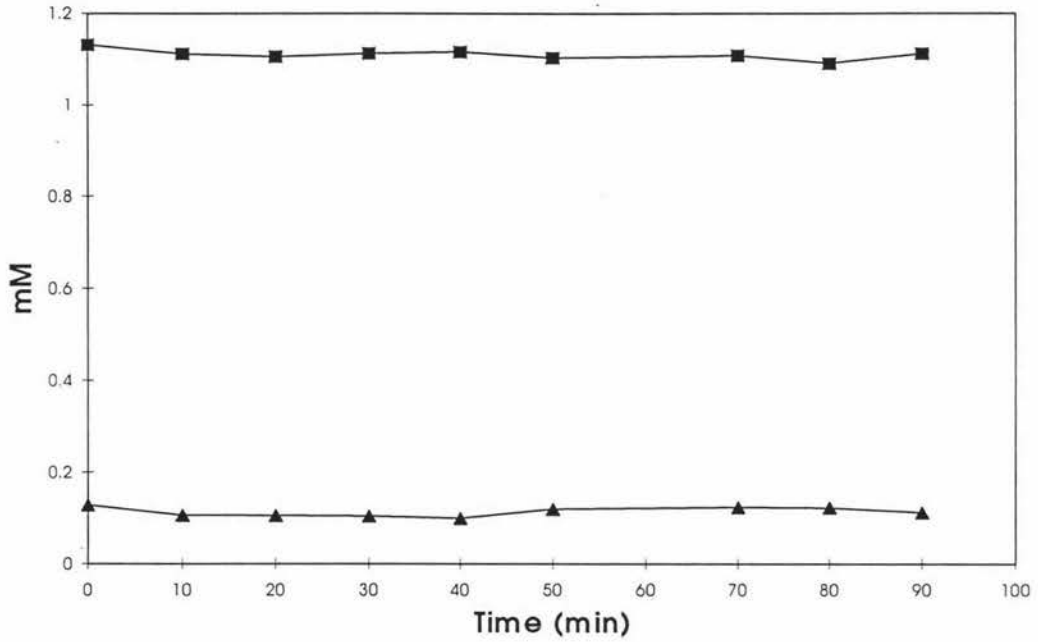


**Figure 4.1.3** Changes in the concentrations of acetaldehyde and ethanol during **a** resting cell experiment using *Pd. damnosus* CUC-4 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

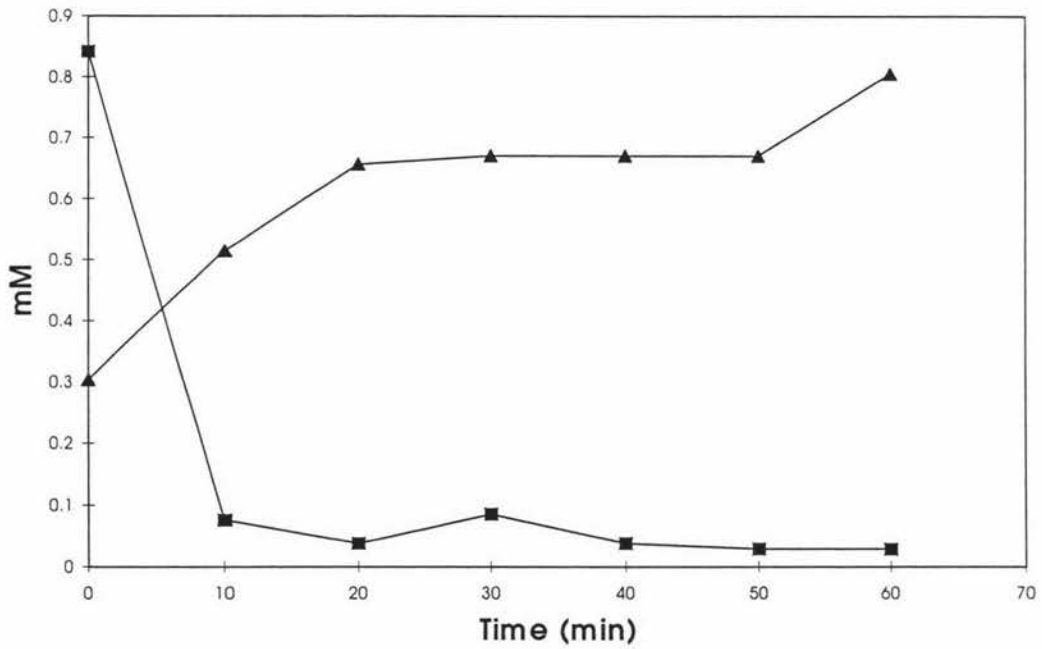


**Figure 4.1.4** Degradation of acetaldehyde and production of ethanol by resting cells of *O. oeni* Lol11 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

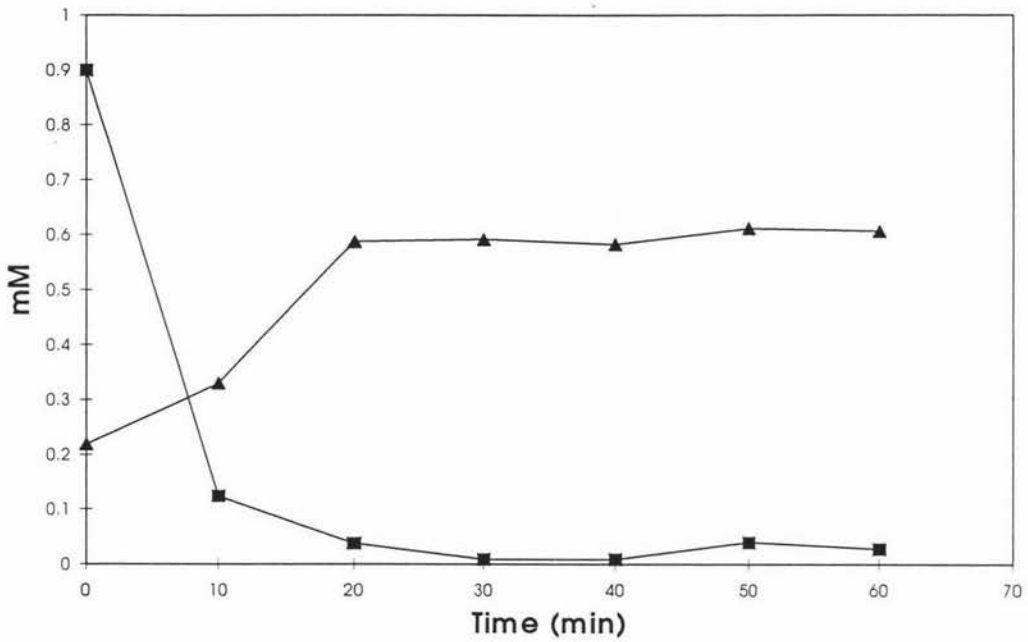


**Figure 4.1.5** Degradation of acetaldehyde and production of ethanol by resting cells of *O. oeni* 2001 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

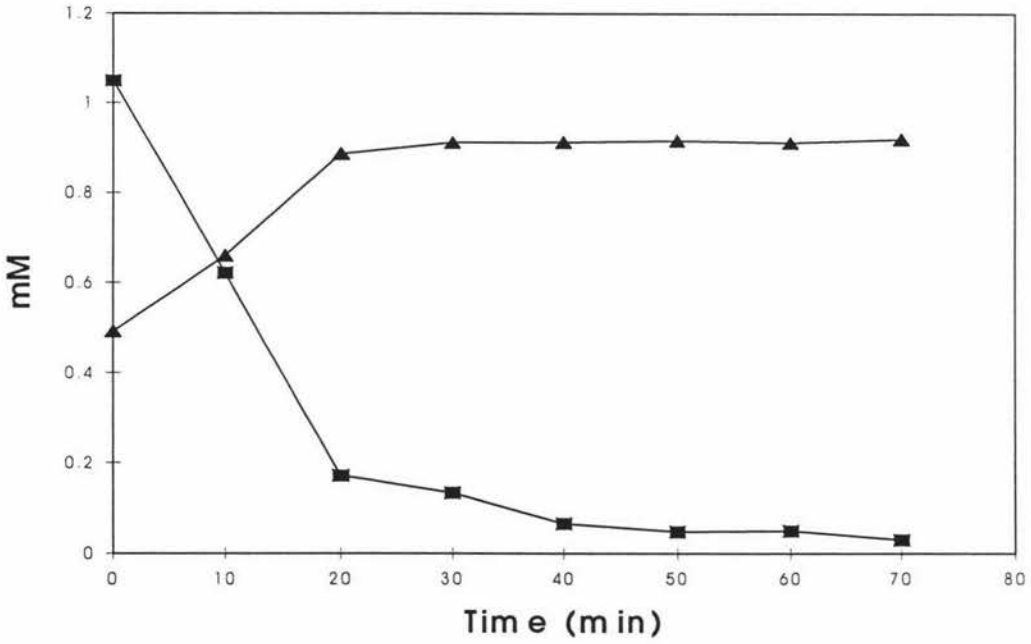


**Figure 4.1.6** Degradation of acetaldehyde and production of ethanol by resting cells of *O. oeni* VFO in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

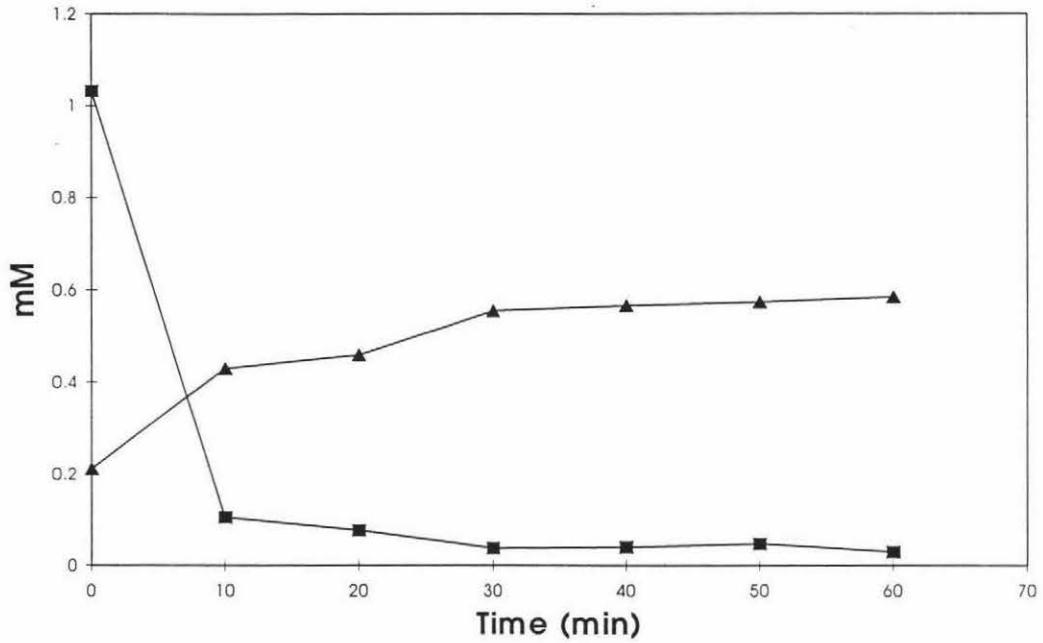


**Figure 4.1.7** Degradation of acetaldehyde and production of ethanol by resting cells of *O. oeni* EQ54 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

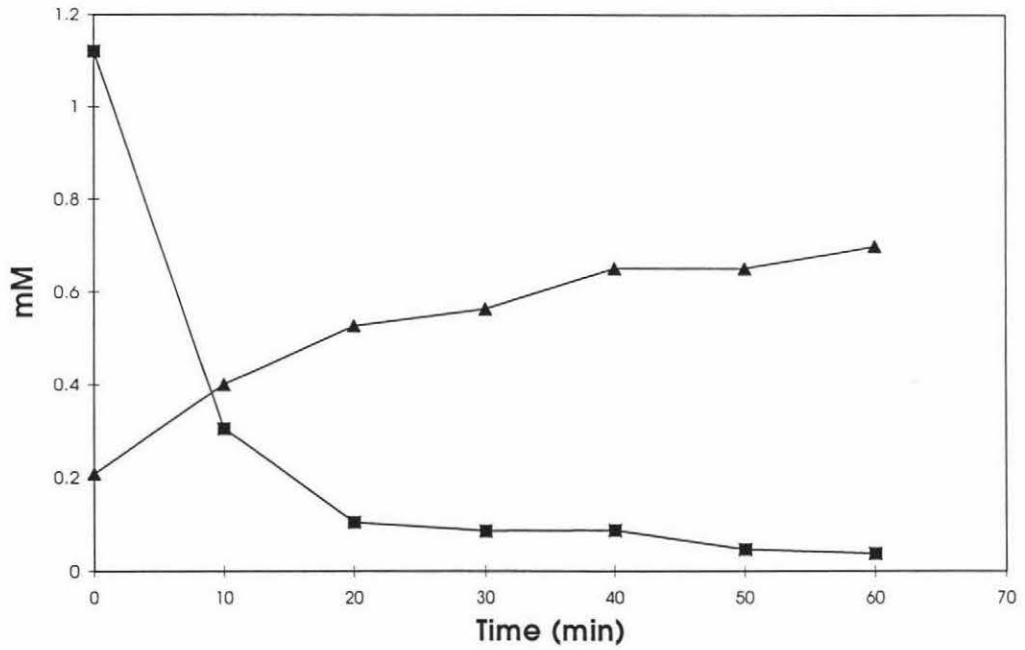


**Figure 4.1.8** Degradation of acetaldehyde and production of ethanol by resting cells of *Lb. Buchneri* CUC-3 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

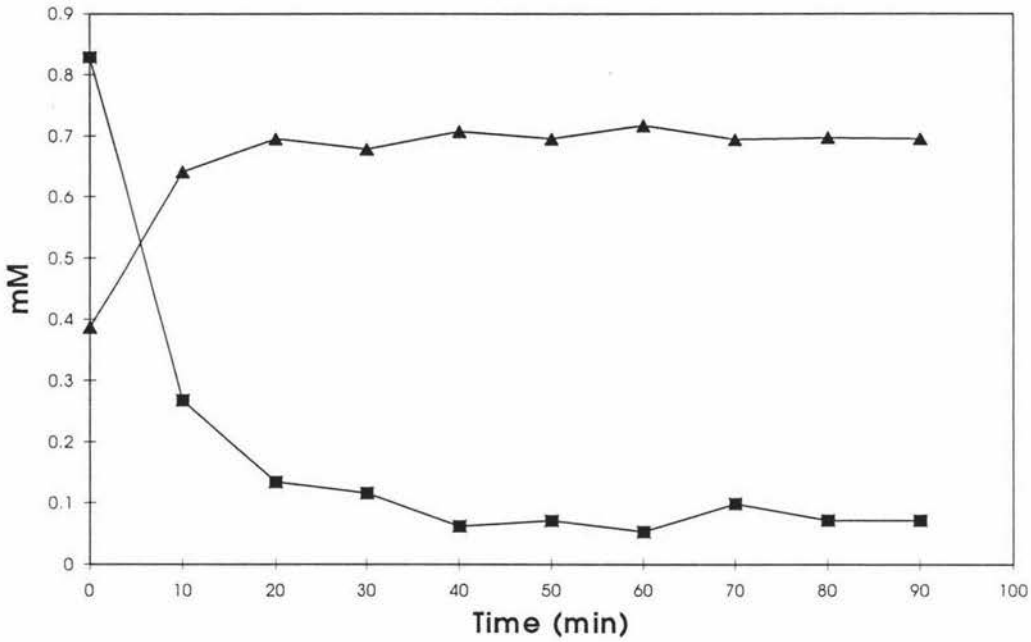


**Figure 4.1.9** Degradation of acetaldehyde and production of ethanol by resting cells of *O. oeni* MCW in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

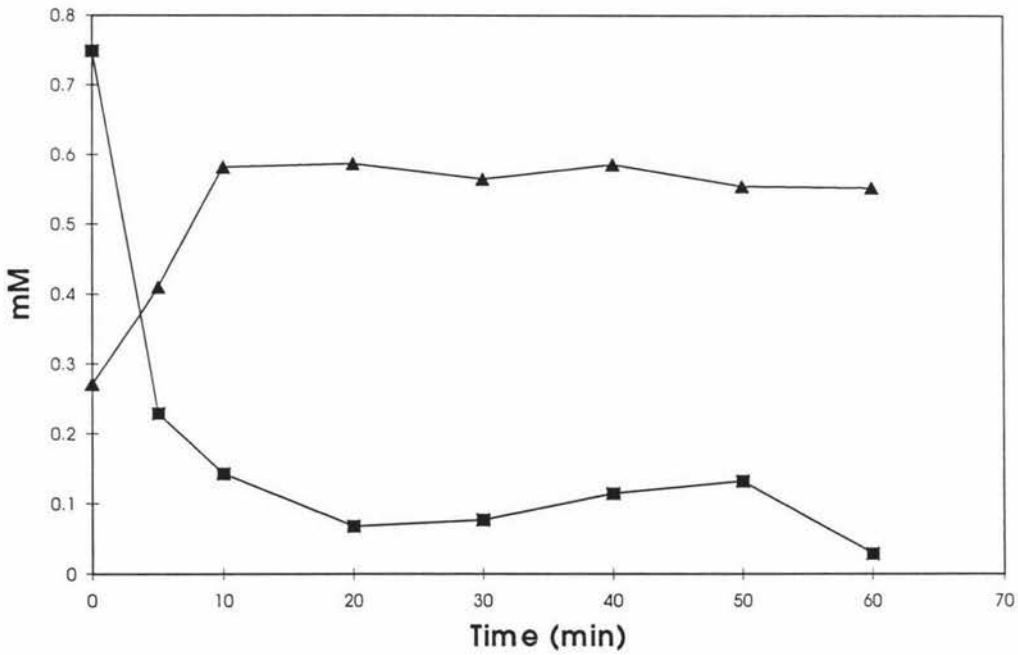


**Figure 4.1.10** Degradation of acetaldehyde and production of ethanol by resting cells of *O. oeni* ML34 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

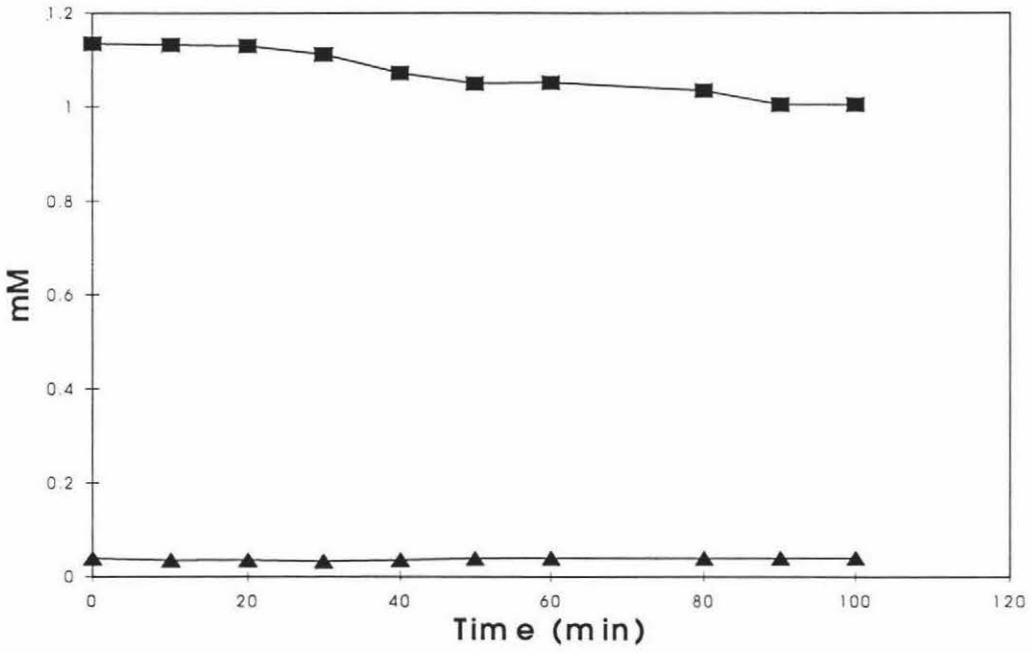


**Figure 4.1.11** Degradation of acetaldehyde and production of ethanol by resting cells of *Pd. sp* 44.40 in a tartrate buffer (pH 3.6) at 30°C. Symbols; ■ acetaldehyde; ▲ ethanol.

