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Growth and Metabolism of Lactic Acid Bacteria in a Model Wine System and a Red Wine with Emphasis on Carbohydrate Metabolism

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Technology (Food Technology) in the Faculty of Technology at Massey University New Zealand

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ABSTRACT

Studies were conducted to investigate the application of capillary gas-liquid chromatography in analysis of wine carbohydrates, and the growth and metabolism of wine lactic acid bacteria in a synthetic model wine system.

1. Analysis of carbohydrates in wine using capillary gas-liquid chromatography

Wine carbohydrates were analysed by capillary gas liquid chromatography of their acetate and aldononitrile acetate derivatives. A wide range of aldoses, polyols and disaccharides (30 compounds) were analysed in 55 minutes, using a single injection. All the derivatives were well-separated except for ribose and rhamnose, which almost co-eluted. The method recovered spiked carbohydrates at 86 to 110% and had adequate reliability. This technique may be applied routinely to the analysis of other alcoholic and non-alcoholic beverages.

2. Growth and metabolism of wine lactic acid bacteria

Malic acid and pH values had determinative effects on the growth of wine lactic acid bacteria. Malic acid stimulated the growth rate and cell population of L. oenos 122 and 252 at pH 4 and allowed their growth at pH 3.2. The absence of malic acid at pH 3.2 inhibited the growth of L. oenos 122 and 252. The stimulatory effect of malic acid on growth was more striking at pH 3.2. This effect was not caused by the pH increases

resulting from malic acid degradation. Malic acid had only a small stimulation on the growth rate of L. plantarum 49 and P. parvulus 93 at pH 4 and their growth was suppressed at pH 3.5, irrespective of malic acid. These results imply that pH 3.5 is a critical value for the bacteriological stability of wine after malolactic fermentation.

This study confirmed that sugars served as the main growth substrates for wine lactic acid bacteria and polyols did not act as growth substrates, with the exception of mannitol. Glucose and trehalose were the preferred substrates for all the bacteria tested. The significance of trehalose in relation to yeast autolysis in induction of malolactic fermentation was discussed. Wine lactic acid bacteria varied in the ability to utilise substrates. Malic acid, citric acid and arginine did not serve as single energy sources.

Malolactic fermentation had a profound impact on substrate utilization by L. oenos 122 and 252, yet seemed not to affect the substrate utilization of L. plantarum 49 and P. parvulus 93. The presence of malic acid resulted in an increased utilization of sugars by L. oenos 122 and 252, and decreased utilization of arabinose by L. oenos 252. Trehalose utilization by L. oenos 252 was not influenced by malolactic fermentation. The increased utilization of sugars may be the biological functions of malolactic fermentation.

pH exerted a marked effect on the metabolism of L. oenos 122 and 252. More sugars were utilized at pH 4 and above than at pH 3.31 and below. L. oenos 122 attacked only a very minor amount of glucose and a portion of malic and citric acids at pH below 3.31. L. oenos 252 also used only

a small quantity of sugars except for glucose, which was used completely, but degraded all malic and citric acid at pH below 3.42. These results strongly suggest that the degradation of malic acid, citric acid and arginine required the presence of fermentable sugars. This implies that the absence of fermentable sugars in wine may prevent malolactic fermentation. These results also justify the benefits of malolactic fermentation at low pH values (below 3.3).

The role of wine lactic acid bacteria in formation of biogenic amines was clarified. L. plantarum 49 was the only organism which reduced the levels of tyrosine and phenylalanine dramatically, indicating that this bacterium may be a potential producer of tyramine and phenylethylamine. P. parvulus 93 did not markedly decrease the levels of any amino acids. Arginine was catabolised only by L. oenos 122 and 252 with the formation of ornithine and ammonia. Arginine was not degraded at low pH values (below 3.5), suggesting that arginine may not play any role in energy supply at low pH values. L. oenos 122 and 252 did not significantly reduce the concentrations of other amino acids.

The role of malolactic fermentation may lie in energy generation. Two potential energy-yielding mechanisms of malolactic reaction were proposed: ATP production through pyruvic acid cleavage (substrate level phosphorylation, pseudo-malolactic fermentation) and chemiosmotic ATP synthesis via formation of extra lactic acid (non-substrate level phosphorylation, real malolactic fermentation). It is speculated that L. oenos 122 may employ the pyruvic acid cleavage pathway and generation of superfluous lactic acid may be adopted by L. oenos 252, L. plantarum 49 and P. parvulus 93. The biological function of the extra lactic acid

could be accounted for by the chemiosmotic theory that postulates energy (ATP) production through efflux of metabolic end-products (e.g., lactic acid). The origin of the superfluous lactic acid remains to be investigated. These findings suggest that the criteria for selection of starter cultures be redefined.

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Chapter One
Introduction

Wine is produced by the alcoholic fermentation of grape juice carried out by yeasts such as Saccharomyces cerevisiae. Wine may also support a secondary fermentation, the malolactic fermentation (MLF), the conversion of L-malic acid to L-lactic acid and carbon dioxide by strains of lactic acid bacteria (LAB) (Kunkee, 1967). The malolactic fermentation normally occurs after completion of the alcoholic fermentation.

The malolactic fermentation is not unique to wine. It also occurs in the fermentation of other foods and beverages which contain L-malic acid such as cider (Beech, 1972). Since its discovery, MLF has become the focus of research of many oenologists and microbiologists (Vaughn, 1955; Kunkee, 1967; Pilone and Kunkee, 1972; Lafon-Lafourcade, 1983; Davis et al, 1986a,b). The occurrence and growth of LAB in wine and the practical implications of MLF have been reviewed recently by Wibowo et al (1985) and Davis et al (1985). Briefly, the beneficial effects of MLF include acidity reduction, flavour complexity and microbial stability. However, the microbial stability is now in question, since it has been recently shown that other LAB will grow after MLF (Davis et al, 1986a,b). The reduction in wine acidity, resulting from the decarboxylation of L-malic acid, is of great advantage in cold wine regions, such as New Zealand, where the wines are often too tart. The deacidification itself softens the excessive acidity of cool climate wines. Malolactic fermentation may also bring about flavour complexity of wines through the production of

flavour compounds, such as acetoin and diacetyl (Fornachon and Lloyd, 1965).

With increased recognition of the impact of MLF on wine quality, winemakers are seeking to control the MLF either by encouraging its rapid induction or inhibiting it completely. One of the problems in inducing MLF is the lack of proper MLF starter cultures with known characteristics. Spontaneous MLF is often too slow and difficult to control and may not possess suitable characteristics for producing good quality wines.

Extensive research on the occurrence and growth of LAB in wines has been undertaken throughout the world, but little has been done on the physiological and biochemical properties that will determine the ability of LAB to grow in wine and on the biochemical mechanisms by which these LAB affect wine quality (Davis et al, 1988). Furthermore, the reasons why LAB conduct MLF are still unknown, although some speculations have been given, for example, a stimulation in utilization of sugars by MLF (Kunkee and Pilone, 1972), production of ATP from malic acid by phosphoroclastic reaction (Chauvet et al, 1980) and chemiosmotic ATP synthesis through efflux of lactic acid resulting from malic acid decarboxylation (Renault et al, 1988; Cox and Henick-Kling, 1989). Such fundamental knowledge is now becoming increasingly important, because the wine industry is moving towards controlled MLF with specific strains of LAB (Beelman et al, 1979; King et al, 1984; Kunkee, 1984). Basic information on the physiological and biochemical properties of strains of lactic acid bacteria will not only help in the selection for commercial use, but will also indicate the conditions under which these

LAB will be most effectively utilized in the wine industry. Furthermore, an understanding of how they grow in wine and what factors affect this growth may eventually lead to the means of limiting or stimulating such growth.

Therefore, the aim of this project was to investigate the metabolism of LAB so that some basic physiological and biochemical information useful for selection of LAB starter cultures could be obtained. Considerable information is available in the literature on the metabolism of lactic acid bacteria in semi-synthetic media such as MRS (de Man et al, 1960) or media containing tomato juice (Rogosa et al, 1953). Little data, however, are available on their metabolism in wine, apart from the recent reports of Davis et al (1986a,b). It becomes important, however, to have complete control over the composition of the growth media to derive specific information on growth substrates and end-products. Therefore, a major part of this work involves the use of a model wine system of totally defined composition which simulates the growth conditions during conservation of wine after alcoholic fermentation.

Associated with this is the requirement of a sensitive and accurate carbohydrate assay which can be used with this model wine system and with wine. Therefore, another major aim of this project was the development of a suitable capillary gas-liquid chromatography method for this purpose.

Much research has considered the carbohydrate metabolism of lactic acid bacteria from dairy products (Lawrence et al, 1976). By contrast, however, little research has focussed specifically on the wine LAB,

particularly when they are actually growing in wine. Much of their basic biochemistry is unknown. Clearly, there is need for further information on what compounds serve as energy sources, under what conditions they are utilized and how this affects wine quality.

Special emphasis was placed on the carbohydrate metabolism of wine LAB during the course of this research, since the metabolism of carbohydrates by LAB is an area of wine biochemistry which is still poorly understood. Also, the understanding of the metabolism of particular carbohydrates could be an important criterion for selection of LAB for induction of MLF.

Chapter Two
Literature Review

2.1 Presence and significance of carbohydrates in wine

A wide range of carbohydrates is present in wine, including monosaccharides, oligosaccharides, polysaccharides and polyols. Amerine (1954) reviewed the carbohydrates of wine. Among the carbohydrates reported were glucose, fructose, sucrose, arabinose, rhamnose, araban, methyl pentosan, dextran, pectin and other polysaccharides. Glycerol, 2,3-butanediol and mannitol were the polyols covered in his review. In addition to the five monosaccharides, Melamed (1962) found xylose. Esau and Amerine (1964) identified 11 sugars in a Cabernet Sauvignon wine and reported the presence of raffinose, lactose, maltose, galactose, ribose, D-glycero-D-manno-octulose, manno-heptulose and altro-heptulose in addition to those sugars already mentioned. Esau and Amerine (1966) quantitated these residual sugars in wine. Rice et al (1968) confirmed the 11 sugars found by Esau and Amerine (1964) in New York State wines. Ribereau et al (1972) reviewed carbohydrates in wine and apart from the carbohydrates described above, the following polysaccharides and polyols were reported as being present in wine: galactan, xylan, fructosan, meso-inositol, sorbitol, erythritol, and arabitol. Trehalose is reported to be the principal disaccharide in wine (Bertrand et al, 1975). The same authors also reported the probable presence of isomaltose, melibiose and gentiobiose and the presence of erythritol, fructose, glucose, mannitol, meso-inositol, perseitol, sucrose and lactose. Washuttle et al (1973) studied the polyols in several foods and found only sorbitol in red and white wines. Only one report showed the

existence of xylitol in some wines (Markinen and Soderling, 1980). Olano (1983) detected trehalose, mannitol, sorbitol and myo-inositol in sherry. The presence of a large number of sugars, including mannose, cellobiose, melibiose, raffinose and fucose, has been determined in a Hungarian dry wine and three sweet table wines by Drawert et al (1976). Amerine et al (1980) have summarized the composition of must and wine, including carbohydrate components. The specific carbohydrate composition of a wine varies greatly, depending on the vintages, grape variety, vinification practices, the kind of storage and microbiology of the fermentation (Amerine, 1954; Drawert et al, 1976; Amerine et al, 1980).