## 4.2. Metabolism of Sulphur Dioxide Bound Acetaldehyde by Resting Cells

In wine, the majority of acetaldehyde will be in a bound form. Sulphur dioxide binds strongly to acetaldehyde and in wine, almost all acetaldehyde is bound to sulphur dioxide (if sufficient $SO_2$ is present in the wine). Therefore, it is important to determine the ability of wine LAB to degrade $SO_2$ bound acetaldehyde.

### 4.2.1. Materials And Methods

#### 4.2.1.1. Media Preparation

Media was prepared as described in section 3.1.

#### 4.2.1.2. Culture Preparation

Cultures were prepared as in 4.1.

4.2.1.3. Experimental Procedure

The experimental procedure was similar to that used in section 4.1.1.3 except that sulphur dioxide ($SO_2$) bound acetaldehyde was used. $SO_2$ bound acetaldehyde was prepared by adding $SO_2$ to an acetaldehyde solution at an equimolar concentration. Sulphur dioxide was added to the acetaldehyde solution through the addition of potassium metabisulphite. To determine that all the acetaldehyde was bound to $SO_2$, free $SO_2$ was measured. This was conducted using the method given in section 3.4.1. However, if excess free $SO_2$ is present in the acetaldehyde solution, then this will inhibit the cells. Therefore, an effort was made to remove as much free $SO_2$ as possible. This involved lowering the pH of the $SO_2$ bound acetaldehyde to about 1.5 using HCL (5 M) and then purging with air. At pH 1.5, the majority of $SO_2$ is in the molecular gas form and therefore it is possible to remove the free $SO_2$ by purging with air. The pH of the acetaldehyde solution was then raised back to about 4.0 by the addition of 5 M NaOH. This allowed the removal of free $SO_2$ down to a level of about 4 mg/L. This solution was added to the cells to begin the reaction as per section 4.1.1.3. Samples were analysed for acetaldehyde concentration as described in section 3.5.1. The enzymatic method for analysis of acetaldehyde measures both free and bound acetaldehyde (total acetaldehyde).

### 4.2.2. Results

Figure 4.2.1 shows the degradation of $SO_2$ bound acetaldehyde by *Lb. buchneri* CUC-3 and *O. oeni* MCW. Compared to the positive control, there was a significant reduction in acetaldehyde over time for both bacteria. This shows that both *Lb. buchneri* CUC-3 and *O. oeni* MCW were able to metabolise the acetaldehyde moiety of the $SO_2$ bound acetaldehyde.

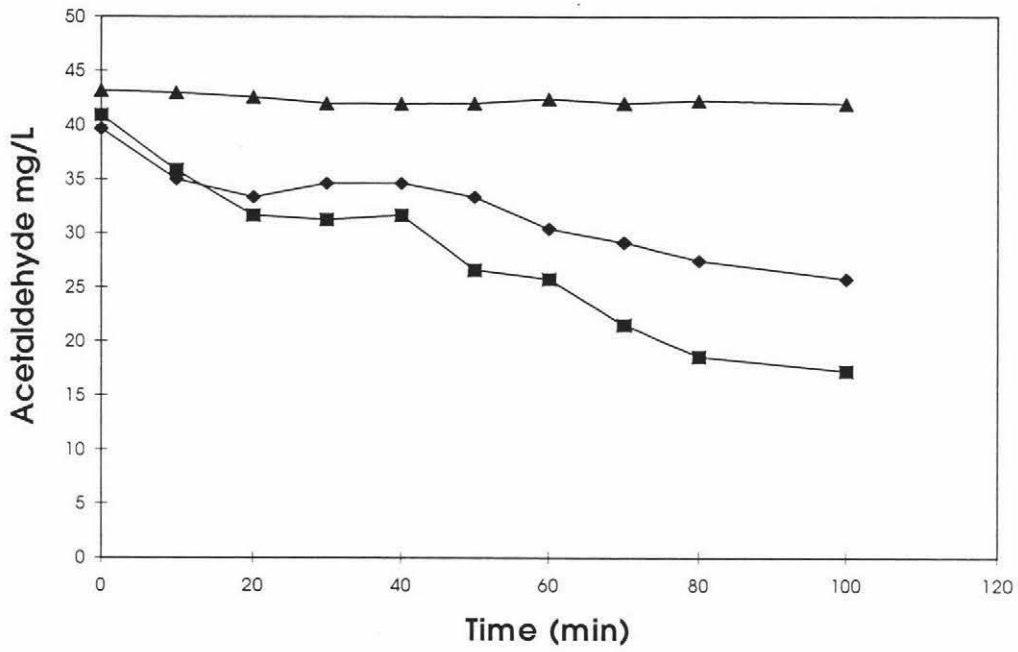


**Figure 4.2.1** Degradation of $SO_2$ bound acetaldehyde by resting cells of *Lb. buchneri* CUC-3 and *O. oeni* MCW in a tartrate buffer (pH 3.6) at 30°C. Dry weights ranged from 3 to 6 mg. Symbols; ◆ MCW; ■ CUC-3; ▲ control.

## 4.3. Discussion

### 4.3.1. Metabolism of Free Acetaldehyde by Resting Cells

The results of the survey have shown that 9 out of the 11 strains were able to metabolise acetaldehyde in a buffered system at wine pH. This included both heterofermentative strains (MCW, EQ54, ML34, VFO, Lol11, 2001, CUC-3, MHP) and the homofermentative strain (CUC-1). The two strains unable to metabolise acetaldehyde were both homofermentative pediococci. Dry weights for theses strains were very similar to the dry weights for the other strains tested and so cell concentration can not explain this negative result. It seems that under these conditions neither *Pd. damnosus* CUC-4 or *Pd. sp* 44.40 were able to metabolise acetaldehyde.

The metabolism of acetaldehyde by these bacteria corresponded with the production of ethanol and acetic acid. On a molar basis, the products ethanol and acetic acid account for the majority of acetaldehyde utilised. Therefore, these results demonstrate the ability of wine LAB to metabolise acetaldehyde, producing ethanol and acetic acid as final products. This metabolism of acetaldehyde to form ethanol and acetic acid has

been shown to occur in dairy LAB. Lees & Jago (1976a) and Liu *et al,* (1997) have shown that the dairy LAB *Leuconostoc mesenteroides* subsp. *cremoris* was able to metabolise acetaldehyde, converting it to ethanol and acetic acid. Lees & Jago (1976a) have shown that *Leuconostoc cremoris*, contained the enzymes aldehyde dehydrogenase (Al-DH) and alcohol dehydrogenase (ADH). Al-DH converts acetaldehyde to acetic acid and ADH converts acetaldehyde to ethanol. Therefore, it was thought that wine LAB would contain these enzymes if they were capable of converting acetaldehyde to ethanol and acetic acid. This hypothesis was explored in Chapter 8.

The formation of ethanol and acetic acid by wine LAB is not expected to have any major impact on wine flavour. This is because ethanol is desirable in wine. Although high levels of acetic acid is not desirable in wine, the amount of acetic acid produced by the metabolism of acetaldehyde by wine LAB may be too small to affect the overall flavour and quality of the wine and is dependent on the concentration of acetaldehyde. The removal of acetaldehyde from the wine, however, is expected to have an impact on wine flavour. This is discussed in section 5.3.

It was thought that acetaldehyde might act as an electron acceptor during anaerobic fermentation (Collins & Speckman, 1974). This proposed model is shown in Figure 4.3.1. During heterofermentation, acetaldehyde may be reduced to ethanol and thus recycle $NAD^+$. This recycling of $NAD^+$, through the reduction of acetaldehyde, would mean that acetyl-phosphate would be available to be converted to acetic acid through the action of the enzyme acetate kinase. This is an ATP generating process and so would be energetically advantageous for the bacteria. This proposed energetic advantage is explored in more detail in Chapter 6. The model is supported by evidence from this survey, in that acetaldehyde metabolism results in ethanol and acetic acid production. However, during the resting cell experiment the heterofermentative pathway may not have been operating as no sugar was present. Therefore, the production of ethanol and acetic acid may be a result of the oxidation and reduction of acetaldehyde by the enzymes aldehyde dehydrogenase and alcohol dehydrogenase alone. The homofermentative wine LAB strains *Pd. damnosus* CUC-4 and *Pd. sp.* 44.40 did not metabolise acetaldehyde. However, the homofermentative wine LAB strain *Lb. delbrueckii* CUC-1 was also able to do so producing ethanol and acetic acid. This may mean that the enzymes responsible for this metabolic process are strain

dependent and not restricted to just heterofermentative wine LAB, but may also be present in homofermentative wine LAB. This hypothesis is supported by evidence from experiments carried out with dairy LAB where homofermentative dairy LAB metabolised acetaldehyde (Lees & Jago, 1978). Further experiments using additional homofermentative wine LAB will need to be undertaken to clarify this issue.

These results were obtained using resting cells in a buffered system at a wine pH. This experimental system closely mimics the situation in wine after MLF has occurred (stationary phase), but further experiments were needed to confirm whether or not wine LAB are able to metabolise acetaldehyde in wine during growth. These experiments and the results obtained are documented in Chapter 5.

### 4.3.2. Metabolism of $SO_2$ Bound Acetaldehyde by Resting Cells

The results show that in a buffered system at wine pH, resting cells of *O. oeni* MCW and *Lb. buchneri* CUC-3 were able to degrade $SO_2$ bound acetaldehyde. Metabolism of bound acetaldehyde occurred at a much slower rate than that of free acetaldehyde. Dry weights were similar and so this may have been due to inhibition by both bound and unbound $SO_2$. Free $SO_2$ would have been released as the bacteria metabolised the acetaldehyde moiety of $SO_2$ bound acetaldehyde. The level of free $SO_2$ present during the experiment was unable to be measured as the technique for the measurement of free $SO_2$ is not sensitive enough when working with small sample volumes as was the case here. It is also known that acetaldehyde bound $SO_2$ can inhibit growth of wine LAB. In both cases, the degree of inhibition is species and strain dependent (Fornachon, 1963; Hood, 1983; Delfini & Morsiani, 1992). In this particular experiment, it can be seen that *Lb. buchneri* CUC-3 was less sensitive to inhibition by $SO_2$ (bound and unbound) than *O. oeni* MCW. Because almost all the acetaldehyde would be bound to $SO_2$, degradation of acetaldehyde indicates the ability of the bacteria to degrade $SO_2$ bound acetaldehyde. Therefore, the observed metabolism of acetaldehyde by *Lb. buchneri* CUC-3 and *O. oeni* MCW indicates their ability to degrade $SO_2$ bound acetaldehyde.

These results are of practical importance in winemaking as $SO_2$ is used as an antioxidant and antimicrobial agent. $SO_2$ can also be added to wine to mask the adverse flavour effects of excess acetaldehyde. The ability of wine LAB to degrade

$SO_2$ bound acetaldehyde would affect the level of $SO_2$ that is needed to be added to the wine. If the bacteria are able to grow in the presence of acetaldehyde bound $SO_2$, they can metabolise acetaldehyde, releasing free $SO_2$. This release of free $SO_2$ may play a role in causing stuck or sluggish MLF as the levels of free $SO_2$ may be sufficient to limit further growth of the wine LAB. Acetaldehyde bound $SO_2$ may act as a reservoir of free $SO_2$. This removal of acetaldehyde by bacterial metabolism will also mean that less $SO_2$ will be needed to be added to the wine. These results have demonstrated the possibility of reducing $SO_2$ use in wine through the use of wine LAB that are able to metabolise acetaldehyde.

These results were obtained using resting cells in a buffered system at a wine pH. This experimental system closely mimics the situation in wine, but further experiments were needed to confirm whether or not wine LAB are able to metabolise $SO_2$ bound acetaldehyde in wine. These experiments and the results obtained are documented in section 5.2

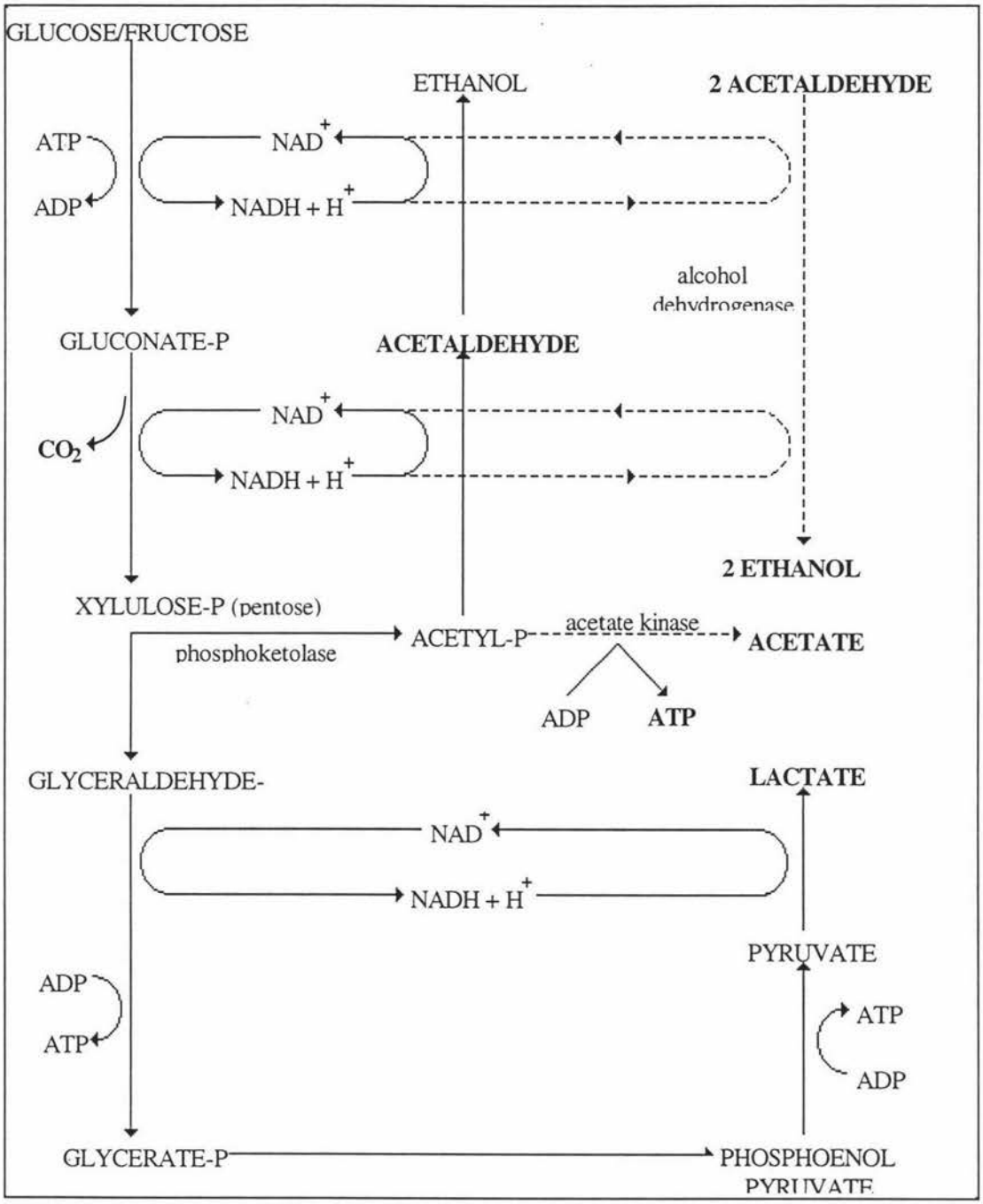


**Figure 4.3.1** Heterofermentation with acetaldehyde

# Chapter 5 Metabolism of Acetaldehyde by Wine LAB in Wine

## 5.1. Metabolism of Free Acetaldehyde in Wine

### 5.1.1. Materials and Methods

#### 5.1.1.1. Media Preparation

The wine was prepared as described in section 3.7. The wine was divided into two lots and the pH of one was adjusted to 3.3 while the other was adjusted to pH 3.6 using 5 M NaOH and 5 M HCl solutions. 200 mL aliquots of the wine were dispensed into 250 mL bottles.

#### 5.1.1.2. Culture Preparation

The wine LAB used were commercially available freeze dried cultures of *O. oeni* VFO from Chr. Hansen (Copahagen, Denmark) and *O. oeni* EQ54 from Lallemand (Toulouse, France). These cultures were stored at 4°C until needed. The cultures were rehydrated as per the manufacturers instructions.

#### 5.1.1.3. Experimental Procedure

The wine was inoculated by addition of the wine LAB at 5 x $10^5$ cells/mL. 1.6 mL samples were immediately taken and OD was measured using a visible light spectrophotometer (Nova Tech) at 650 nm. The samples were then centrifuged (10,000 *g* for 5 minutes) and the supernatant was used to determine the concentration of acetaldehyde (see 3.5.1).

The inoculated wines were incubated at 18°C and samples were taken periodically to determine the OD, acetaldehyde concentration and L-malic acid concentration using the method described in section 3.5.

### 5.1.2. Results

Figure 5.1.1 shows the production of acetaldehyde by and the growth of *S. bayanus* Première Cuvée during the alcoholic fermentation. Since $SO_2$ was not added, it was presumed that most acetaldehyde produced would exist in free form. It can be seen that acetaldehyde levels reached as high as 110 mg/L before there is some re-uptake by the yeast. Acetaldehyde levels then increase presumably to auto-oxidation of ethanol and phenolic compounds in the wine (Wildenrandt & Singleton, 1974). The production of acetaldehyde occurred during the exponential growth phase of the yeast, while re-uptake occurred during the stationary phase of growth. The glucose level at the end of fermentation was below 2 g/L and the wine was considered to be 'dry'.

Figure 5.1.2 and Figure 5.1.3 show the growth of *O. oeni* EQ54 and the associated metabolism of acetaldehyde and L-malic acid during MLF in wine at pH 3.3 and pH 3.6. It can be seen that there is a rapid reduction in acetaldehyde concentration from around 89 mg/L to below 5 mg/L. This can be attributed to the metabolism of acetaldehyde by *O. oeni* EQ54. Acetaldehyde and L-malic acid were catabolised simultaneously during active growth. Similar results were obtained for *O. oeni* VFO (Figure 5.1.4 and Figure 5.1.5). These results demonstrate that both *O. oeni* EQ54 and *O. oeni* VFO were able to metabolise acetaldehyde during MLF in wine.

Figure 5.1.6 gives an overview of the fate of acetaldehyde during both alcoholic fermentation and MLF. It can be seen that during alcoholic fermentation, the yeast produced over 100 mg/L acetaldehyde. There is some reuptake of acetaldehyde by the yeast before acetaldehyde levels increased slightly, presumably due to auto-oxidation of ethanol and phenolic compounds. But the acetaldehyde concentrations rapidly decreased during MLF. This was likely due to metabolism of the acetaldehyde by the wine LAB.

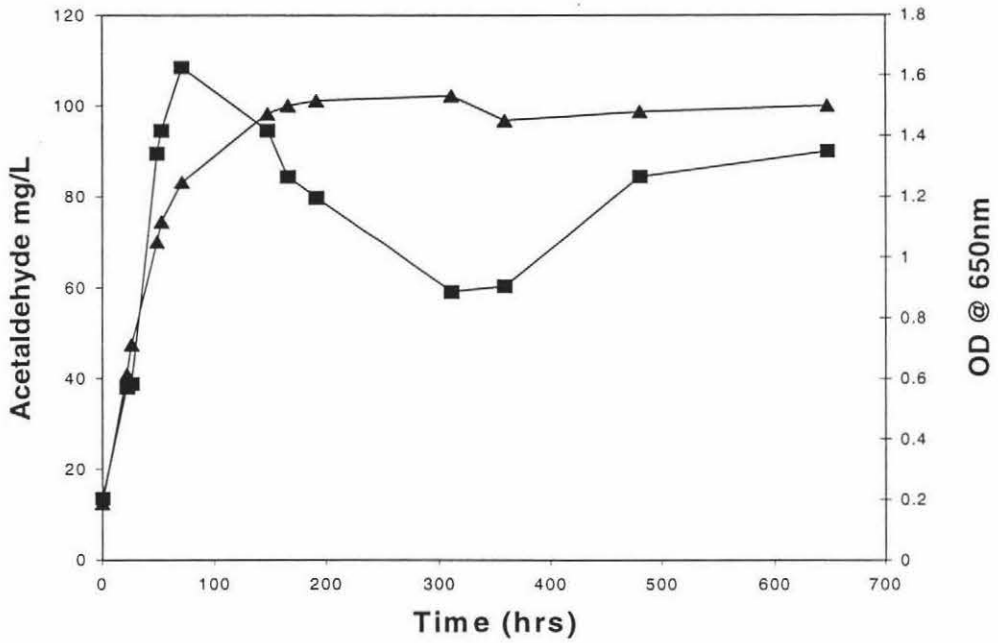


**Figure 5.1.1** Growth and production of acetaldehyde by *S. bayanus* Première Cuvée during alcoholic fermentation. Symbols; ▲ optical density (OD); ■ acetaldehyde.

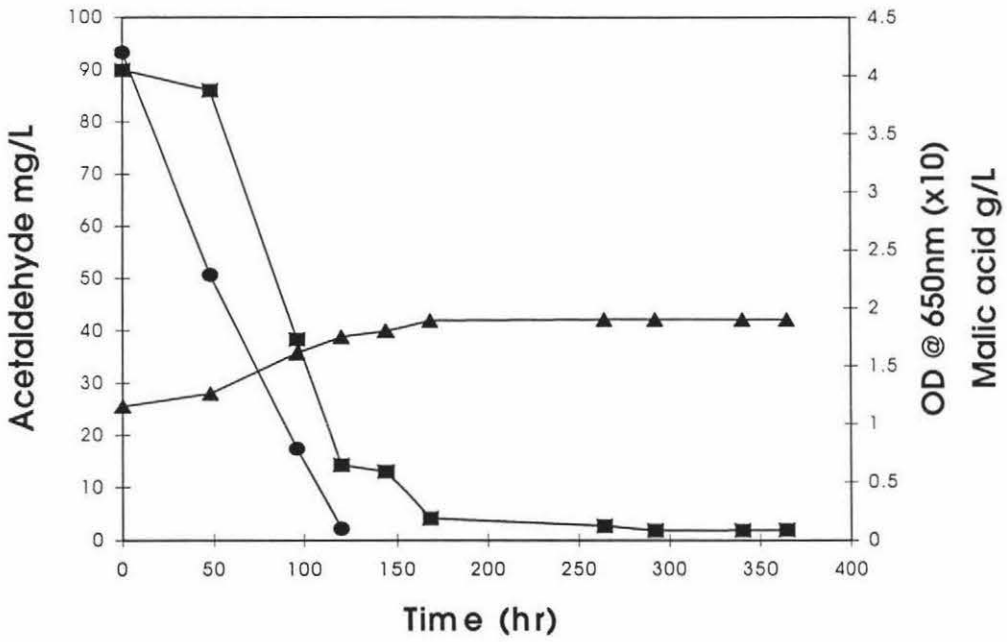


**Figure 5.1.2** Growth and metabolism of acetaldehyde and malic acid by *O. oeni* EQ54 in a laboratory vinified wine at pH 3.3. Symbols; ▲ OD; ■ acetaldehyde; ● malic acid.

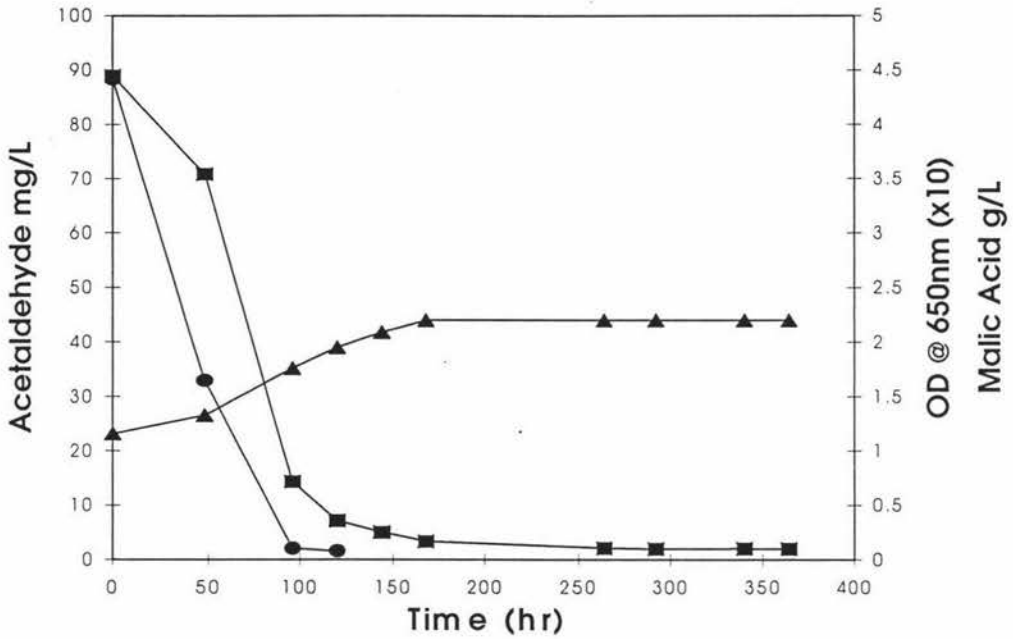


**Figure 5.1.3** Growth and metabolism of acetaldehyde and malic acid by *O. oeni* EQ54 in a laboratory vinified wine at pH 3.6. Symbols; ▲ OD; ■ acetaldehyde; ● malic acid.

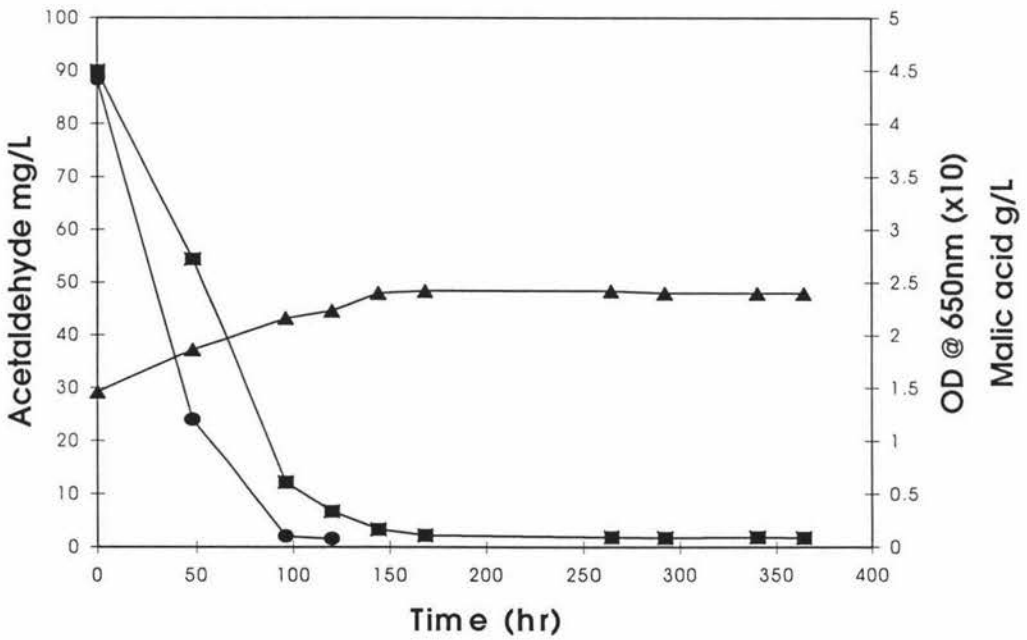


**Figure 5.1.4** Growth and metabolism of acetaldehyde and malic acid by *O. oeni* VFO in a laboratory vinified wine at pH 3.3. Symbols; ▲ OD; ■ acetaldehyde; ● malic acid.

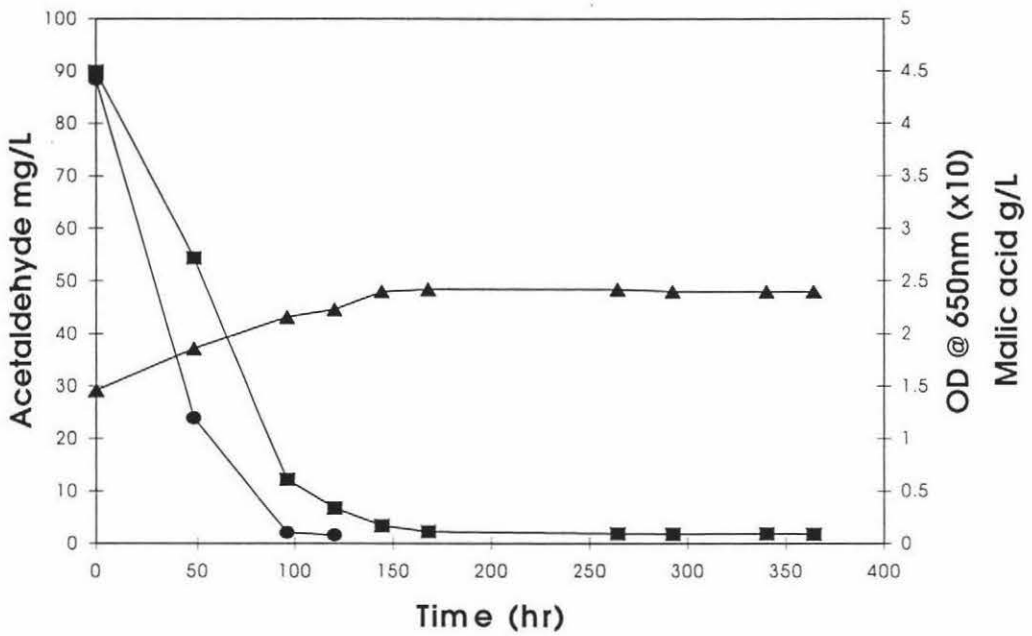


**Figure 5.1.5** Growth and metabolism of acetaldehyde and malic acid by *O. oeni* VFO in a laboratory vinified wine at pH 3.6. Symbols; ▲ OD; ■ acetaldehyde, ● malic acid.

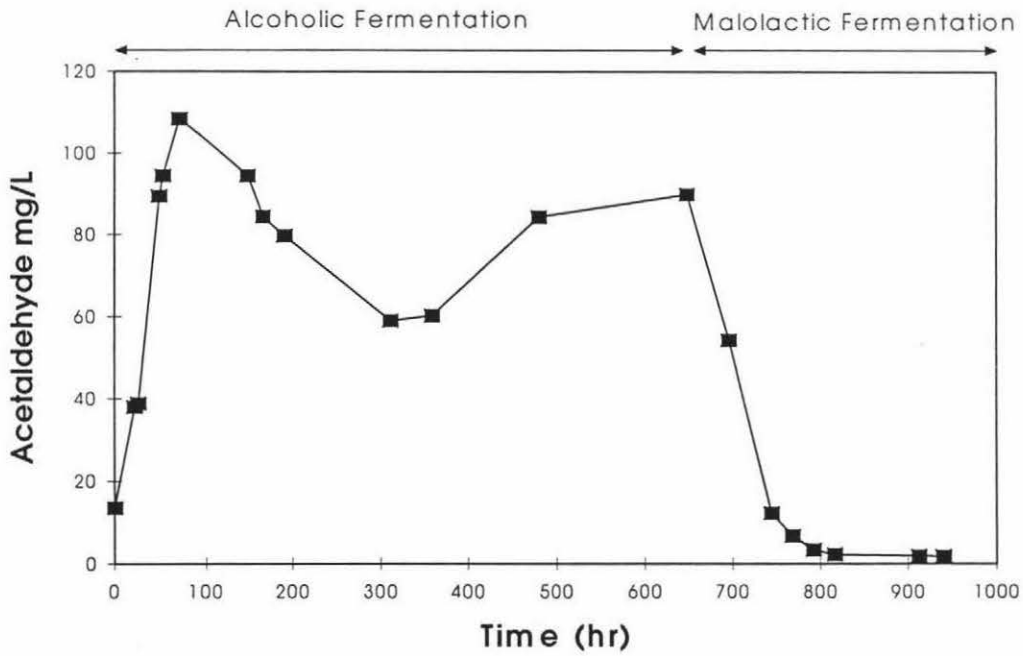


**Figure 5.1.6** Acetaldehyde concentration during alcoholic fermentation by *S. bayanus* Première Cuvée and MLF by *O. oeni* EQ54 in wine at pH 3.6.

## 5.2. Metabolism of Sulphur Dioxide Bound Acetaldehyde by Wine LAB in Wine

### 5.2.1. Materials and Methods

#### 5.2.1.1. Media Preparation

The wine was prepared as per section 3.7. The wine was divided into two lots and the pH of one was adjusted to 3.3 while the other was adjusted to pH 3.6. 200 mL of the wine was dispensed into 250 mL bottles. The acetaldehyde of the wine was measured (see section 3.5.1) and $SO_2$ was added to the wine (as potassium metabisulphite) at a level where all the acetaldehyde would be $SO_2$ bound (equimolar amounts of acetaldehyde and $SO_2$).

#### 5.2.1.2. Culture Preparation

Cultures were prepared as described in section 5.1.1.2

#### 5.2.1.3. Experimental Procedure

The wine was inoculated by addition of the wine LAB at approximately 5 x $10^5$ cells/mL. 1.6 mL samples were immediately taken and OD was measured using a visible light spectrophotometer (Nova Tech) at 650 nm. The sample was then centrifuged (10,000 *g* for 5 minutes) and the supernatant was used to determine the concentration of acetaldehyde in the wine (see section 3.5.1).

The wine was incubated at 18°C and regular samples were taken to determine the OD and the acetaldehyde concentration.

### 5.2.2. Results

Figure 5.2.1 shows the growth of *O. oeni* EQ54 and the changes in acetaldehyde concentration during MLF in wine at pH 3.3 with $SO_2$ added. This strain grew very slowly during the first 800 hrs of incubation and during this time there was an increase in the acetaldehyde concentration. This slow growth may be due to the presence of $SO_2$ (free and bound) and low pH. After 800 hrs, there was more rapid growth and a

further increase in the acetaldehyde level. This increase in acetaldehyde may be due to auto-oxidation of ethanol and phenolic compounds in the wine (Wildenrandt & Singleton, 1974). The production of acetaldehyde by auto-oxidation of ethanol and phenolic compounds is shown in Figure 5.2.5. These results show production of acetaldehyde in wine which has not been inoculated by wine LAB. This uninoculated control was treated in the same way as the inoculated wine.

Figure 5.2.2 shows the changes in OD of *O. oeni* EQ54 and the acetaldehyde concentration during MLF in wine at pH 3.6 with $SO_2$ added. These results show that initially there was slow growth and an increase in the acetaldehyde concentration. However, after about 400 hrs there was more rapid growth and a reduction in the concentration of acetaldehyde. This indicates the ability of *O. oeni* EQ54 to degrade $SO_2$ bound acetaldehyde in wine at pH 3.6 as almost all the acetaldehyde present in the wine would be $SO_2$ bound. Similar patterns of growth and changes in acetaldehyde concentrations was observed for *O. oeni* VFO (Figure 5.2.3 and Figure 5.2.4)

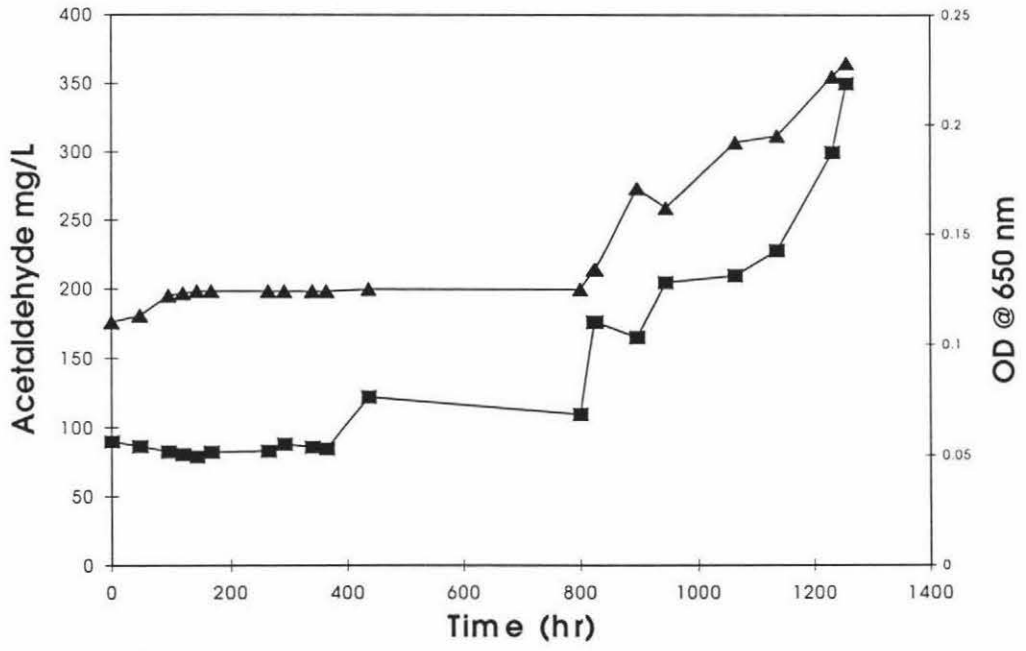


**Figure 5.2.1** Growth of *O. oeni* EQ54 and changes in acetaldehyde concentration in a laboratory vinified wine at pH 3.3 with sulphur dioxide added. Symbols; ▲ OD; acetaldehyde ■.

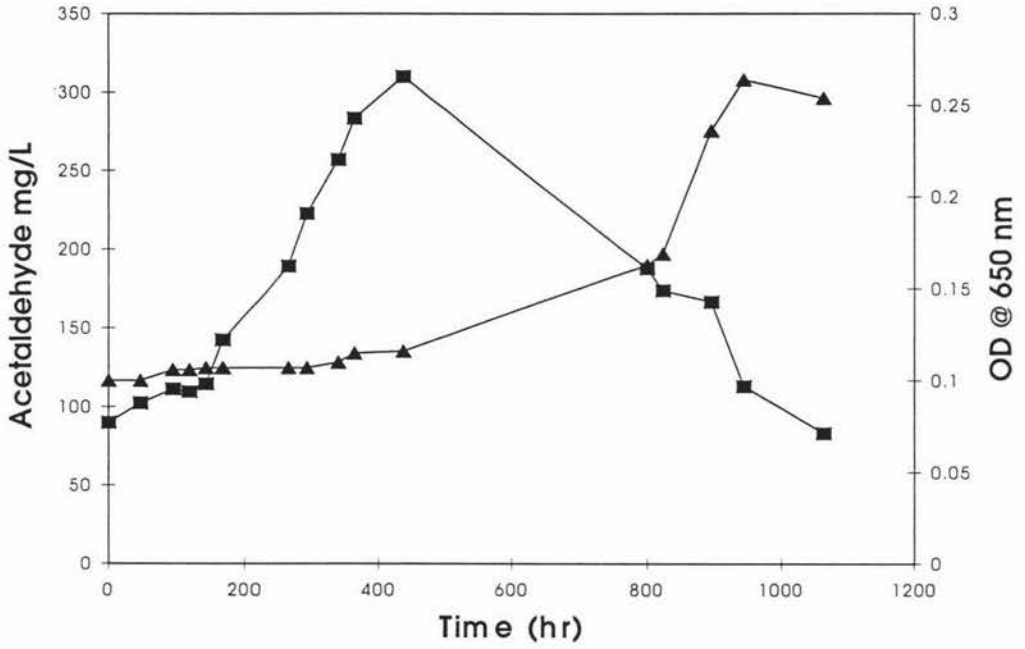


**Figure 5.2.2** Growth of *O. oeni* EQ54 and changes in acetaldehyde concentration in a laboratory vinified wine at pH 3.6 with sulphur dioxide added. Symbols; ▲ OD; ■ acetaldehyde.