Most dry wines contain 1-3 g/L of hexoses and pentoses (Melamed, 1962). The range of concentrations of individual carbohydrates of wines as found by several workers are collected in Table 2.1.

Table 2.1 Reported concentrations of some carbohydrates in dry wine

Carbohydrate	Concentration (mg/L)
Glycerol	3 -- 15 (g/L)
Erythritol	10 -- 20
Ribose	Trace -- 620
Rhamnose	Trace -- 400
Arabinose	10 -- 1000
Fucose	Trace -- 90
Xylose	Trace -- 1460
Arabitol	10 -- 30
Xylitol	0 -- 135
Mannose	0 -- 370
Galactose	Trace -- 2490
Fructose	10 -- 2500
Glucose	88 -- 5000
Myo-inositol	220 -- 736
Mannitol	10 -- 40
Glucitol	Trace -- 150
Sucrose	0 -- 600
Trehalose	0 -- 611
Maltose	0 -- 17
Lactose	0 -- 35
Melibiose	0 -- 10
Cellobiose	0 -- 70
Raffinose	0 -- 10

Sources of data: Melamed (1962), Esau and Amerine (1966), Ribereau-Gayon et al (1972), Washuttle et al (1973), Bertrand et al (1975), Drawert et al (1976), Amerine and Ough (1980), Amerine et al (1980), Markinen and Soderling (1980) and Olano (1983).

The presence of residual unfermented and non-fermentable carbohydrates in wine is of great significance to the biological and chemical stability of wine. The unfermented carbohydrates may be metabolized by yeasts and bacteria, if present. Residual carbohydrates after alcoholic fermentation may be metabolized by lactic acid bacteria. The secondary fermentation by yeasts and the growth of lactic acid bacteria, thereby utilising residual carbohydrates during maturation/aging and in bottles, can pose serious problems such as gasiness and haze formation (Davis et al, 1985). Details of carbohydrate metabolism will be presented later in this chapter. The presence of residual sugars may contribute to the browning of the wine (Esau and Amerine, 1964).

2.2 Occurrence, properties and metabolism of lactic acid bacteria in wine

2.2.1 Occurrence of lactic acid bacteria conducting malolactic fermentation

The lactic acid bacteria as presently constituted consist of four genera: Lactobacillus, Leuconostoc, Pediococcus and Streptococcus (Jay, 1980). However, only the former three genera occur in wine since this medium is quite selective due to its low pH and alcohol concentration (Lafon-Lafourcade, 1983). Wibowo et al (1985) reviewed the taxonomy of lactic acid bacteria in wine. Leuconostoc oenos is the sole species within the genus Leuconostoc that occurs in wine. Within the genus Pediococcus, only P. damnosus, P. parvulus and P. pentosaceous occur in wine. Numerous species of Lactobacillus have been isolated from wine such as the homofermentative species L. plantarum and L. casei, and the heterofermentative species L. brevis, L. hilgardii, L. fructivorans.

The common characteristics of lactic acid bacteria include: gram-positive, catalase-negative, nonsporing, microaerophilic, mesophilic with optimal growth temperature being 25^0C, optimal pH 5.5. They are nutritionally fastidious, requiring preformed amino acids, B-vitamins, purines and pyrimidines. They are only weakly proteolytic and lipolytic. (Bergey's Manual of Systematic Bacteriology, Vol 2, 1986; Kandler, 1983)

Besides the general features mentioned above, wine lactic acid bacteria possess some other important properties relevant to their growth in wine:

1. tolerance to 10 - 15% ethanol;

2. growth at pH values as low as 3.0;

3. tolerance to at least 50 mg/L of total SO_2;

4. ability to grow at low cellar temperatures (in the range of 10 to 20^0C);

5. ability to grow under very low oxygen tension;

(Fleet, 1985).

These properties become important when selecting strains for induction of malolactic fermentation. Strains of L. oenos are particularly desirable as agents for carrying out MLF in wine because these organisms can attack the malic acid at a pH of 3.0, but only attack sugars at pHs of 3.4 and above (Goswell, 1986). The search for "better" strains of L. oenos which are more active at low pH and low temperature in wine still continues (Henick-Kling, 1988). However, detailed knowledge of how these factors (ethanol, pH, SO_2, O_2 and temperature) affect the metabolism of wine lactic acid bacteria is lacking. This area certainly demands further research.

2.2.2 Metabolism of organic acids by wine lactic acid bacteria

The major organic acids of wine include malic, tartaric, lactic, citric and acetic acid, and the minor organic acids include formic, propionic, butyric, oxalic, pyruvic, quinic and α-ketoglutaric acids (Amerine and Joslyn, 1970; Amerine et al, 1980). Some of the organic acids orginate from the must (malic, tartaric and citric acid), but other organic acids result from microbial metabolism. Davis and Reeves (1988) discussed acid formation during fermentation and conservation of wine. The concentrations of major organic acids in wine are given in Table 2.2.