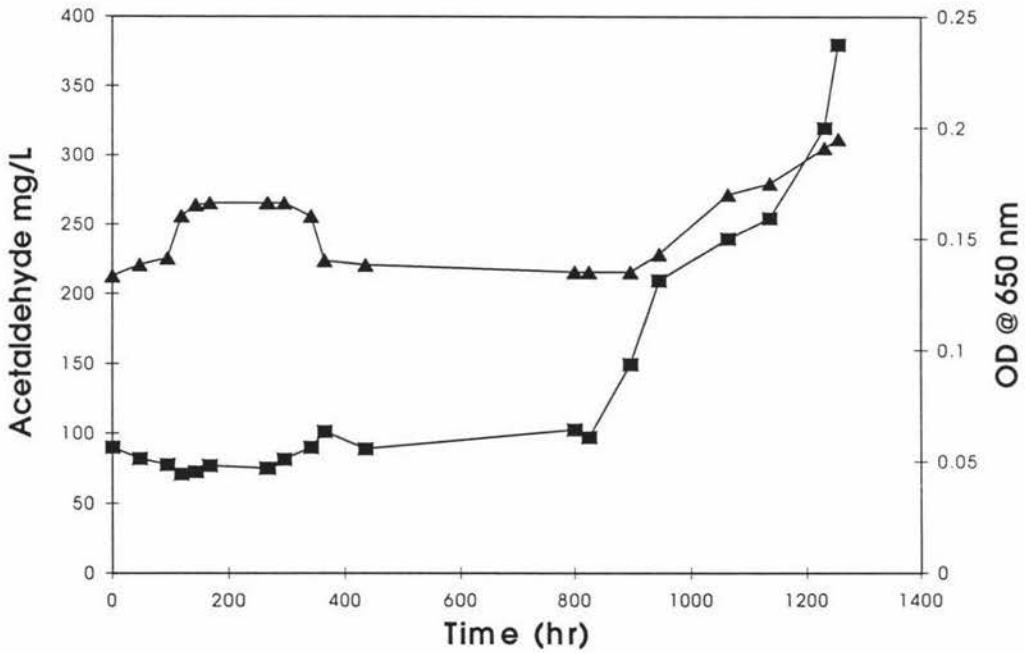


**Figure 5.2.3** Growth of *O. oeni* VFO and changes in acetaldehyde concentration in a laboratory vinified wine at pH 3.3 with sulphur dioxide added. Symbols; ▲ OD; ■ acetaldehyde.

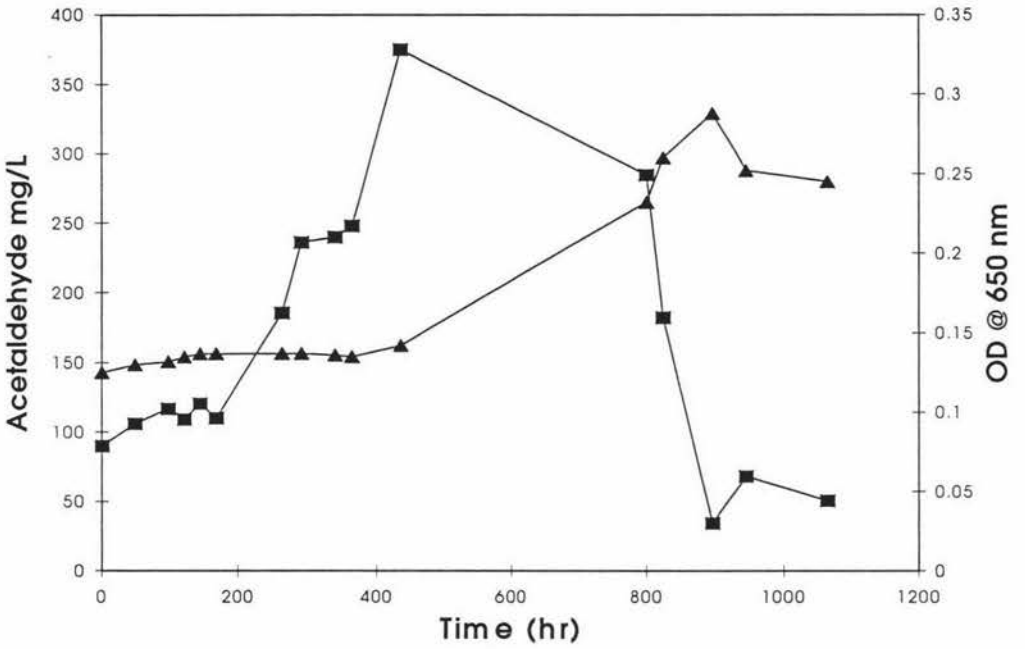


**Figure 5.2.4** Growth of *O. oeni* VFO and changes in acetaldehyde concentration in a laboratory vinified wine at pH 3.6 with sulphur dioxide added. Symbols; ▲ OD; ■ acetaldehyde.

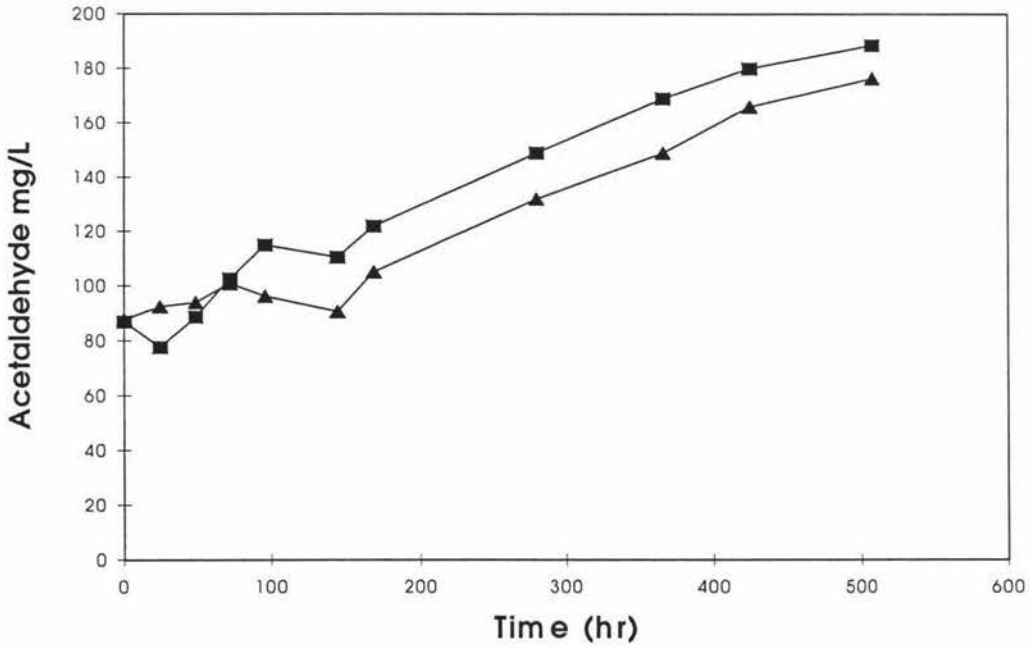


**Figure 5.2.5** Changes in acetaldehyde concentration of a laboratory vinified wine at pH 3.3 and pH 3.6 (Uninoculated controls). Symbols; ▲ acetaldehyde at pH 3.3, ■ acetaldehyde at pH 3.6

## 5.3. Discussion

### 5.3.1. Vinification

It has been shown that acetaldehyde is excreted into the environment mainly during the first stage of fermentation (growth phase) (Ciani, 1997) and can also be recatabolised by the yeast (Farris *et al*, 1983). The results presented here are consistent with these findings. During the growth phase of the yeast, there is considerable production of acetaldehyde. There is also a reuptake of some of this acetaldehyde during stationary phase followed by an increase in the acetaldehyde concentration. The increased formation of acetaldehyde during stationary phase was presumably due to the auto-oxidation of ethanol and phenolic compounds present in the wine (Wildenrandt & Singleton, 1974) and demonstrated in Figure 5.2.5.

### 5.3.2. Metabolism of Free Acetaldehyde in Wine

Previously, it had been shown that both *O. oeni* EQ54 and *O. oeni* VFO were able to metabolise acetaldehyde in a buffered system at wine pH (section 4.1). The results from section 5.1 show that these bacteria are also capable of metabolizing acetaldehyde in wine during MLF. Both strains were able to metabolise acetaldehyde in wine, rapidly reducing the initial concentration of 89 mg/L to a level below the detectable limit. This metabolism of acetaldehyde coincided with malic acid degradation. The results from the buffer system (Chapter 4) and the wine system were very consistent confirming the validity of the buffer system (resting cell experiments).

Wine LAB metabolised acetaldehyde produced by yeast during the alcoholic fermentation (Figure 5.1.6). The pH had no impact on the ability of wine LAB to metabilise acetaldehyde as almost all the acetaldehyde was metabolised by 100-120 hr in wine at pH 3.3 and pH 3.6. This metabolism of acetaldehyde has many practical implications as related to the production of wine. As has been previously stated (section 2.2) acetaldehyde can affect both the physical and chemical properties of wine. The effects can be both positive and negative in nature. In winemaking currently, excess acetaldehyde (giving a green grassy flavour) is masked by the addition of sulphur dioxide. However, the metabolism of acetaldehyde by wine LAB

may be a better option for the removal of this compound. If wine LAB remove the acetaldehyde, then less sulphur dioxide will need to be added for it to accomplish its role as an antimicrobial and antioxidative agent (Romano & Suzzi, 1993). This is because sulphur dioxide is usually added to a level where a certain amount of free sulphur dioxide is present in the wine. If acetaldehyde is removed, then there is one less compound that will bind sulphur dioxide and so less sulphur dioxide will be required to reach the same level of free sulphur dioxide. This is beneficial in wine production as the consumer today is wanting products which have reduced amounts of added chemicals. The use of wine LAB to remove acetaldehyde from wine is an example of how the levels of chemicals added to wine can be reduced. However, in red wine, acetaldehyde plays a role in colour developement by combining with anthocyanins in the wine to form stable colour pigments (Timberlake & Bridle, 1976; Somers & Wescombe, 1987). Therefore, the total removal of acetaldehyde in red wines may not be desirable. This makes strain selection critical. For white wine production, the winemaker would add a bacterial strain that was able to metabolise acetaldehyde and thus removing it from the wine. But for red wine production, the winemaker may add a bacterial strain unable to degrade acetaldehyde or control the timing of MLF by allowing some colour development before beginning MLF. The survey of strains (section 4.1) produced only two strains unable to degrade acetaldehyde. Unfortunately, both of these were *Pediococcus* strains. In winemaking, pediococci are considered to be spoilage organisms, so the addition of these bacteria to wine is not recommended. A more complete survey for the ability (or lack of ability) of wine LAB to degrade acetaldehyde is needed to increase the options available for strain selection by the winemaker.

### 5.3.3. Metabolism of Bound Acetaldehyde in Wine

It had previously been shown that both *O. oeni* EQ54 and *O. oeni* VFO were able to degrade acetaldehyde in wine at both pH 3.3 and pH 3.6 (section 5.1). In this experiment, the ability of these bacteria to degrade $SO_2$ bound acetaldehyde in wine at pH 3.3 and pH 3.6 was investigated. It appeared that pH had a dramatic impact on the ability of wine LAB to degrade $SO_2$ bound acetaldehyde. The results show that at pH 3.3, neither bacterial strain was able to degrade $SO_2$ bound acetaldehyde within the incubation period. Compared to the pattern of acetaldehyde formation and utilisation

at pH 3.6, acetaldehyde utilisation may have been observed if further incubation had been allowed.

There was an initial lack of growth, followed by a period of more rapid growth and an increase in acetaldehyde concentration. The initial lack of growth may be due to inhibition by bound and free $SO_2$ (Fornachon, 1963; Hood, 1983; Delfini & Morsiani, 1992). Even low levels of free $SO_2$ can have very strong inhibitory effects on wine LAB in wine. As the bacteria metabolise the acetaldehyde, $SO_2$ is released. This free $SO_2$ is then able to inhibit or kill the bacteria (Fornachon, 1963; Delfini & Morsiani, 1992). Fornachon (1963) and Hood (1983) showed that acetaldehyde bound $SO_2$ can be inhibitory to wine LAB. The secondary growth of the culture (after about 800 hrs) may indicate that the fermentation was originally stuck or sluggish (due to initial inoculation into wine containing $SO_2$) and so the culture was inhibited, not killed, by the original levels of free and bound $SO_2$. The observed increase in acetaldehyde may be due to production of acetaldehyde through the auto-oxidation of ethanol and phenolic compounds present in the wine. This process may have been accelerated due to the stirring of the wine needed to resuspend the bacteria each time an OD measurement was taken. The results from the uninoculated control (Figure 5.2.5) indicate that this was the case as the uninoculated control also showed an increase in acetaldehyde over time. These controls were subjected to the same treatment as the inoculated wine. The increase in acetaldehyde levels may also be due to the production of acetaldehyde by wine LAB. It has been shown that some dairy LAB are able to produce acetaldehyde from glucose (Lees & Jago, 1976a) but it is unclear if wine LAB are able to do the same.

At pH 3.6, both bacterial strains were able to degrade acetaldehyde. After an initial lack of growth and an increase in acetaldehyde concentration, there was growth of the bacteria and a decrease in acetaldehyde levels. This decrease in acetaldehyde may indicate the ability of *O. oeni* EQ54 and *O. oeni* VFO to degrade $SO_2$ bound acetaldehyde. However, because of the increase in acetaldehyde, not all of the acetaldehyde would have been bound to $SO_2$ and so the bacteria may have been degrading free acetaldehyde. Therefore, a firm conclusion cannot be drawn that *O. oeni* EQ54 and *O. oeni* VFO are indeed able to degrade $SO_2$ bound acetaldehyde in wine. It is possible that a combination of both free and bound acetaldehyde was metabolised, but this is yet to be substantiated.

It took the cultures growing in wine containing $SO_2$ bound acetaldehyde much longer to reach 'maximum' growth then those cultures growing in wine containing free acetaldehyde (section 5.1.2). The presence of $SO_2$ bound acetaldehyde resulted in very slow growth, especially at pH 3.3. At the lower pH of 3.3, a greater proportion of $SO_2$ may be in the free form. This is due to the fact that the levels of each form of $SO_2$ in solution are pH dependent and at lower pH, the molecular form of $SO_2$ predominates over the bisulphite form (which binds strongly to acetaldehyde) (Zoecklein *et al*, 1995). These results suggest the possible role of $SO_2$ bound acetaldehyde in sluggish MLF especially at low pH

Future experiments are needed where the production of acetaldehyde through auto-oxidation of ethanol and phenolic compounds can be minimised so as not to interfere with the results. This would involve reducing the head space in the bottles containing the wine, and using separate bottles for OD and acetaldehyde measurements. This will mean that there will be no need to stir the bottle from which only acetaldehyde levels are being measured. The phenolic compounds could also be removed from the wine through fining with polyvinylpolypyrrolidone (PVPP). These precautions should reduce significantly the production of acetaldehyde through the auto-oxidation of ethanol and phenolic compounds present in the wine. The experiment was run for six weeks. There is a possibility that if the experiment using wine at pH 3.3 was allowed to continue further, then a decrease in acetaldehyde levels may have been observed as it was with wine at pH 3.6. This is because, at lower pH, growth of the wine LAB may have been much more restricted then at pH 3.6. This sluggish growth can be seen in the results (Figure 5.2.1 and Figure 5.2.3) and if the experiment was allowed to continue, further growth of the wine LAB may have resulted in a decrease in acetaldehyde levels. Further experiments would also include the use of more bacterial strains, including those that are more $SO_2$ tolerant than those used in this experiment. The ability of wine LAB to produce acetaldehyde from glucose should also be explored.

# Chapter 6 Effect of Acetaldehyde on Growth Characteristics of Wine LAB

## 6.1. Growth in VJG Broth

### 6.1.1. Materials and Methods

#### 6.1.1.1. Media Preparation

VJG broth was prepared as per section 3.1.3. Nine mL aliquots of broth were then dispensed into capped 15 mL test tubes followed by sterilisation by autoclaving in a pressure cooker at 15 psig for 15 minutes. Solutions of acetaldehyde were prepared at 500, 1000 and 3000 mg/L by dilution with DI water of a stock acetaldehyde solution (5000 mg/L). The stock solution was prepared as described in section 4.1.1.1. All acetaldehyde solutions were sterile filtered. 1 mL of each of the 500, 1000, 3000 mg/L solutions were dispensed into the screw-capped test tubes containing 9 mL VJG broth to give final acetaldehyde concentrations of 50, 100, and 300 mg/L. This was done in triplicate so that there were three replicates at each concentration.

#### 6.1.1.2. Culture Preparation

Cultures were prepared as described in section 4.1.1.2. The culture was used to inoculate the experimental VJG broth.

LAB are slow growing microorganisms and therefore, to optimise the time spent recording results, it was necessary to determine the length of each growth phase. Ideally, strains for subculturing should bein the exponential growth phase so that the lag phase of the subsequent growth is short. Therefore, a preliminary experiment was undertaken to follow the growth of *Lb. buchneri* CUC-3 to determine when this strain would be in the exponential growth phase.

A screw-capped test tube containing 9 mL VJG broth was inoculated with 1 mL of culture (incubated overnight at 30°C). The test tubes used fit the well of the

spectrophotometer so that the optical density (OD) could be measured directly. The tubes were immediately measured for OD using a visible light spectrophotometer (Spectronic 20) at 600 nm. The tubes were then incubated at 30°C and the OD was measured every 30 min until a growth curve was obtained. The optimum time for inoculation of a culture was obtained from these results. It was found that after 8 hours *Lb. buchneri* CUC-3 had reached exponential growth phase. This procedure was also carried out for the other wine LAB to be tested. *Lb. delbrueckii* CUC-1 needed to be pre-incubated for 8 to 10 hr, *Pd. damnosus* CUC-4 for 15 to 20 hr and *O. oeni* MCW for over 24 hr.

6.1.1.3. Experimental Procedure

Cultures were incubated at 30°C until they reached the exponential phase of growth (section 6.1.1.2) and were then used to inoculate the broth in screw-capped test tubes that had been prepared as per section 6.1.1.1 and that fit the spectrophotometer as described in section 6.1.1.2. Tubes containing no acetaldehyde (9 mL VJG + 1 mL sterile DI water) were also inoculated as a control. Tubes were inoculated by adding 0.5 mL of culture (in exponential growth phase) to each tube, which gave an initial change in OD (ΔOD) reading of about 0.1 (except for MCW). Tubes were then incubated at 30°C and OD readings were taken every 30 min. All measurements were carried out using a Spectronic 20 visible light spectrophotometer at 600 nm. Measurements continued until a suitable growth curve had been obtained such that a straight line from the exponential growth phase could be calculated. It was found that MCW grew too slowly if the initial inoculum size was only 0.5 mL and that no appreciable growth was obtained after 12 hr. Therefore a larger inoculum was used for strain MCW. Tubes containing 4.5 mL of VJG + 0.5 mL of acetaldehyde were used (still giving acetaldehyde at 0, 50, 100, 300 mg/L). These tubes were inoculated with 5 mL of culture giving an initial ΔOD reading of over 0.1 (compared to the uninoculated control). This larger inoculum size gave more significant results within a suitable time frame. When the experiment was completed, the acetaldehyde concentration in each tube was measured as described in section 3.5.1.

6.1.1.4. Statistical Analysis

Results were plotted as ΔOD versus time and as Log ΔOD versus time. A regression was performed on the linear portion of the curve (exponential phase). The slope of the curve (obtained from the regression) was used to calculate maximum growth rates. Cultures in the exponential phase of growth obey the following equation:

$\log_{10}N = kt/2.303 + \log_{10}N_0$ (Ingraham *et al*, 1983)

This equation describes a straight line (y = ax + b). By plotting $\log_{10}N$ against *t* (time), one obtains a straight line that intercepts the ordinate at $\log_{10}N_0$ and has a slope of k/2.303. From the slope of the graphs, one can calculate the specific growth rate k of the culture as slope = k/2.303, therefore, k = slope x 2.303.
From this calculation, doubling time per hour, (μ), can be calculated using the following formula:

$\mu = k/0.693$ (Ingraham *et al*, 1983)

Doubling rates per hour were then compared for each acetaldehyde level per strain. An ANOVA analysis was carried out on the data and the least significant difference was calculated to determine if the differences between the μ at each acetaldehyde concentration were significant or not.

### 6.1.2. Results

Figure 6.1.1 shows the growth curves for *O. oeni* MCW grown in VJG broth at various acetaldehyde concentrations. From these curves, doubling times and maximum growth rates were calculated (see section 6.1.1.4). The mean maximum growth rates for each culture grown at the various acetaldehyde concentrations are given in Table 6.1.1. The data is graphically presented in Figure 6.1.2. For *O. oeni* MCW, one batch of tubes was accidentally inoculated at a higher acetaldehyde concentration of 3000 mg/L instead of 300 mg/L. The results for the other strains tested are shown in Table 6.1.3, Table 6.1.5 and Table 6.1.7. The data is also illustrated in Figure 6.1.3, Figure 6.1.4 and Figure 6.1.5. For these strains, only acetaldehyde concentrations of 0, 50, 100 and 300 mg/L were used. The tables also show the difference between the means and the calculated least significant difference.

These results show that for *O. oeni* MCW there is a statistically significant difference between the 0 mg/L & 300 mg/L means, the 0 mg/L & 3000 mg/L means, the 50 mg/L & 3000 mg/L means, the 100 mg/L & 3000 mg/L means and the 300 mg/L & 3000 mg/L means. These differences indicate that there was inhibition of growth at the 300 mg/L and 3000 mg/L acetaldehyde concentrations. For the other strains tested (*Lb. delbrueckii* CUC-1, *Lb. buchneri* CUC-3 and *Pd. damnosus* CUC-4), the results show that there was inhibition of growth only at the 300 mg/L acetaldehyde concentration.

The acetaldehyde concentration in each tube was measured before and after growth. The mean of the results for each tube at each acetaldehyde concentration can be seen in Table 6.1.2, Table 6.1.4, Table 6.1.6 and Table 6.1.8. These results show that there was degradation of acetaldehyde at all concentrations but less so at the higher levels. The uninoculated controls showed no significant decrease in acetaldehyde, indicating that the degradation of acetaldehyde was due to the inoculated culture. The degradation of acetaldehyde by *Pd. damnosus* CUC-4 was unexpected, as in previous experiments (section 4.1) it had been shown that this strain was unable to metabolise acetaldehyde in a resting cell experiment at pH 3.6 in a buffered system. It was thought that for this strain, the degradation of acetaldehyde may be energy dependent and so a further resting cell experiment was carried out, identical to the experiment carried out in section 4.1 but glucose was added as an energy source. Figure 6.1.6 shows that even with glucose present, *Pd. damnosus* CUC-4 was unable to metabolise acetaldehyde.

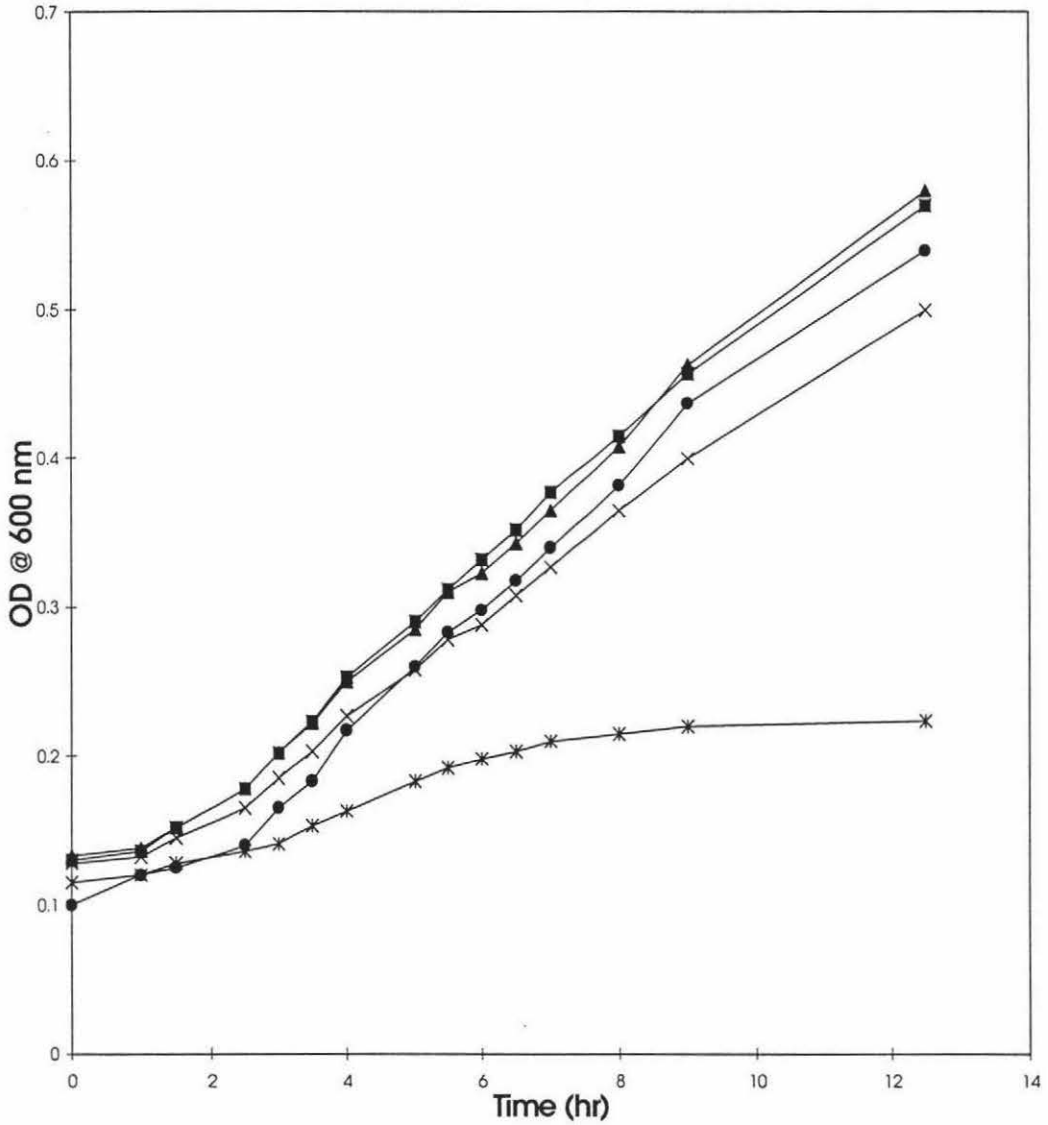


**Figure 6.1.1** Growth of *O. oeni* MCW in VJG broth at various acetaldehyde concentrations. Results are averages of three separate experiments. Symbols; ● 0 mg/L acetaldehyde; ▲ 50 mg/L acetaldehyde; ■ 100 mg/L acetaldehyde; × 300 mg/L acetaldehyde; ✶ 3000 mg/L acetaldehyde.

**Table 6.1.1** Growth rates of *O. oeni* MCW at different acetaldehyde concentrations

| Acetaldehyde Concentration mg/L | Maximum Growth Rate $\mu_{max}$ (mean) |
|---|---|
| 0 | 0.244 |
| 50 | 0.207 |
| 100 | 0.201 |
| 300 | 0.192 |
| 3000 | 0.116 |
| | |
| Mean Differences | |
| Mean(0mg)- Mean(50mg) | 0.037 |
| Mean(0mg)- Mean(100mg) | 0.043 |
| Mean(0mg)- Mean(300mg) | 0.052 |
| Mean(0mg)- Mean(3000mg) | 0.128 |
| Mean(50mg)- Mean(100mg) | 0.006 |
| Mean(50mg)- Mean(300mg) | 0.015 |
| Mean(50mg)- Mean(3000mg) | 0.091 |
| Mean(100mg)- Mean(300mg) | 0.009 |
| Mean(100mg)- Mean(3000mg) | 0.085 |
| Mean(300mg)- Mean(3000mg) | 0.076 |
| | |
| Least Significant Difference = 0.051 | |

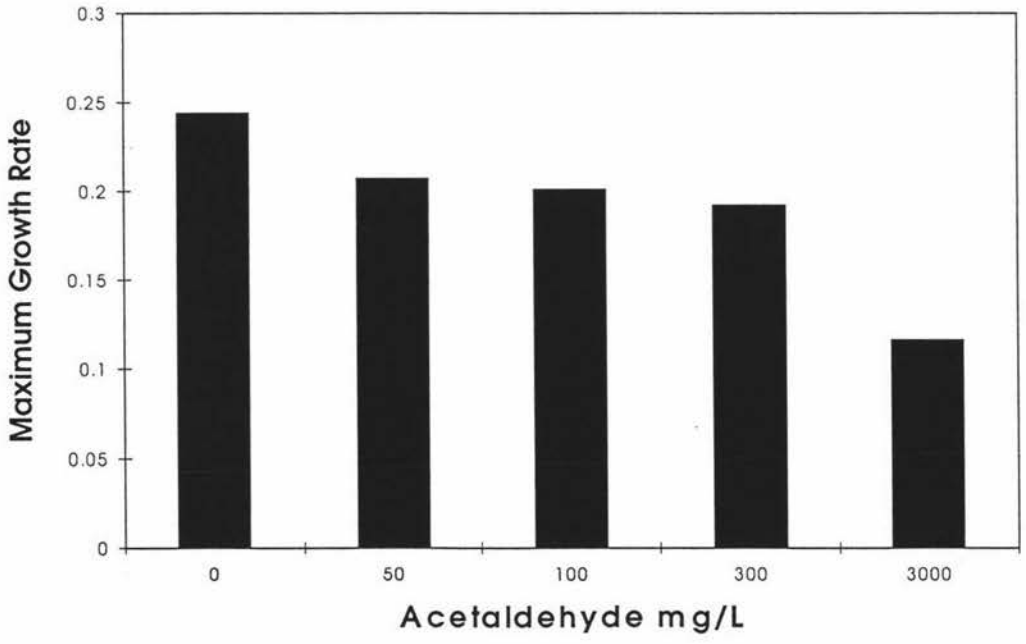


**Figure 6.1.2** Maximum growth rates ($\mu_{max}$) of *O. oeni* MCW grown in VJG broth at various acetaldehyde concentrations.

**Table 6.1.2** Initial and final acetaldehyde concentrations in VJG broth inoculated with *O. oeni* MCW

| Initial Acetaldehyde Conc. (mg/L) | Final Acetaldehyde Conc. (mg/L) | % degraded |
|---|---|---|
| 0 | 0 | 0 |
| 50 | 8.2 | 83.6 |
| 100 | 10.5 | 89.5 |
| 300 | 188.5 | 37.2 |
| 3000 | 2654.25 | 11.5 |

**Table 6.1.3** Growth rates of *Lb. delbrueckii* CUC-1 at different acetaldehyde concentrations

| Acetaldehyde Concentration mg/L | Maximum Growth Rate $\mu_{max}$ (mean) |
|---|---|
| 0 | 0.417 |
| 50 | 0.413 |
| 100 | 0.406 |
| 300 | 0.261 |
| | |
| Mean Differences | |
| Mean (0mg/L) - Mean (50mg/L) | 0.004 |
| Mean (0mg/L) - Mean (100mg/L) | 0.011 |
| Mean (0mg/L) - Mean (300mg/L) | 0.156 |
| Mean (50mg/L) - Mean (100mg/L) | 0.007 |
| Mean (50mg/L) - Mean (300mg/L) | 0.152 |
| Mean (100 mg/L) - Mean (300mg/L) | 0.145 |
| | |
| Least Significant Difference = 0.142 | |

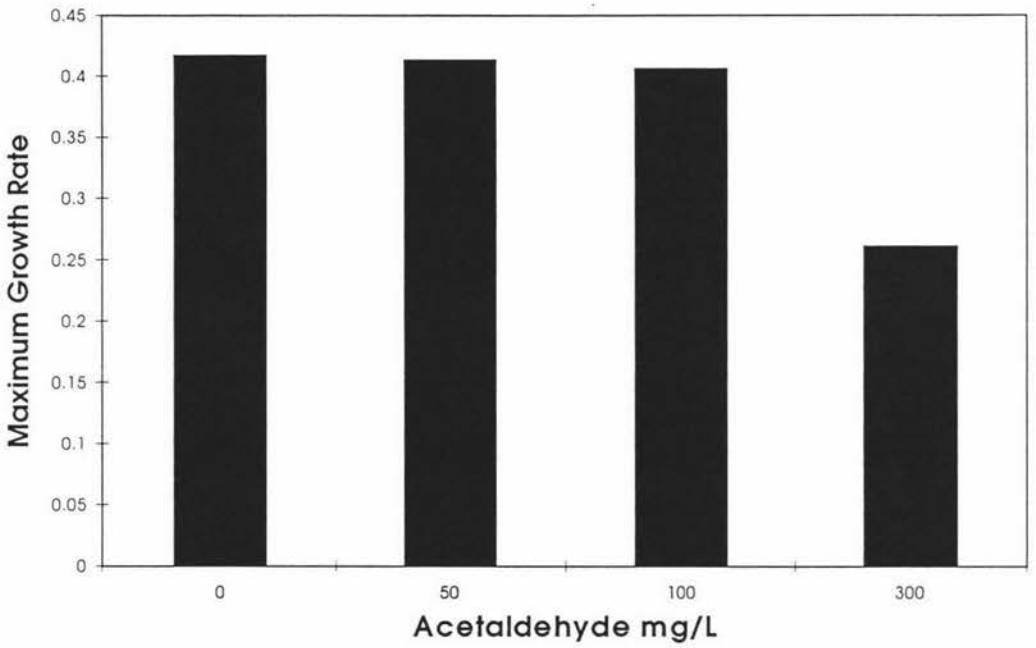


**Figure 6.1.3** Maximum growth rates ($\mu_{max}$) of *Lb. delbruecki* CUC-1 grown in VJG broth at various acetaldehyde concentrations.

**Table 6.1.4** Initial and final acetaldehyde concentrations in VJG broth inoculated with *Lb. delbruecki* CUC-1

| Initial Acetaldehyde Conc. (mg/L) | Final Acetaldehyde Conc. (mg/L) | % degraded |
|---|---|---|
| 0 | 0 | 0 |
| 50 | 1.9 | 96.2 |
| 100 | 11.5 | 88.5 |
| 300 | 192.6 | 35.8 |

**Table 6.1.5** Growth rates of *Pd. damnosus* CUC-4 at different acetaldehyde concentrations.

| Acetaldehyde Concentration mg/L | Maximum Growth Rate $\mu_{max}$ (mean) |
|---|---|
| 0 | 0.28 |
| 50 | 0.282 |
| 100 | 0.282 |
| 300 | 0.199 |
| | |
| Mean Differences | |
| Mean (0mg)- Mean (50mg) | 0.002 |
| Mean (0mg)- Mean (100mg) | 0.002 |
| Mean (0mg)- Mean (300mg) | 0.081 |
| Mean (50mg)- Mean (100mg) | 0 |
| Mean (50mg)- Mean (300mg) | 0.083 |
| Mean (100mg)- Mean (300mg) | 0.083 |
| | |
| Least Significant Difference = 0.0194 | |

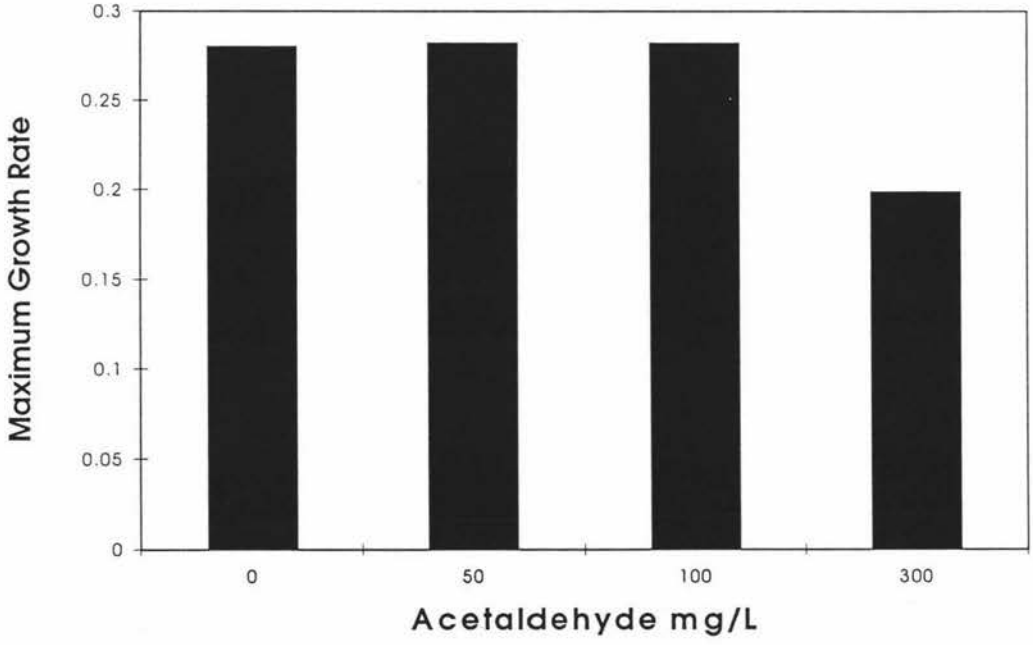


**Figure 6.1.4** Maximum growth rates ($\mu_{max}$) of *Pd. damnosus* CUC-4 grown in VJG broth at various acetaldehyde concentrations.