Table 2.2. Reported concentrations of major organic acids in wine

Acid	Concentration (g/L)
Tartaric	1 -- 6
Malic	0 -- 6
Lactic	1 -- 5
Citric	0 -- 0.5
Acetic	0.3 -- 0.5
Succinic	0.5 -- 1.5

Sources of data: Amerine and Joslyn (1970), Amerine et al (1980).

Although a number of organic acids are present in wine, only a few of them are metabolized by wine lactic acid bacteria (Radler, 1975).

L-malic acid degradation

The conversion of L-malic acid to L-lactic acid and carbon dioxide, i.e., the so-called malolactic fermentation, is a well known reaction among wine lactic acid bacteria. The malolactic fermentation is not restricted to wine lactic acid bacteria. Lactic acid bacteria from ciders, for instance, can also conduct malolactic fermentation (Beech, 1972). The reason for the malolactic conversion is still a puzzle, although some hypotheses have been suggested for its role. The current view is that the malolactic enzyme converts L-malic acid to L-lactic acid and CO_2 without the formation of true pyruvic acid as an intermediate.

Kunkee (1967) has calculated the stoichiometry of the conversion of malic acid to lactic acid and anhydrous carbon dioxide. This yields a standard free-energy of -6 Kcal/mole, i.e., it is slightly endothermic, but there is no way the bacteria can trap this energy biochemically as there is no net change in redox state and no formation of high energy compounds such as ATP. There has been no report regarding the use of

malic acid as a sole carbon and energy source by wine lactic acid bacteria (Kunkee, 1967; Pilone and Kunkee, 1972). Nevertheless, malic acid has been shown to stimulate the growth rate of Leuconostoc oenos ML 34 and this stimulation could not be accounted for by the change in pH which accompanies malolactic fermentation. This growth rate stimulation was even more striking at low pH (below pH 4) (Pilone and Kunkee, 1976). They found a formation of extra lactic acid that greatly exceeded that expected from the fermentation of glucose and malic acid. This implied a stimulation in utilization of sugars. The mechanism of stimulation is as yet unknown. These areas require further investigation.

There are, however, sporadic reports concerning ATP production from malic acid. Chauvet et al (1980) state that the phosphoroclastic cleavage of pyruvic acid via inorganic phosphate produces enough ATP to permit the growth of Leuconostoc oenos from malic or citric acids. Brechot et al (1984) suggest that malic acid helps the growth of Leuconostoc oenos because ATP is produced from malic acid by phosphoroclastic reaction. However, these authors could not present any experimental evidence to support the phosphoroclastic cleavage of pyruvic acid by wine lactic acid bacteria. Renault et al (1988) tried to explain the stimulation of growth observed during MLF based on the chemiosmotic theory. The chemiosmotic theory postulates that another form of energy (chemiosmotic ATP synthesis) could be generated by translocation of protons through the membrane coupled to end-product efflux. They believe that this theory could be applicable to lactate efflux because malic acid decarboxylation also produces a lactate efflux. However, no experimental proof has been given in terms of ATP formation from lactic acid efflux resulting from the degradation of

malic acid, citric acid or sugars in wine LAB. The latest work of Cox and Henick-Kling (1989) may provide indirect experimental evidence for the chemiosmotic theory in elucidation of the role of MLF. They have demonstrated that MLF does yield ATP in whole cells of L. oenos, while this energy-yielding mechanism did not occur in cell extract. Likewise, lactate efflux, as a result of carbohydrate fermentation, would also yield ATP. Furthermore, malic acid cannot act as a sole energy source. Therefore, the importance of ATP generation from malic acid degradation is in question.

Most workers focussed their attention on the energy generation from the reaction of malic acid to lactic acid. The malolactic reaction itself may not provide any biologically available energy for the organism, but it may lead to more efficient utilization of other compounds such as sugars, so that extra ATP may yield from sugar catabolism, resulting in the stimulation of growth. Currently, it is believed that the malolactic enzyme involved in malolactic reaction has bifunctional activity. During malolactic fermentation, the major portion of malic acid is directly decarboxylated to lactic acid while a small amount of pyruvic acid (and reduced coenzyme) is formed as an end product, rather than as an intermediate (Morenzoni, 1974; Kunkee, 1975). It is suspected that this small amount of pyruvic acid has extremely important consequences on the intermediary metabolism of wine lactic acid bacteria (Morenzoni, 1974; Kunkee, 1975). Nevertheless, Kunkee (1975) believed that the small amount of pyruvic acid formed, or acetyl phosphate arising from it, would be too small to provide enough energy to bring about any detectable effect on growth yield.

Kunkee (1967) also gave some other explanations. From an evolutionary point of view, the increase in pH of weakly buffered wine solutions afforded by MLF would be advantageous for bacterial growth. Metabolism of malic acid also may provide some sort of detoxification, as has been shown with citric acid (Harvey and Collins, 1963).

Tartaric acid degradation

The decomposition of tartaric acid by lactic acid bacteria is observed rarely and appears to occur in a few species of Lactobacillus such as L. plantarum and L. brevis (Radler, 1975). The metabolic pathways of tartaric acid have been determined and the end-products are: lactic, acetic and succinic acids, and carbon dioxide.