**Table 6.1.6** Initial and final acetaldehyde concentrations in VJG broth inoculated with *Pd. damnosus* CUC-4

| Initial Acetaldehyde Conc. (mg/L) | Final Acetaldehyde Conc. (mg/L) | % degraded |
|---|---|---|
| 0 | 0 | 0 |
| 50 | 6.5 | 87 |
| 100 | 7.9 | 92.1 |
| 300 | 205.8 | 31.4 |

**Table 6.1.7** Growth rates of *Lb. buchneri* CUC-3 at different acetaldehyde concentrations.

| Acetaldehyde Concentration (mg/L) | Maximum Growth Rate $\mu_{max}$ (mean) |
|---|---|
| 0 | 0.322 |
| 50 | 0.343 |
| 100 | 0.346 |
| 300 | 0.163 |
| | |
| Mean Differences | |
| Mean (0mg/L) - Mean (50mg/L) | 0.021 |
| Mean (0mg/L) - Mean (100mg/L) | 0.029 |
| Mean (0mg/L) - Mean (300mg/L) | 0.159 |
| Mean (50mg/L) - Mean (100mg/L) | 0.003 |
| Mean (50mg/L) - Mean (300mg/L) | 0.18 |
| Mean (100mg/L) - Mean (300mg/L) | 0.183 |
| | |
| Least Significant difference = 0.159 | |

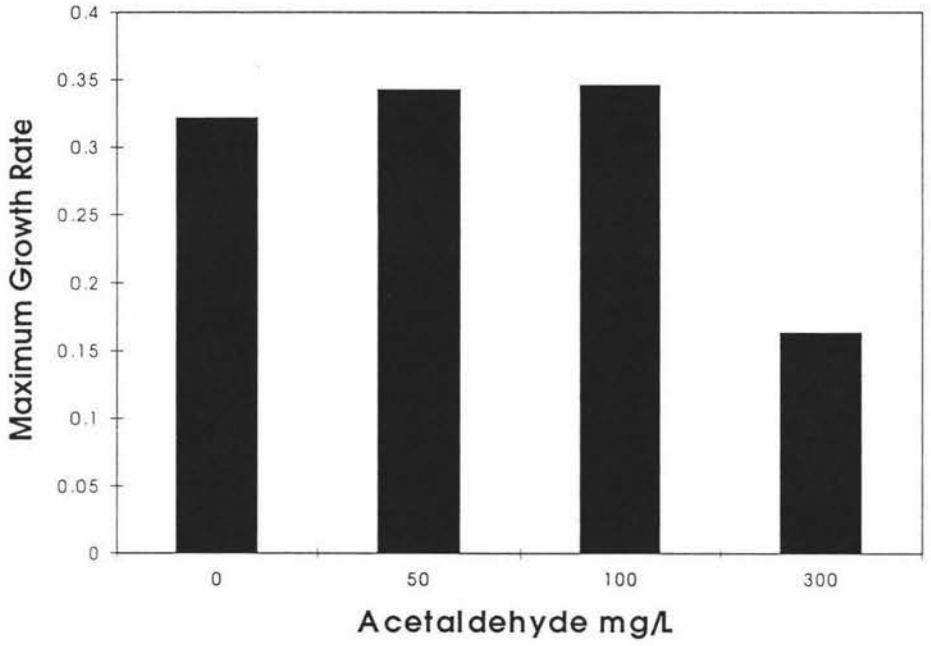


**Figure 6.1.5** Maximum growth rates ($\mu_{max}$) of *Lb. buchneri* CUC-3 grown in VJG broth at various acetaldehyde concentrations.

**Table 6.1.8** Initial and final acetaldehyde concentrations in VJG broth inoculated with *Lb. buchneri* CUC-3

| Initial Acetaldehyde Conc. (mg/L) | Final Acetaldehyde Conc. (mg/L) | % degraded |
|---|---|---|
| 0 | 0 | 0 |
| 50 | 5.2 | 89.6 |
| 100 | 10.9 | 89.1 |
| 300 | 220.5 | 26.5 |

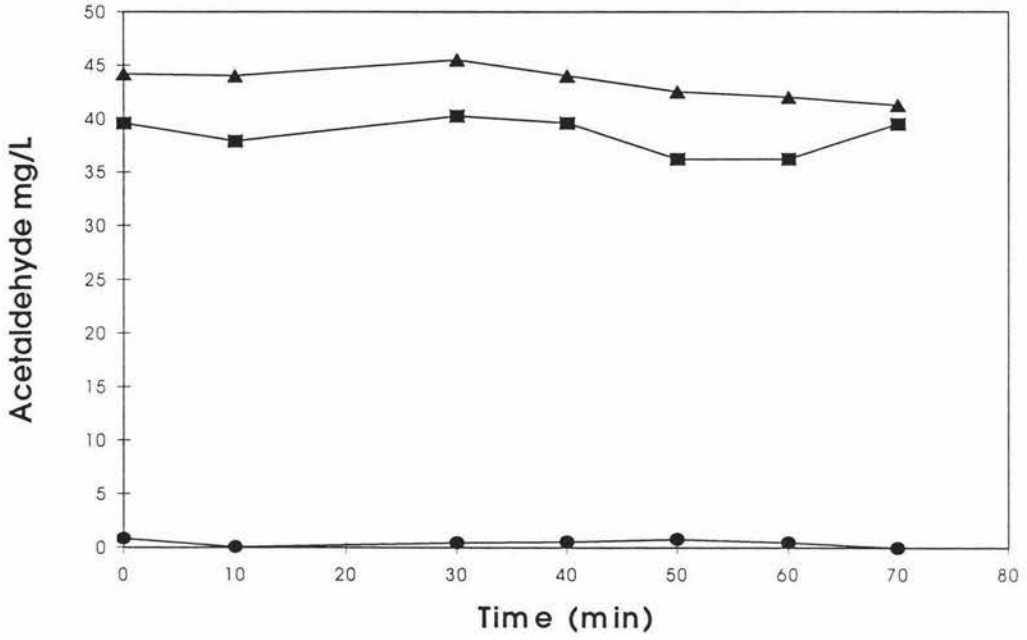


**Figure 6.1.6** Changes in the acetaldehyde concentration in a cell suspension of *Pd. damnosus* CUC-4 with glucose added at 1 g/L. Symbols; ■ acetaldehyde; ▲ positive control (acetaldehyde only); ● negative control (cell suspension only).

## 6.2. Growth in Wine

### 6.2.1. Materials and Methods

#### 6.2.1.1. Media Preparation

The wine used was prepared as per section 3.7. The wine contained approximately 90 mg/L acetaldehyde. An attempt was made to remove the acetaldehyde from 500 mL of the wine using a vacuum distiller. This was successful in lowering the acetaldehyde level to approximately 50 mg/L. The wine was dispensed in 10 mL aliquot's into screw-capped test tubes. These test tubes fit directly into the spectrphotometer as described in section 6.1.1.2. The acetaldehyde in these test tubes was then adjusted (by the addition of stock acetaldehyde solution) so that there were four replicas each at 50, 100 and 300 mg/L concentration.

6.2.1.2. Culture Preparation

The wine LAB used was a commercially available freeze dried culture of *O. oeni* VFO obtained from Chr. Hansen (Cophagen, Denmark). It was stored at 4°C until needed. The culture was rehydrated according to the manufacturer's instructions.

6.2.1.3. Experimental Procedure

At each acetaldehyde concentration, three replica tubes were inoculated with wine LAB at 5 x $10^5$ cells/mL (section 6.2.1.2). The fourth test tube at each concentration was uninoculated and served as a control. The OD for each tube was recorded. All tubes were incubated at 18°C and OD readings were taken regularly until a growth curve was obtained. At the end of incubation, the acetaldehyde concentration in each tube was measured as described in section 3.5.1. The data from the growth curves were analysed as per section 6.1.1.4.

### 6.2.2. Results

Figure 6.2.1 shows the growth of *O. oeni* VFO grown in wine (pH 3.6) at various acetaldehyde concentrations. From these results, doubling times and maximum growth rates were calculated. Maximum growth rates for each concentration are presented in Table 6.2.1 and are shown in Figure 6.2.2. Also shown in Table 6.2.1 are the mean values of the maximum growth rates at each acetaldehyde concentration. The least significant difference was calculated using results from an ANOVA analysis and this was used to determine if the differences between the means were significant or not. None of the differences between the means of the maximum growth rates at each acetaldehyde concentration were significant (i.e., none of the differences were larger then the least significant difference). Table 6.2.2 shows the initial and final acetaldehyde concentration of the wine in which *O. oeni* VFO was grown. These results show that at all concentrations there was almost complete degradation of acetaldehyde. Uninoculated controls showed no significant decrease in acetaldehyde concentration over time indicating that the degradation of the acetaldehyde was due to bacterial metabolism.

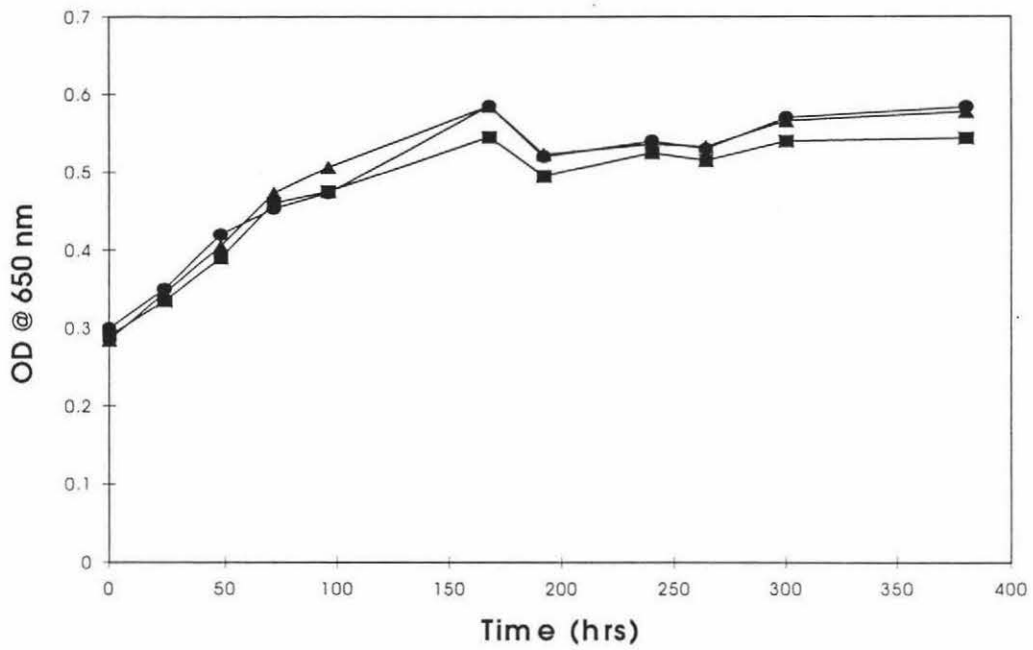


**Figure 6.2.1** Growth of *O. oeni* VFO in wine (pH 3.6) at various acetaldehyde concentrations. Results are averages of three experiments. Symbols; ● 50 mg/L acetaldehyde; ▲ 100 mg/L acetaldehyde; ■ 300 mg/L acetaldehyde.

**Table 6.2.1** Growth rates of *O. oeni* VFO at different acetaldehyde concentrations

| Acetaldehyde Concentration mg/L | Maximum Growth Rate $\mu_{max}$ (mean) |
|---|---|
| 50 | 0.00543 |
| 100 | 0.00619 |
| 300 | 0.00501 |
| | |
| Mean Differences | |
| Mean(50 mg/L - 100 mg/L) | 0.00076 |
| Mean (50 mg/L - 300 mg/L) | 0.00042 |
| Mean (100 mg/L - 300 mg/L) | 0.00118 |
| | |
| Least Significant Difference = 0.001221 | |

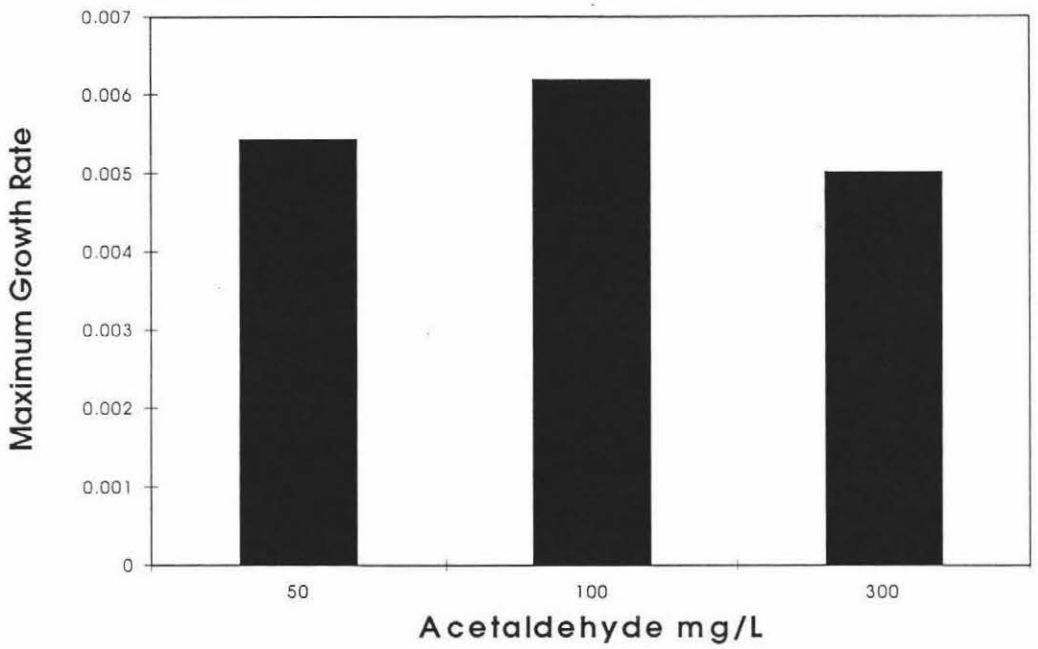


**Figure 6.2.2** Maximum growth rates ($\mu_{max}$) of *O. oeni* VFO grown in wine (pH 3.6) at various acetaldehyde concentrations.

**Table 6.2.2** Initial and final acetaldehyde concentrations in wine (pH 3.6) inoculated with *O. oeni* VFO

| Initial Acetaldehyde Conc. (mg/L) | Final Acetaldehyde Conc. (mg/L) | % degraded |
|---|---|---|
| 50 | 2.9 | 94.2 |
| 100 | 4.1 | 95.9 |
| 300 | 4.5 | 98.5 |

## 6.3. Discussion

### 6.3.1. Growth in VJG Broth

These results demonstrate that acetaldehyde at concentrations of 300 mg/L or above in VJG broth, was inhibitory to the growth of the four bacterial strains tested. For all four strains, there was no significant inhibition at the 300 mg/L concentration of acetaldehyde. There was no significant stimulation of growth for any of the strains at any acetaldehyde concentration. The inhibitory affect of acetaldehyde at high

concentrations (> 100 mg/L) has been demonstrated in dairy LAB (El-Gendy *et al*, 1983). It is thought to be due to the toxic effects of acetaldehyde at high concentrations. The lack of stimulation of growth at the lower concentrations of acetaldehyde was unexpected. This was because it had been demonstrated that for dairy LAB, there was stimulation of growth at acetaldehyde levels below 100 mg/L (Lindsay *et al*, 1965). The proposed model (Figure 4.3.1) demonstrated that the reduction of acetaldehyde to ethanol would result in the production of extra ATP through the conversion of acetyl phosphate to acetic acid. This ATP production should result in increased growth of the bacterial culture. However, this stimulatory effect of acetaldehyde was not observed here. The nutrient rich medium in which the cultures were grown (VJG broth) may account for this discrepancy. It was thought that any energetical advantage that could be gained through the metabolism of acetaldehyde would be masked due to the growth of the cultures in a nutrient rich medium. However, in the harsher environment of wine, this may not be the case. Consequently, further work was undertaken to determine the effect of acetaldehyde on the growth of wine LAB in wine (section 6.2).

After incubation, the acetaldehyde concentration in all of the inoculated broth cultures was lower, demonstrating that the bacteria were able to metabolise acetaldehyde during growth. The metabolism of acetaldehyde by *Pd. damnosus* CUC-4 was unexpected as a previous experiment using resting cells had shown that this bacteria was unable to degrade acetaldehyde (section 4.1). Further resting cell experiments using glucose as an energy source yielded the same results as in previous resting cell incubations (section 4.1). This may indicate that for *Pd. damnosus* CUC-4, other factors present in the VJG broth but absent in the resting cell experiments may be required for acetaldehyde metabolism. The enzymes may also be inducible and so the bacteria may require exposure to acetaldehyde for a period of time before the enzymes are produced. Further resting cell experiments using bacteria which have been pre-incubated in media containing acetaldehyde need to be undertaken to ascertain if the enzymes are inducible or not. Differences in pH may also have played a role in the observed results. The pH of the VJG broth was 5.5 while the pH of the wine was 3.6. This difference may have attributed to the ability of CUC-4 to utilise acetaldehyde in the broth but not in the wine. Further experiments in broth should be undertaken using broth at a wine pH.

Any inhibition or stimulation of wine LAB could affect MLF. Inhibition by acetaldehyde may cause the MLF to be sluggish or become stuck while stimulation of wine LAB by acetaldehyde may speed up the MLF. Therefore, it is important to know which bacterial strains are inhibited or stimulated by acetaldehyde, and at what concentrations. Further experiments need to be carried out using other commercially available malolactic starter strains and these experiments should be carried out in wine.

### 6.3.2. Growth in Wine

The results from this experiment have shown that there was no statistically significant inhibition or stimulation of growth for *O. oeni* VFO in wine at any of the acetaldehyde concentrations tested (up to 300 mg/L). The lack of inhibition at 300 mg/L concentration of acetaldehyde was unexpected as in VJG broth it had been shown that there was inhibition of growth at 300 mg/L concentration (section 6.1). This lack of inhibition may be due to the complex nature of wine. In wine, there are many more compounds able to bind to acetaldehyde which may reduce the toxicity of acetaldehyde at higher concentrations. These interactions of acetaldehyde in wine may also explain the lack of growth stimulation of *O. oeni* VFO when grown at lower levels of acetaldehyde. Also, as it was not possible to remove all of the acetaldehyde from the wine prior to the experiment (using the vacuum distiller), a comparison between wine with no acetaldehyde and that with varying concentrations of acetaldehyde was not possible. For future experiments, either synthetic wine (containing no acetaldehyde) or wine produced using a yeast which does not produce acetaldehyde should be used. The fact that there was no significant inhibition of growth at any acetaldehyde concentration indicates that in wine (where acetaldehyde concentrations typically range from 20 to 200 mg/L), acetaldehyde alone may not cause a stuck or sluggish fermentation.

Although the initial acetaldehyde concentrations of the wine were known (as measured), the acetaldehyde levels were not monitored during the course of the experiment. These acetaldehyde levels may indeed have increased at some point due to auto-oxidation of ethanol and phenolic compounds present in the wine. Auto-oxidation would have been accelerated by the stirring necessary to resuspend the bacteria when taking OD measurements. Therefore, comparisons between the growth rates at each concentration may not be meaningful. In future experiments, care should

be taken to remove as much air as possible from each tube (i.e. fill each tube to the top with wine to eliminate any excess air) and to minimise the amount of stirring when taking OD readings. *O. oeni* VFO was able to metabolise (almost completely) the acetaldehyde which was present in the wine showing that metabolism of acetaldehyde did indeed occur.

# Chapter 7 Simultaneous Incubation of Resting Cells of Yeast and LAB

## 7.1. Production of Acetaldehyde by Resting Cells of Yeast

Experiments were performed to assess the suitability of *S. bayanus* Première Cuvée for use in simultaneous resting cell experiments. A wine yeast that was able to produce acetaldehyde under resting cell conditions in a buffered system at wine pH was required.

### 7.1.1. Materials and Methods

#### 7.1.1.1. Media Preparation

YM broth and slopes were prepared as in section 3.2.1.

#### 7.1.1.2. Culture Preparation

Cultures were prepared as in section 3.7.2

#### 7.1.1.3. Experimental Procedure

Cells were harvested as per section 4.1.1.3 except that YM broth was used instead of VJG broth. A resting cell experiment was carried out as per section 4.1.1.3. For this experiment, however, one vial had acetaldehyde added to the yeast cells at 50 mg/L while the other vial had glucose added to the yeast cells to give 2 g/L glucose in the vial. A 50% glucose solution was used. Dry weight, and acetaldehyde concentrations were measured as per sections 3.6 and 3.5.1 respectively.

### 7.1.2. Results

Figure 7.1.1 shows the production and re-assimilation of acetaldehyde by resting cells of *S. bayanus* Première Cuvée with glucose added at 2 g/L. Acetaldehyde is produced by the yeast during the first 30 min. The acetaldehyde is then utilised by the yeast. Acetaldehyde levels reached as high as 58 mg/L and then dropped down to below 20 mg/L. As a comparison, the yeast was also able to utilise the added acetaldehyde under similar conditions (Figure 7.1.1). In this experiment, acetaldehyde was added at 50 mg/L but no glucose was added.

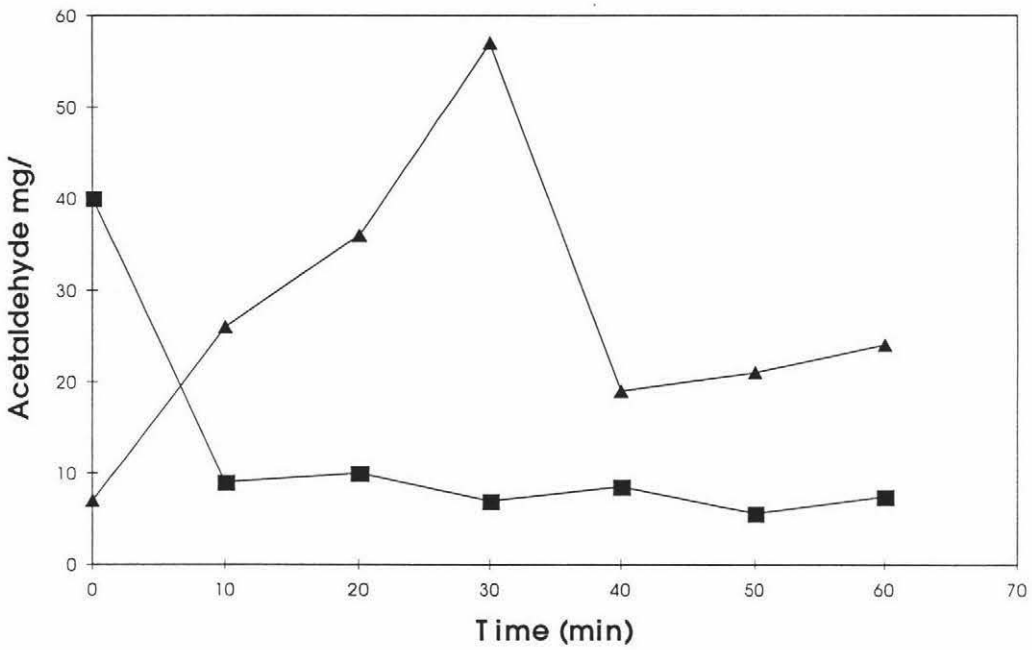


**Figure 7.1.1** Production of acetaldehyde by resting cells of *S. bayanus* Première Cuvée with glucose added at 2 g/L ▲, and utilisation of added acetaldehyde by the same yeast (resting cells) in the absence of glucose ■. Dry weights were over 10 mg in both experiments.

## 7.2. Simultaneous Incubation Using Resting Cells of Yeast and Wine LAB

The previous experiments had shown that the resting cells of wine yeast *S. bayanus* Première Cuvée were able to produce acetaldehyde (section 7.1) and that the resting cells of wine LAB *O. oeni* Lol11were able to metabolise acetaldehyde (section 4.1). Therefore, a resting cell experiment was carried out in which resting cells of *S. bayanus* Première Cuvée and *O. oeni* Lol11 were present together to simulate a simultaneous fermentation. The aim of this experiment was to ascertain if the wine LAB was capable of metabolising acetaldehyde produced by the yeast during co-incubation with a view to understanding the metabolic interactions between the yeast and the wine LAB.

### 7.2.1. Materials and Methods

#### 7.2.1.1. Media preparation

YM broth and slopes were prepared as per section 3.2.1. AMRS broth and agar stabs were prepared as per section 3.1.1. VJG was prepared as per section 3.1.3.

#### 7.2.1.2. Culture Preparation

Cultures were prepared as described in section 3.7.2.

#### 7.2.1.3. Experimental procedure

A resting cell experiment was carried out as per 4.1.1.3. However, one vial contained 9 mL of yeast cell suspension + glucose (2 g/L in vial) while the other vial contained 4.5 mL yeast cell suspension + 4.5 mL malolactic bacteria cell suspension + glucose (2 g/L in vial). A 50% glucose solution was used. Sampling was carried out as per section 4.1.1.3 and dry weight and acetaldehyde were measured as per sections 3.6 and 3.5.1 respectively.

### 7.2.2. Results

Figure 7.2.1 shows changes in acetaldehyde concentration during the simultaneous incubation of resting cells of *S. bayanus* Première Cuvée and *O. oeni* Lol11. The results show that when only the yeast was present (plus glucose), there was an initial increase in the acetaldehyde concentration followed by utilisation of acetaldehyde by the yeast as was observed in section 7.1. When both yeast and bacteria were present together (plus glucose), there was no significant increase in acetaldehyde concentration. These results indicate that the bacteria metabolised the acetaldehyde produced by the yeast. Ethanol production was also measured in both cases to ensure that the yeast cells were viable. In both cases, ethanol was produced in similar amounts (Figure 7.2.1). This suggests that the yeast were viable and able to metabolise glucose, thus producing ethanol.

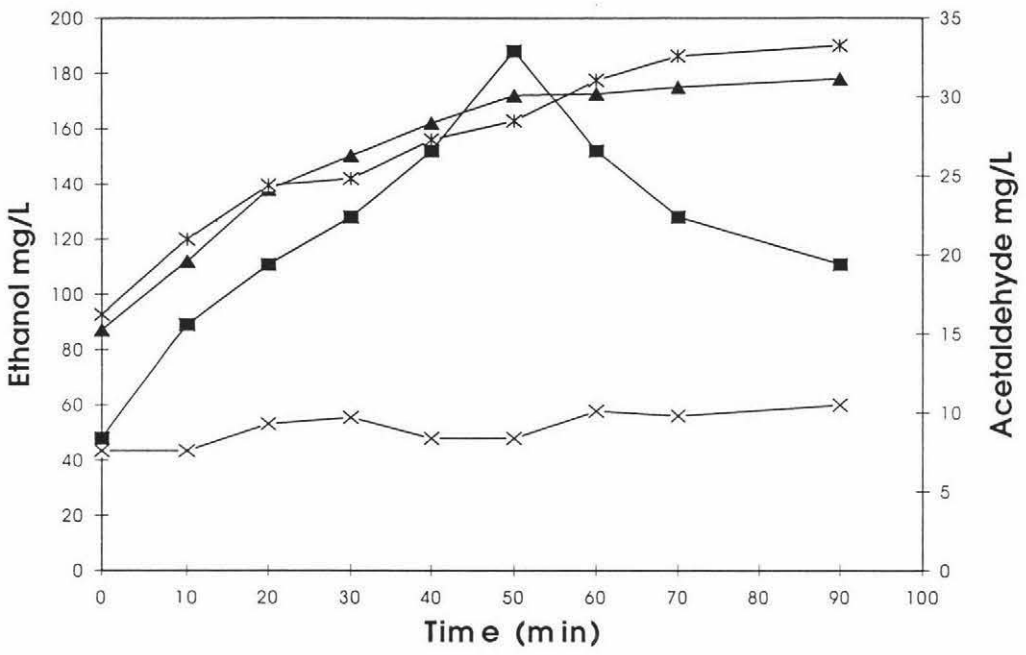


**Figure 7.2.1** Changes in acetaldehyde and ethanol concentrations during simultaneous incubation of resting cells of *S. bayanus* Première Cuvée and *O. oeni* Lol11 (resting cell experiment). Symbols; ■ Yeast + Glucose (acetaldehyde); × Yeast + Bacteria + Glucose (acetaldehyde); ▲ Yeast + Glucose (ethanol); ✳ Yeast + Bacteria + Glucose (ethanol).

## 7.3. Discussion

### 7.3.1. Production of Acetaldehyde by Resting Cells of Yeast

The results from this experiment have demonstrated the ability of the wine yeast, *S. bayanus* Première Cuvée, to produce acetaldehyde. This yeast was also able to utilise acetaldehyde as demonstrated in Figure 7.1.1. *S. bayanus* Première Cuvée is suitable for use in a simultaneous resting cell experiment with wine LAB as it was able to produce acetaldehyde in a buffered system at wine pH. It has been shown that acetaldehyde is excreted into the environment mainly during the first stages of fermentation (growth phase) (Ciani, 1997) and can also be recatabolised by the yeast (Farris *et al*, 1983). These results are consistent with these findings.

These results also demonstrate that yeast are able to produce acetaldehyde while in a resting state and also during growth in wine as has been previously shown (section 5.1.2). These results also demonstrate the ability of *S. bayanus* Première Cuvée to

utilise acetaldehyde while in a resting state as well as in wine (section 5.1.2). The pattern of acetaldehyde formation and utilisation by resting cells of *S. bayanus* Première Cuvée is consistent with that of growing cells of *S. bayanus* Première Cuvée in wine (Figure 5.1.1). This verifies the validity of using resting cells as a simple research model.

### 7.3.2. Simultaneous Incubation Using Resting Cells of Yeast and Wine LAB

The results from this experiment demonstrate that resting cells of *O. oeni* Lol11 were indeed able to degrade acetaldehyde that had been produced by resting cells of *S. bayanus* Première Cuvée during a simultaneous incubation. These results have practical significance for wine making as the wine industry is looking towards the use of simultaneous fermentation as a way to speed up the wine making process. In a simultaneous fermentation, both the alcoholic fermentation and MLF are inoculated at the same time. It is, therefore, important to understand the interactions between the yeast and bacteria and the influence of such interactions on the wine. This experiment has shown that for these two organisms, a product of one organism (acetaldehyde) can be metabolised by the other. This may influence the final flavour of the wine.

These results were obtained using resting cells in a buffered system at a wine pH. This experimental system closely mimics the situation in wine. Further experiments using growing cells need to be performed in wine to confirm whether or not these interactions also occur in wine. Further combinations of wine yeast and malolactic bacteria should also be tested. Results from these experiments should identify the compatibility or incompatibility of yeast and wine LAB for simultaneous fermentation

# Chapter 8 Enzyme Activity

Alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (Al-DH) are the two enzymes thought to be responsible for the conversion of acetaldehyde into ethanol and acetic acid which was shown in section 4.1. If enzyme activity from cell extracts of the strains used in section 4.1 (*Lb. hilgardii* MHP and *Lb. delbrueckii* CUC-1) were measured and corresponded to the relative amounts of ethanol and acetic acid being produced from acetaldehyde, then this would help determine that the activities of ADH and Al-DH were responsible for the amount of ethanol and acetic acid formed. Various activities of ADH and Al-DH have been found in dairy LAB (Lees & Jago, 1978), suggesting that these two enzymes are indeed involved in the metabolism of acetaldehyde. Therefore, an attempt was made to measure the activity of the enzymes ADH and Al-DH were measured to correlate enzyme activities with formation of ethanol and acetic acid from acetaldehyde. Saturating substrate levels were used so that the maximum activity of each enzyme could be compared.

## 8.1.1. Materials and Methods

### 8.1.1.1. Media Preparation

Media was prepared as in section 3.1

### 8.1.1.2. Culture Preparation

The malolactic bacteria used were *Lb. hilgardii* MHP and *Lb. delbrueckii* CUC-1. These strains were maintained at 4°C after growth as stabs in AMRS agar. For routine use, subcultures of strains were prepared in AMRS broth (one loop full in 5 mL broth) and stored at 4°C without prior incubation (growth). When a culture was required, it was removed from the cold room and incubated at 30°C until growth was visible as indicated by turbidity changes. Cells were harvested by centrifugation at 5,000 rpm for 10 min. Cells were washed in phosphate buffer (see section 3.3.2) and centrifuged at 5,000 rpm for a further 10 min. The cells were then resuspended in 20 mL of phosphate buffer and placed in an ice bath. Cells were ruptured by three passes

through a French Press. The cell extract was then centrifuged at 14,000 rpm for 20 min. The supernatant was decanted off and stored in an ice bath.

8.1.1.3. Experimental Procedure

Enzyme activity was measured using a Cary Spectrophotometer connected to a computer. The buffers used were the same as used in section 3.5.1 for Al-DH and 3.5.2 for ADH. For determining enzyme activity, 2 mL of buffer was added to a cuvette. To this, 0.5 mL of acetaldehyde/ethanol (depending on which enzyme is being assayed) was added. Absorbance at 340 nm was followed until stable readings had been obtained. Then, 0.5 mL of the cell extract supernatant was added to the cuvette to start the reaction and absorbance readings were monitored (once every 12 seconds) until a curve was obtained. Total protein of the cell extract was determined using the Bradford protein assay (section 3.4.2.)

### 8.1.2. Results and Discussion

Activities of ADH and Al-DH were not detectable in cell free extracts of any of the strains examined.. At first it was thought that the protein concentration of the cell extract was too low to allow measurable enzyme activity. However, the total protein concentration of the cell extract was measured and found to be 1.8 mg/mL $NADP^+$ was used in a subsequent experiment instead of $NAD^+$ (which was present in the buffers being used). Again, no significant activities were obtained. Larger amounts of substrate, buffer, and cell extract were then used but still no significant activities were obtained. The activities of ADH and Al-DH from a commercial source were assayed to ensure that the assay system worked. The results (not shown) demonstrated that the assay system being used was capable of detecting the activities of Al-DH and ADH. The failure to detect the activities of Al-DH and ADH does not mean that these enzymes are not present in wine LAB. It is possible that the conditions used were not optimum for the activity of these two enzymes. There may be other factors that are required. The commercial ADH and Al-DH enzymes used to ensure that the assay system worked were obtained from yeast. The bacterial ADH and Al-DH involved in the metabolism of acetaldehyde may require different conditions then the yeast enzymes. Further work is needed to optimise the assay conditions for bacterial ADH and Al-DH.

# Chapter 9 General Discussion

This research has definitvely demonstrated the ability of wine LAB to metabolise free and bound acetaldehyde while in a resting and growing state. Nine out of eleven strains metabolised free acetaldehyde while in a resting state. The survey of strains contained both heterofermentative and homofermentative LAB and included *Oenococcus*, *Lactobacillus* and *Pediococcus* strains. Acetaldehyde consumption and/or depletion during MLF had previously been observed by Somers & Wescombe, (1987). The results from this research definitively showed that acetaldehyde can be metabolised by wine LAB and this metabolism would account for the depletion of acetaldehyde during MLF. The products formed from this metabolism were ethanol and acetic acid. These products are not expected to have any major impact on wine flavour because ethanol is desirable in wine. Although acetic acid is not desirable in wine, the amount of acetic acid produced would be too small to affect the overall flavour and quality of the wine. The removal of acetaldehyde from the wine, however, is expected to have an impact on wine flavour. Excess acetaldehyde is undesirable in wine as it has a sour, grassy, green apple like aroma. Therefore, from a sensory point of view, the removal of acetaldehyde by wine LAB is desirable. *Lb. buchneri* CUC-3 and *O. oeni* MCW were surveyed for their ability to metabolise $SO_2$ bound acetaldehyde and were indeed able to degrade this bound form of acetaldehyde. In wine, $SO_2$ is added to mask excess acetaldehyde as it binds strongly to acetaldehyde. This bound $SO_2$ is less effective in its other roles as an antimicrobial and antioxidant agent. The metabolism of $SO_2$ bound acetaldehyde releases free $SO_2$ which may be sufficient to limit further growth of the wine LAB and may play a role in sluggish and stuck MLF. The inhibition by acetaldehyde bound $SO_2$ of wine LAB has been previously reported (Hood, 1983; Delfini & Morsiani, 1992). Therefore, bound $SO_2$ may act as a reservoir of free $SO_2$.

Free acetaldehyde in wine was metabolised by *O. oenos* VFO and *O. oenos* EQ54 at pH 3.3 and pH 3.6. It appears that pH has no effect on the metabolism of free acetaldehyde. The metabolism of acetaldehyde coincided with the degradation of malic acid during growth of the wine LAB. The metabolism of free acetaldehyde by wine LAB may be an alternative to $SO_2$ use for the removal of excess acetaldehyde from wine to improve wine aroma. Currently, the negative effects of excess

acetaldehyde are masked by the addition of $SO_2$. However, today there is a tendency among winemakers to reduce $SO_2$ use to meet consumer demand because of health implications (Yang *et al*, 1985). Acetaldehyde plays a role in the colour development of red wine, so the complete removal of acetaldehyde by wine LAB may not be desired in red wine. However, there will always be some production of acetaldehyde due to auto-oxidation of ethanol and phenolic compounds. This makes strain selection crucial as the winemaker would need to know if the wine LAB being used for MLF was able to degrade acetaldehyde or not. Timing of MLF is also important. It may be necessary to delay the induction of MLF to allow some colour development before removing the acetaldehyde through MLF.

The degradation of $SO_2$ bound acetaldehyde by *O. oenos* VFO and *O. oenos* EQ54 in wine at pH 3.6 was demonstrated. After a period of slow growth, the wine LAB increased in growth rate and degraded the bound acetaldehyde. The slow growth in the presence of $SO_2$ bound acetaldehyde, compared to the faster growth with free acetaldehyde at same pH, shows the inhibitory effect of acetaldehyde bound $SO_2$. This is consistent with previous reports (Hood, 1983; Delfini & Morsiani, 1992). Some work has been done on the inhibitory effect of acetaldehyde bound $SO_2$ on LAB growth (Fornachon, 1963; Hood, 1983; Delfini & Morsiani, 1992). These reports suggested the metabolism of the acetaldehyde moiety by LAB, thus releasing free $SO_2$ which leads to the inhibition of the bacteria. However no reports have emerged demonstrating that LAB can indeed metabolise the acetaldehyde moiety of $SO_2$ bound acetaldehyde. This research has provided the first evidence that LAB can indeed catabolise $SO_2$ bound acetaldehyde, therefore releasing free $SO_2$. The delay in growth of the wine LAB when in the presence of $SO_2$ bound acetaldehyde suggests a possible role of $SO_2$ bound acetaldehyde in causing stuck and sluggish MLF. This was further demonstrated in wine at pH 3.3. At this lower pH, growth of wine LAB in the presence of $SO_2$ bound acetaldehyde was even further delayed than that at pH 3.6. In fact, degradation of $SO_2$ bound acetaldehyde was shown not to occur in wine at pH 3.3 during incubation. At pH 3.3, there was an extended period of slow growth and perhaps if the experiment had been allowed to continue for a longer period of time, acetaldehyde degradation might have been observed. The increased inhibition of growth at pH 3.3 compared to pH 3.6 may be explained by the fact that there might have been more free $SO_2$ present at pH 3.3 than at pH 3.6. This is due to the fact that the levels of each form of $SO_2$ in solution are pH dependent and at lower pH, the

molecular form of $SO_2$ predominates over the bisulphite form (which binds strongly to acetaldehyde) (Zoecklein *et al*, 1995). At both pH 3.3 and pH 3.6, there was considerable inhibition of growth of the wine LAB in the presence of $SO_2$ bound acetaldehyde when compared to growth in the presence of free acetaldehyde.

Growth rate experiments demonstrated that in VJG broth acetaldehyde at 300 mg/L inhibited the growth of all four wine LAB tested. There was no observed stimulation of growth for any of the wine LAB tested. The inhibitory affect of acetaldehyde is thought to be due to the toxic effects of acetaldehyde at high concentrations (Jones, 1989) and has been demonstrated in dairy LAB (El-Gendy *et al,* 1983). All wine LAB tested degraded the acetaldehyde during incubation. It was thought that this degradation of acetaldehyde might stimulate the growth of the wine LAB. This is because the proposed model for acetaldehyde metabolism results in the production of extra ATP (Chapter 4). Stimulation of growth by acetaldehyde has been demonstrated in dairy LAB. Collins & Specks (1974) and Schmitt & Divies (1990) showed that at low levels acetaldehyde stimulated the growth of dairy LAB. This was not observed, however, for the wine LAB used in this experiment (*O. oenos* MCW, *Lb. delbrueckii* CUC-1, *Lb. buchneri* CUC-3 and *Pd. damnosus* CUC-4). Perhaps the nutrient rich media (VJG broth) might have masked any energetical advantage that could be gained through the metabolism of acetaldehyde. Therefore, further experiments investigating the effect of acetaldehyde on the growth of wine LAB in wine were undertaken. Wine is a much harsher environment for growth. The results from this experiment showed that there was no statistically significant inhibition or stimulation of growth of *O. oenos* VFO at any of the acetaldehyde concentrations tested (up to 300 mg/L). This lack of inhibition or stimulation may be due to the complex nature of wine.