Citric acid degradation

As mentioned earlier, Chauvet et al (1980) believe that ATP is produced from citric acid to permit the growth of Leuconostoc oenos. Harvey and Collins (1963) regarded the detoxification effect of citric acid degradation as acetoin formation from excess intracellular pyruvic acid not required for synthesis of cell material. Bacteria that can metabolize citric acid can use it as an energy source via pyruvic acid oxidation, since high energy phosphate is formed (Hager et al, 1954, cited by Kunkee, 1967), but no reports have demonstrated the use of citric acid by wine LAB as a sole carbon energy source. The citric acid degradation is associated with the increase of volatile acidity since acetic acid is produced from citric acid together with pyruvic acid, from which lactic acid, succinic acid, acetoin, 2,3-butanediol and diacetyl can be formed (Lafon-Lafourcade, 1983).

2.2.3 Metabolism of amino acids by wine lactic acid bacteria

Formation of biogenic amines in wines

In recent years, there has been considerable interests in the presence of amines in wines and the role of lactic acid bacteria in their formation.

Significance

Amines are basic nitrogenous substances. A large number of amines occur in meat, fish, vegetables, dairy products, bakery products, poultry products and beverages. Reviews of amines in various foods have been published by Maga (1978) and Smith (1980-1981). Amines are of concern mainly due to their biological and potentially medical significance because they may be toxic and cause headache, migraine, brain haemorrhage, heart attack and hypertension, particularly when monoamine oxidase (MAO)-inhibitory drugs are administered. For details of the episodes, the reader is referred to Blackwell et al (1965a,b). Alcoholic beverages have received special attention since there is some evidence to suggest that ethanol potentiates the effects of the amines by directly or indirectly inhibiting the amine oxidase (Maynard and Schenker, 1962; Marquardt and Werringloer, 1965).

Amines in wine and beer

Of the amines found in wine and beer, tyramine and histamine are often encountered, together with minor amounts of ethanolamine, putrescine, cadaverine and agmatine (Marquardt and Werringloer, 1965; Sen, 1969; Ough, 1971; Zappavigna and Cerutti, 1973; Subden et al, 1979; Zee et al, 1981a; Zee et al, 1983). There are reports showing a higher histamine

concentrations in red wines than in white wines (Marquardt and Werringloer, 1965; Zee et al, 1983), but no significant difference in cadaverine and tyramine concentrations between red wines and white wines (Zee et al, 1983). The histamine concentration is normally less than 10 mg/L (Ough,1971; Zappavigna and Cerutti, 1973; Subden et al 1979). Zee et al (1981a; 1983) found only histamine, tyramine, putrescine, cadaverine and agmatine in wines and beers.

Formation pathways of amines

The formation pathways of amines, as reviewed by Maga (1978), include: 1) amino acid decarboxylation; 2) aldehyde amination; 3) phospholipid decomposition; 4) thermal amino acid decomposition (bakery products) and 5) trimethylamine oxide conversion (fishery products). Among these pathways, amino acid decarboxylation is the most common mode of synthesis of amines (Smith, 1980-1981), and may be responsible for the biogenesis of amines in wines, beers and related alcoholic beverages. It has been postulated and demonstrated that numerous amines can be formed through this route. For instance, theoretically phenylalanine can be decarboxylated into 2-phenylethylamine (Chaytor et al, 1975). Tyramine formation has been correlated to tyrosine, which in turn can result from protein breakdown (Kristofferson, 1963). Likewise, histamine can be produced from histidine (Dierick et al, 1974).

The role of lactic acid bacteria in the biogenesis of amines

The biogenesis of amines in wine and beer is still a matter of controversy, since there have been no convincing demonstrations of the ability of LAB to form amines in wine and beer. Many authors believe that LAB are responsible for the generation of amines in wine on the

basis of observations that wines having undergone MLF have a higher histamine content than those which have not. Marquardt and Werringloer (1965) attributed histamine formation from histidine to growth of lactobaccilli and colibacilli in wine. They further commented that histamine was not a by-product of alcoholic fermentation, i.e., yeast would not lead to histamine formation from histidine. But Quevaviller and Maziere (1969) reported contrary results (cited by Ough, 1971). They found histamine was formed during alcoholic fermentation by yeast. Rivas-gonzalo et al (1983) reported tyramine concentration was concomitant to alcoholic fermentation of yeast. Malolactic fermentation, fermentation temperature and bentonite fining did not significantly affect the average amount of histamine in commercial wine samples (Ough, 1971). Contrary to this statement, Subden et al (1979) noted the disparities between the histamine concentrations of thermally vinified, hot press wine (1.20 mg/L), and those fermented on the skins (2.71 mg/L). The same authors also noticed that amelioration and bentonite fining reduced the histamine concentration. They suggested that hot-pressing killed off the microflora responsible for histidine decarboxylation, but gave no explanations for reduction in histamine by amelioration and bentonite fining. Mayer et al (1971) consider that histamine is formed by lactic acid cocci during MLF (quoted by Smith, 1980). They also stated that Leuconostoc oenos, occasionally long rods (bacilli) and acetic acid bacteria were not implicated, suggesting histamine formation might be prevented by controlling the bacterial flora during MLF and conservation. The enzyme histidine decarboxylase was shown to occur in Lactobacillus spp (Rodwell, 1953) and also in Pediococcus cerevisiae B16 (Radler, 1975). Weiller and Radler (1976) studied 28 strains of Pediococcus cerevisiae and found only one strain

was able to decarboxylate histidine to histamine. The same authors also found several strains of Lactobacillus brevis that were able to form 4-aminobutyric acid and ornithine from glutamic acid and arginine, respectively. Lactobacillus and Pediococcus are implicated in the decarboxylation of amino acids to corresponding amines in beer through contamination of yeast and raw material (Rice and Koehler, 1976; Zee et al, 1981a). Lactobacillus brevis produced putrescine and tyramine, but reduced agmatine content in wort and Saccharomyces uvarum did not produced amines in brewing (Zee et al, 1981b).

Buteau et al (1984) undertook experiments to elucidate the production of amines by Leuconostoc oenos and Pediococcus cerevisiae. Their results indicated that yeasts rather than LAB produced amines in wine. They also claimed that temperature, pH and type of microorganisms present influenced the content of various amines in different ways. The recent work of Ough et al (1987) and Delfini (1989) show that wine LAB have little to do with histamine production. Conversely, Umezu (1979) demonstrated amine oxidation by lactobacilli from Sake. However, these particular species and strains are not commonly found in wines.

Future work

Overall, there have been no consistent findings about the biogenesis of amines in wine and beer. Thus, the role of LAB in the formation of amines is still unclear. In the main, most researchers conducted their studies using wine or beer as media under uncontrolled conditions. Hence, these studies would be influenced by many factors: species and strains of microorganisms present, contamination, interactions among microorganisms, vinification practices, etc. Further investigations are

required under strictly controlled conditions similar to those of wine or beer, for example, in a synthetic medium, to elucidate the role of LAB in the biogenesis of amines in an effort to select suitable strains for induction of MLF. Factors affecting decarboxylase activity and, therefore, amine formation also require investigation.

Arginine metabolism

The metabolism of arginine has been found to generate ATP. The multienzyme system and reaction sequence have been determined in bacteria such as Streptococcus faecalis, Clostridium spp and Halobacterium salinarum (Bauchop and Elsden, 1960; Doelle, 1969; Sokatch, 1969). Bauchop and Elsden (1960) found that the $Y_{arginine}$ for S. faecalis was 10.5 grams cell dry weight per mole arginine, which corresponds with the formation of one mole of ATP per mole of arginine fermented. Fundamentally, arginine metabolism follows the equations below:

$$\text{Arginine} + H_2O \rightarrow \text{citrulline} + NH_3 \quad (1)$$

$$\text{Citrulline} + H_3PO_4 \rightarrow \text{ornithine} + \text{carbamyl phosphate} \quad (2)$$

$$\text{Carbamyl phosphate} + ADP \rightarrow ATP + CO_2 + NH_3 \quad (3)$$

Sum: $\text{Arginine} + H_2O + ADP + H_3PO_4 \rightarrow$

$$\text{ornithine} + 2NH_3 + CO_2 + ATP$$

The three enzymes involved in the catabolism of arginine include: (1) arginine deiminase; (2) ornithine transcarbamylase; (3) carbamate kinase.

The above pathways of arginine breakdown and enzymes involved have been found to operate in Lactobacillus brevis from brewery sources, but there

are no reports of this mechanism functioning in beer pediococci (Rainbow, 1975; 1981). Jonsson et al (1983) demonstrated the transformation of arginine to ornithine and ammonia by a Lactobacillus plantarum from fish. Several authors emphasize the need for glucose to implement the growth of organisms on arginine (Bauchop and Elsden, 1960; Jonsson et al, 1983).

By contrast, arginine metabolism of wine LAB has been poorly studied. Consistent trends, however, have emerged for the decrease in arginine concentration corresponding to the increase in ornithine concentration during growth of L. oenos in wine (Kuensch et al, 1974). These workers suggested that this reaction was linked to the urea cycle. The same phenomenon was also noted by Mayer et al (1973). However, Moore and Rainbow (1955) detected no urea as a product of arginine-ornithine transformation. According to Bender (1985), the urea cycle is an energy-utilizing, rather than energy-yielding reaction series. The decrease in arginine after MLF was also observed by Davis et al (1986). Pediococcus does not possess the ability to transform arginine to ornithine (Kuensch et al, 1974).

The effects of arginine metabolism on wine quality have not been properly investigated. It is known from the above equations that, apart from one mole of ATP produced, two moles of ammonia and one mole of ornithine are generated. What effects do these compounds have on wine quality? Does the formation of ammonia raise wine pH and if so, by how many units? Conversely, a stabilizing effect of ornithine on wine has been reported since ornithine has a pronounced inhibitory effect on the growth of wild yeasts (Mayer et al, 1973).

Further research should focus on the elucidation of the multiple enzyme system operating in the arginine metabolism of wine LAB, and the role of arginine metabolism in MLF. The effects of arginine metabolism on wine quality also demand further studies.

2.2.4. Production of flavour compounds by wine LAB

In this section, only a brief account of flavour modification and complexity of wine brought about by malolactic bacteria will be given. The biochemical pathways of flavour compound formation and factors influencing their production have been discussed elsewhere (Speckman and Collins, 1968; Eschenbruch and Dittrich, 1970; Collins, 1972; Cogan et al, 1981; El-Gendy et al, 1983).