Any inhibition or stimulation of wine LAB by acetaldehyde could affect MLF. Stimulation of wine LAB could result in a faster MLF while inhibition of wine LAB could result in a stuck or sluggish MLF.

Simultaneous incubation experiments demonstrated the ability of resting cells of *S. bayanus* Première Cuvée to produce acetaldehyde from added glucose and to utilise added acetaldehyde. Resting cells of this wine yeast were subsequently used in a simultaneous incubation with resting cells of *O. oenos* Lol11. The results from the incubation demonstrated that resting cells of *O. oenos* Lol11 were able to degrade the

acetaldehyde produced by resting cells of *S. bayanus* Première Cuvée. This demonstrates an interesting and significant metabolic interaction between yeast and the LAB. The concentration of acetaldehyde produced by the yeast is crucial. High levels of acetaldehyde may cause inhibition of the wine LAB while at lower levels, acetaldehyde produced by the yeast may stimulate growth of the wine LAB. The presence or absence of $SO_2$ is also important. For simultaneous fermentation, it may be unnecessary to add $SO_2$ to mask excess acetaldehyde as the wine LAB will metabolise it as the yeast produces it. However, if $SO_2$ is added as an antioxidant and antimicrobial agent, then the resulting acetaldehyde bound $SO_2$ could lead to a stuck or sluggish MLF. The wine industry is looking towards the use of simultaneous fermentation as a way to speed up the wine making process, and so these results are of significance as it is important to understand what interactions there are between the yeast and the bacteria and how these interactions will influence the wine.

# Chapter 10 Summary and Future Work

## 10.1. Summary

Using resting cells, nine of eleven LAB strains tested were able to metabolise free acetaldehyde in a buffered system at wine pH (3.6). Metabolism of free acetaldehyde resulted in the production of ethanol and acetic acid. The two bacterial strains unable to degrade acetaldehyde were *Pd. damnosus* CUC-4 and *Pd. sp* 44.40.

Resting cells of *O. oeni* MCW and *Lb. buchneri* CUC-3 were able to metabolise $SO_2$ bound acetaldehyde although at a reduced rate (compared to free acetaldehyde metabolism).

Both *O. oeni* EQ54 and *O. oeni* VFO were able to degrade free acetaldehyde in wine at pH 3.3 and pH 3.6 during MLF. The acetaldehyde in the wine was produced by *S. bayanus* Première Cuvée.

In wine at pH 3.6, both *O. oeni* EQ54 and *O. oeni* VFO were able to metabolise $SO_2$ bound acetaldehyde after initial sluggish growth. Neither *O. oeni* EQ54 nor *O. oeni* VFO was able to metabolise $SO_2$ bound acetaldehyde in wine at pH 3.3 within the incubation period.

No stimulation of growth from acetaldehyde metabolism was found, but there was inhibition of growth when grown in VJG broth containing acetaldehyde at 300 mg/L concentration. All strains metabolised acetaldehyde during growth. The growth of *O. oeni* VFO was neither inhibited nor stimulated when grown in wine containing acetaldehyde at up to 300 mg/L concentration.

During a simultaneous incubation of resting cells of *S. bayanus* Première Cuvée and *O. oeni* VFO, the LAB was able to metabolise the acetaldehyde produced by *S. bayanus* Première Cuvée.

## 10.2. Future Work

Acetaldehyde is an important sensory compound in wine and little is known about its effect on wine LAB and vice versa. This study has opened many areas requiring more research. Future work into acetaldehyde metabolism by wine LAB and its oneological significance should involve the following:

1) Undertaking of a more complete survey for the ability of wine LAB to degrade both free and $SO_2$ bound acetaldehyde including homofermentative species (using both resting and growing cells).

2) Identification of all products formed from the metabolism of acetaldehyde by wine LAB.

3) Investigation of the metabolism of $SO_2$ bound acetaldehyde by wine LAB in wine; taking care to reduce increases in acetaldehyde through auto-oxidation of ethanol and phenolic compounds. Experiments should be allowed to run for an extended period in case the MLF becomes stuck or sluggish especially at low pH and/or in synthetic wine or minimal media.

4) Further investigation of the effects of acetaldehyde on growth of wine LAB in wine using commercially available strains. Wine containing no initial acetaldehyde should be used and care should be taken to reduce the occurrence of auto-oxidation of ethanol and phenolic compounds during incubation.

5) Investigate whether the enzymes ADH and Al-DH are inducible in *Pd. damnosus* CUC-4. Pre-incubate culture in media containing acetaldehyde.

6) Optimise the assay conditions for ADH and Al-DH so that the activities of these two enzymes can be obtained. The results from this experiment will help confirm whether or not ADH and Al-DH are responsible for the conversion of acetaldehyde to ethanol and acetic acid in wine LAB.

7) Investigate the effect of simultaneous incubation of yeast and wine LAB on acetaldehyde in wine. This experiment should also look into the impact of acetaldehyde production on growth of wine LAB and malic acid degradation. The effect of timing of LAB inoculation on LAB growth and malic acid degradation should be investigated as should the addition of $SO_2$ and the effect it has on the MLF.

# Chapter 11 Literature Cited

Adachi, O., Tayama, K., Shinagawa, K. & Ameyama, M. (1980). Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. *Agricultural and Biological Chemistry*, **44**, 503-515.

Amerine, M.A. & Ough, C.S. (1974). *Methods for Analysis of Musts and Wines*.New York: John Wiley & Sons, Inc.

Amerine, M.A., Berg, H.W., Kunkee, R.E., Ough, C.S., Singleton, V.L. & Webb, A.D. (1980). *The Technology of Wine Making*. Connecticut: AVI Publishing Company Inc.

Ameyana, M. & Adachi, O. (1982). Aldehyde dehydrogenase from acetic acid bacteria, membrane-bound. *Methods of Enzymology*, **89**, 491-497.

Bakker, J. & Timberlake, C.F. (1986) The mechanism of colour changes in aging port wine. *American Journal of Enology and Viticulture,* **37**, 288-292.

Bakker, J., Picinelli, A & Bridle, P. (1993). Model wine solutions: colour and composition changes during aging. *Vitis,* **32**, 111-118.

Bartowsky, E.J. & Henschke, P.A. (1995). Malolactic fermentation and wine flavour. *The Australian Grapegrower and Winemaker*, April, **No. 374a**, 83-94.

Bergmeyer, H.U. (1974). *Methods of Enzymatic Analysis*. New York: Academic Press, Inc.

Bisson, L.F. (1999). Stuck and sluggish fermentations. *American Journal of Enology and Viticulture*, **50**, 107-119.

Boulton, R.B., Singleton, V.L., Bisson, L.K. & Kunkee, R.E. (1996). *Principles and Practices of Winemaking*. New York: Chapman and Hall.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* **72**, 248-254.

Burroughs, L.F. & Sparks, A.H. (1973). Sulphite-binding power of wines and ciders. II. Theoretical considerations and calculation of sulphite-binding equilibria. *Journal of the Science of Food and Agriculture*, **24**, 199-206.

Cabranes, C., Moreno, J. & Mangas, J.J. (1998). Cider production with immobilized *Leuconostoc oenos. Journal of the Institute of Brewing*, **104**, 127-130.

Cannon, M.C. & Pilone, G.J. (1993). Interactions between commercial wine yeast and malolactic bacteria. In: *Proceedings of the New Zealand Wine and Grape Symposium: sustainability/Cabernet Sauvignon* (edited by D.T. Jordan). Auckland, New Zealand: New Zealand Society for Viticulture and Oenology. Pp. 85-89

Ciani, M. (1997). Role, enological properties and potential use of non-*Saccharomyces* wine yeast. *Recent Research Developments in Microbiology*, **1**, 317-331.

Cogan, T.M. & Jordan, K.N. (1994). Metabolism of *Leuconostoc* bacteria. *Journal of Dairy Science*, **77**, 2704-2717.

Collins. E.B. & Speckman, R.A. (1974). Influence of acetaldehyde on growth and acetoin production by *Leuconoctoc citrovorum. Journal of Dairy Science,* **57**, 1428-1431.

Cox, D.J. & Henick-Kling, T. (1989). Chemiosmotic energy from malolactic fermentation. *Journal of Bacteriology*, **171**, 5750-5752.

Davis, C.R., Wibowo, D., Lee, T.H. & Fleet, G.H. (1985). Practical implications of malolactic fermentation. A review. *American Journal of Enology and Viticulture.* **36**, 290-301.

Delfini, C. & Morsiani, M.G. (1992). Resistance to sulphur dioxide of malolactic strains of *Leuconostoc oenos* and *Lactobacillus sp.* isolated from wines. *Sciences Des Aliments,* **12**, 493-511.

Dicks, L.M.T., Dellaglio, F. & Collins, M.D. (1995). Proposal to reclassify *Leuconostoc oenos* as *Oenococcus oeni. International Journal of Systematic Bacteriology*, **45**, 395-397.

Drysdale, G.S. & Fleet. G.H. (1989). The effect of acetic acid bacteria upon the growth and metabolism of yeasts during the fermentation of grape juice. *Journal of Applied Bacteriology,* **67**, 471-481.

Edwards, C.G., Haag, K.M., & Collins, M.D. (1998) Identification of some lactic acid bacteria associated with sluggish/stuck fermentations. *American Journal of Enology and Viticulture*, **49**, 445-448.

Edwards, C.G., Reynolds, A.G., Rodriguez, A.V., Semon, M. & Mills, J. (1999). Implication of acetic acid in the induction of slow/stuck grape juice fermentation and inhibition of yeast by *Lactobacillus. sp. American Journal of Enology and Viticulture*, **50**, 204-210.

El-Gendy, S.M., Abdel-Galil, H., Shahin, Y. & Hegazi, F.Z. (1983). Acetoin and diacetyl production by homo- and heterofermentative LAB. *Journal of Food Protection,* **46**, (420-425), 428.

Farris, G.A., Fatichenti, F., Deiana, P. & Madau, G. (1983). Functional selection of low sulphur dioxide acceptor producers among 30 *S. cerevisiae* strains. *Journal of Fermentation Technology,* 61, 201-204.

Fleet, G.H. & Heard, G.M. (1993). Yeasts - growth during fermentation. In: *Wine Microbiology and Biotechnology* (edited by G.H. Fleet). Pp. 27-54. Amsterdam: Harwood Academic Publishers.

Fornachon. J.C.M. (1963). Inhibition of certain LAB by free and bound sulphur dioxide. *Journal of the Science of Food and Agriculture,* **14**, 857-862.

Fornachon. J.C.M. (1968). Influence of different yeasts on the growth of lactic acid bacteria in wine. *Journal of the Science of Food and Agriculture,* **19**, 374-378.

Francia-Aricha, E.M., Guerra, M.T., Rivas-Gonzalo, J.C. & Santos-Buelga, C. (1998). New anthocyanin pigments formed after condensation with flavonols. *Journal of Agriculture and Food Chemistry*, **45**, 2262-2266.

Fugelsang, K.C. (1997). *Wine Microbiology*. New York: Chapman and Hall.

Fulcrand, H., Benabdeljalil, C.,Rigaud, J., Cheynier, V. & Moutounet, M. (1998). A new class of wine pigments generated by a reaction between pyruvic acid and grape anthocyanins. *Phytochemistry*, **47**, 1401-1407.

Griffith, R. & Hammond, E.G. (1989). Generation of Swiss cheese flavour components by the reaction of amino acids with carbonyl compounds. *Journal of Dairy Science,* **61**, 581-585.

Henick-Kling, T. (1993). Malolactic Fermentation. In: *Wine Microbiology and Biotechnology* (edited by G.H. Fleet). Pp. 286-326. Amsterdam: Harwood Academic Publishers.

Henschke, P.A. & Jiranek, V. (1993). Yeasts - metabolism of nitrogen compounds. In: *Wine Microbiology and Biotechnology* (edited by G.H. Fleet). Pp. 77-164. Amsterdam: Harwood Academic Publishers.

Herraiz, T., Martin-Alvarez, P.J., Reglero, G., Herraiz, M. & Cabezudo, M.D. (1989). Differences between wines fermented with and without $SO_2$ using various selected yeast. *Journal of the Science of Food and Agriculture,* **49**, 249-258.

Hood, A. (1983). Inhibition of wine LAB by acetaldehyde bound sulphur dioxide. *The Australian Grapegrower and Winemaker,* April, Pp. 34, 37-38, 40, 42-43.

Huang, Y.C., Edwards, C.G., Peterson, J.C. & Hagg, K.M. (1996). Relationship between sluggish fermentations and the antagonism of yeast by lactic acid bacteria. *American Journal of Enology and Viticulture,* **47**, 1-10.

Ingraham, J.L., Maaloe, O. & Meidhardt, F.C. (1983). *Growth of the Bacterial Cell.* Massachusetts: Sinduer Associates, Inc Publishers.

Ishikawa, H., Nobayashi, H. & Tanaka, H. (1990). Mechanism of fermentation performance of *Zymomonas mobilis* under oxygen supply in batch culture. *Journal of Fermentation and Bioengineering*, **70**, 34-40.

Jones, R.P. (1989). Biological principles for the effects of ethanol. *Enzyme and Microbial Technology,***11**, 130-153.

Keenan, T.W. & Lindsay, R.C. (1966). Removal of green flavour from ripened butter cultures. *Journal of Dairy Science,* **49**, 1563-1565.

Keenan, T.W., Lindsay, R.C. & Day, E.A. (1966a). Acetaldehyde utilisation by *Leuconostoc* species. *Applied Microbiology,* **14**, 802-806.

Keenan, T.W., Lindsay, R.C., Morgan, M.E. & Day, E.A. (1966b) Acetaldehyde production by single-strain lactic streptococci. *Journal of Dairy Science,* **49**, 10-14.

Keenan, T.W. & Lindsay, R.C. (1967). Dehydrogenase activity of *Lactobacillus* species. *Journal of Dairy Science,* **50**, 1585-1588.

Keenan, T.W., Parmelee, C.E. & Branen, A.L. (1968). Metabolism of volatile compounds of *Pediococcus cerevisiae* and their occurrence in Cheddar cheese. *Journal of Dairy Science,* **51**, 1737-1742.

King, S.W. & Beelman, R.B. (1986). Metabolic interactions between *Saccharomyces cerevisiae* and *Leuconostoc oenos* in a model grape juice/wine system. *American Journal of Enology and Viticulture,* **37**, 53-60.

Krieger, S.A. (1993). The use of active dry malolactic starter cultures. *Australian and New Zealand Wine Industry Journal*, **8**, 56-62.

Laurent, M.H., Henick-Kling, T. & Acree, T.E. (1994). Changes in the aroma and odor of Chardonnay wine due to malolactic fermentation. *Viticulture and Enology Science*, **49**, 3-10.

Lees, G.J. & Jago, G.R. (1976a). Acetaldehyde: an intermediate in the formation of ethanol from glucose by LAB. *Journal of Dairy Science,* **43**, 63-73.

Lees, G.J. & Jago, G.R. (1978). Role of acetaldehyde in metabolism: A review. *Journal of Dairy Science*, **61**, 1216-1224.

Liu, S.-Q., Davis, C.R. & Brooks, J.D. (1995). Growth and metabolism of selected LAB in synthetic wine. *American Journal of Enology and Viticulture*, **46**, 166-174.

Liu, S.-Q., Asmundson, R.V., Holland, R. & Crow, V.L. (1997). Acetaldehyde metabolism by *Leuconocstoc mesenteroides* subsp. *cremoris* under stress conditions. *International Dairy Journal,* **7**, 175-183.

Liu,S.-Q. & Pilone, G.J. (2000). An overview of formation and role of acetaldehyde in winemaking with emphasis on microbiological implications. *International Journal of Food Science and Technology*, **35**, 49-62.

Margalith, P.Z. (1981). *Flavour Microbiology*. Pp. 233-234. Springfield, Illinois: Charles C. Thomas Publishers.

McCloskey, L.P. & Mahaney, P. (1981). An enzymatic assay for acetaldehyde in grape juice and wine. *American Journal of Enology and Viticulture*, **32**, 159-162.

Millan, C. & Ortega, J.M. (1988). Production of ethanol, acetaldehyde, and acetic acid in wine by various yeast races: role of alcoholic and aldehyde dehydrogenases. *American Journal of Enology and Viticulture,* **39**, 107-112.

Ough, C.S. (1992). *Winemaking Basics*. New York: Food Products Press.

Pilkington, B. & Rose, A.H. (1988). Reactions of *S. cerevisiae* and *Zygosaccharomyces bailii* to sulphite. *Journal of General Microbiology,* **134**, 2823-2830.

Pilone, G.J. & Kunkee, R.E. (1965). Sensory characterisation of wines fermented with several malolactic strains of bacteria. *American Journal of Enology and Viticulture*, **16**, 224-230.

Pilone, G.J. (1995). A New Zealand experience in direct-vat inoculation for malolactic fermentation. *Australian and New Zealand Wine Industry Journal,* **10**, 169-173.

Rasmussen, J.E., Schultz, E., Snyder, R.E., Jones, S.R. & Smith, C.R. (1995) Acetic acid as a causative agent producing stuck fermentations. *American Journal of Enology and Viticulture*, **46**, 278-280.

Ribereau-Gayon, P., Pontallier, P. & Glories, Y. (1983). Some interpretations of colour changes in young red wines during their conservation. *Journal of the Science of Food and Agriculture,* **34**, 505-516.

Romano, P. & Suzzi, G. (1993). Sulphur dioxide and wine microorganisms. In: *Wine Microbiology and Biotechnology* (edited by G.H. Fleet). Pp. 373-393. Amsterdam: Harwood Academic Publishers.

Romano, P., Suzzi, G., Turbanti, L. & Maifreni, M. (1994). Acetaldehyde production in *S. cerevisiae* wine yeasts. *FEMS Microbiology Letters,***118**, 213-218.

Schmitt, P. & Divies, C. (1990). Effect of acetaldehyde on growth, substrates, and products by *Leuconostoc mesenteroides* ssp. *cremoris. Biotechnology Progress*, **6**, 421-424.

Somers, T.C. & Wescombe, L.G. (1987). Evolution of red wines. II. An assessment of the role of acetaldehyde. *Vitis*, **26,** 27-36.

Stanley, G.A. & Pamment, N.B. (1993). Transport and intracellular accumulation of acetaldehyde in *S. cerevisiae. Biotechnology and Bioengineering*, **42**, 24-29.

Stanley, G.A., Douglas, N.G., Every, E.J., Tzanatos, T. & Pamment, N.B. (1993). Inhibition and stimulation of yeast growth by acetaldehyde. *Biotechnology Letters*, **15**, 1199-1204.

Stanley, G.A., Hobley, T.J. & Pamment, N.B. (1997). Effect of acetaldehyde on *S. cerevisiae* subjected to environmental shocks. *Biotechnology and Bioengineering*, **53**, 71-78.

Timberlake, C.F. & Bridle, P. (1976). Interactions between anthocyanins, phenolic compounds, and acetaldehyde and their significance in red wines. *American Journal of Enology and Viticulture*, 27, 97-105.

Walsh, B. & Cogan, T.M. (1973). Diacetyl, acetoin, and acetaldehyde production by mixed-species lactic starter cultures. *Applied Microbiology*, 26, 820-825.

Wibowo, D., Eschenbruch, R., Davis, C.R., Fleet, G.H. & Lee, T.H. (1985). Occurrence and growth of LAB in wine. A review. *American Journal of Enology and Viticulture*, **36**, 302-313.

Wildenrandt, H.L. & Singleton, V.L. (1974). The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. *American Journal of Enology and Viticulture*, **25**, 119-126.

Yang, W.H. & Purchase, E.C. (1985). Adverse reactions to sulfites. *Canandian Medical Association Journal*, **133**, 865-867.

Zoecklein, B.W., Fugelsang, K.C., Gump, B.H. & Nury, F.S. (1995). *Wine Analysis and Production*. New York: Chapman & Hall.