As discussed in the review of Davis et al (1985), one of the most important influences of LAB on wine composition and quality is the production of flavour compounds. Apart from producing lactic acid as the major end-product of carbohydrate metabolism and malic acid degradation, wine LAB are also capable of generating other compounds such as acetic acid, acetaldehyde, acetoin, 2,3-butanediol, diacetyl, and numerous esters and alcohols. Amongst these compounds, acetoin, diacetyl and 2,3-butanediol are of greatest importance to the wine flavour profile, since in small quantities they contribute to the flavour complexity of wine. They may, however, become undesirable at higher concentrations.

It has been shown that wines which have undergone MLF contain significantly more acetoin and diacetyl than wines that have not and the amounts of acetoin and diacetyl formed depend on the species present and

on the composition of the wine (Fornachon and Lloyd, 1965; Rankine et al, 1969; Mascarenhas, 1984). Meunier and Bott (1979) studied the behaviour of different volatile constituents of Burgundy wines during MLF and found that different volatile substances, esters in particular, increased in concentration during MLF. Zeeman et al (1982) found significant increases in fatty acids (lactic, acetic and dodecanoic acid) and esters (3-methyl-n-butyl acetate, n-hexyl acetate, 2-phenyl ethyl acetate and ethyl-n-hexanoate) after MLF. They also found highly significant increases in the concentrations of ethyl lactate and diethyl succinate, and the concentrations of acetoin, diacetyl and 2,3-butanediol were significantly higher than in wines which had not undergone MLF. Le Roux et al (1989) demonstrated the formation of several alcohols (1-butanol, 3-methyl-1-pentanol, 1-hexanol, 6-methyl-5-hepten-2-ol and 1-octanol) and the complete or considerable removal of 2-heptanone, 1,3,5,7-cyclo-octatetraene, 2,4-dimethyl hexane, benzaldehyde, hexanoic acid, propyl benzene and camphor by Leuconostoc oenos ML 34 in MRS medium.

Contrary to these reports, some studies indicate that MLF does not necessarily alter the sensory attributes of wine (Kunkee et al, 1964) and there is no distinct correlation between award-winning wines and the occurrence of MLF (van Wyk, 1976). However, the qualities of wines which win awards are exceedingly complex and in part the result of the skill of the winemaker. It is therefore not surprising that such a correlation has not been found.

Most of these studies were carried out in wine and are consequently affected by a number of factors, such as the interference of other

organisms (yeasts and bacteria). Rigorously controlled studies are needed to elucidate wine flavour modification by LAB and to correlate flavour compound production with species of LAB.

2.3 Carbohydrate metabolism of wine lactic acid bacteria

2.3.1 Biochemistry of carbohydrate metabolism by LAB

The biochemical aspects of carbohydrate metabolism by both homofermentative and heterofermentative lactic acid bacteria are well described (Wood, 1961; Doelle, 1969; Sokatch, 1969; Moat, 1979, Kandler, 1983). The following summary is based on these descriptions.

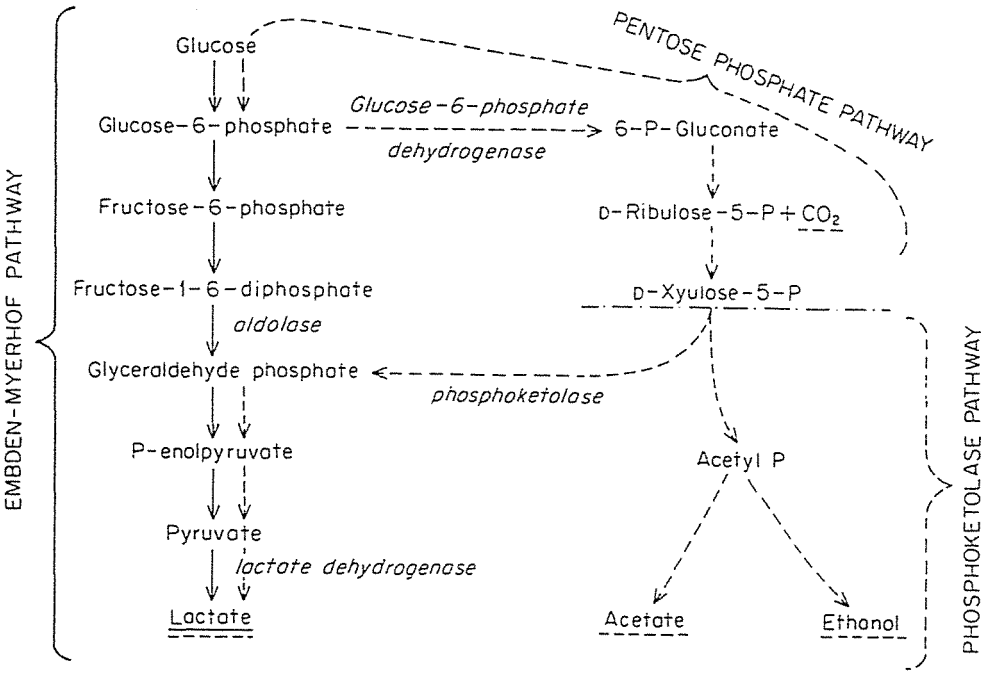

Figure 2.1 Glycolytic pathways in lactic acid bacteria

(after Garvie, 1984)

In general, homofermenters adopt the glycolytic pathway (also referred to as Embden-Myerhoff Pathway/hexose diphosphate pathway), while heterofermenters follow the 6-phosphate-gluconate pathway (also known as HMP/hexose monophosphate pathway) (Figure 2.1). Homofermenters produce 2 moles of lactic acid from 1 mole of hexose with the net formation of 2 ATP molecules. Heterofermenters generate 1 mole of lactic acid, ethanol or acetic acid and carbon dioxide with a net gain of 1 or 2 ATP. Usually heterofermenters form ethanol and thus 1 mole of ATP. Ethanol/acetate formation depends on the redox potential of the system. If an additional hydrogen acceptor, e.g., oxygen or fructose, is involved, no ethanol is formed; oxygen is reduced to hydrogen peroxide or water, and fructose is reduced to mannitol. This phenomenon is characteristic for heterofermentative lactic acid bacteria (Kunkee, 1967; Sharp, 1981). Accompanying the formation of mannitol is a decrease in levels of the other reduced products, glycerol and ethanol (Eltz and Vandemark,1960) and formation of excess acetic acid (Dittrich, 1977; cited by Dicks and Vuuren, 1988), more importantly, one extra mole of ATP is produced.

Pentoses are usually fermented by all heterofermenters and some homofermenters. Pentoses are converted by appropriate enzymes to D-xylulose-5-phosphate which is then fermented to lactate and acetate by way of the remaining reactions of the 6-phosphate-gluconate pathway. This is accompanied by the energy gain of 2 moles of ATP per mole of pentose.

Only very few lactobacilli can ferment pentitols (certain strains of Lactobacillus casei). Pentitol phosphates are first formed and then oxidized by the appropriate dehydrogenase and isomerized to D-xylulose-

5-phosphate, the common intermediate of pentose and glucose fermentation, via the 6-phosphate-gluconate pathway (London and Chase, 1977; 1979).

Hexitol fermentation is rare except for mannitol. Actually, mannitol has been recorded as fermentable by Lactobacillus plantarum (Pederson and Albury, 1969; Beech, 1972; Chen et al, 1983b). According to these reports, it is proposed that mannitol-1-phosphate is first formed and isomerized to fructose-6-phosphate, which is then broken down to 2 moles of lactate following the EMP pathway, with the generation of 2 ATP. Therefore, mannitol and fructose are equally good sources of energy for L. plantarum. As far as can be determined, the biochemical pathways and enzymology of mannitol metabolism have not been experimentally established apart from the suggestions mentioned above.

McFeeters and Chen (1986) and Chen and McFeeters (1986a,b) studied anaerobic mannitol metabolism by L. plantarum. They found that the following electron acceptors were utilized and reduced to the corresponding products: pyruvic acid to lactic acid; acetic acid, acetaldehyde and acetyl phosphate to ethanol; malic acid and fumaric acid to succinic acid; citric acid to succinic acid and ethanol; oxaloacetic acid to lactic acid and ethanol and α-ketobutyric acid to α-hydroxybutyric acid.

2.3.2 Carbohydrate metabolism of wine LAB in semi-synthetic media

Numerous studies have been conducted to investigate the carbohydrate metabolism of wine LAB in semi-synthetic media such as MRS and undefined media such as tomato juice broth (TJB) at optimal pH 5.5 (Pilone and

Kunkee, 1972; Chalfan et al, 1977; Silver and Leighton, 1981; Lafon-Lafourcade et al, 1983; Izuagbe et al, 1985; Dicks and Vuuren, 1988; Davis et al, 1988). All these findings show that there is considerable heterogeneity between species and strains in their ability to ferment carbohydrates. For example, Lafon-Lafourcade et al (1983) found that of 166 strains of Leuconostoc oenos, 71% fermented arabinose, 71% fermented ribose, and 2% fermented xylose; merely 11% of the strains degraded both glucose and fructose. Also, Davis et al (1988) reported that only 55% of 71 strains of Leuconostoc oenos fermented ribose, 27% fermented D-arabinose and 45% fermented sucrose. From these studies, it may be tentatively generalized that, most strains of Leuconostoc oenos ferment ribose, glucose, mannose, fructose, cellobiose, trehalose, aesculin, salicin, arbutin, but few strains ferment rhamnose, xylose, sucrose, maltose, lactose, raffinose, and no strains ferment polyols.

There is little information on carbohydrate fermentation of Pediococcus spp. Of the 17 strains of Pediococcus parvulus examined, it was shown that all fermented glucose, fructose, mannose, α-methyl-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, salicin and arbutin while 94% of them fermented trehalose, cellobiose, gentiobiose, and few fermented pentoses and polyols (Davis et al, 1988).

Based on the report of Dicks and Vuuren (1988), most strains of Lactobacillus spp ferment L-arabinose, ribose, D-xylose, galactose, glucose, fructose, aesculin, maltose and melibiose; some strains ferment mannose, rhamnose, salicin, lactose and trehalose and few strains ferment polyols except for mannitol, which is fermented by L. plantarum.

On the whole, it is difficult to draw any conclusions about carbohydrate fermentation of LAB in semi-synthetic and undefined media except in the case of glucose and fructose, which are most frequently metabolized. Fermentation of other carbohydrates is species and strain dependent. Polyols are normally not fermented with the exception of mannitol, which is reported to be limited to L. plantarum so far.

2.3.3 Carbohydrate metabolism of LAB in wine, cider, beer and other related beverages and foods

Reports on carbohydrate metabolism of LAB in wine to date are scarce, inconsistent and inconclusive. Research of this type has been hindered in the past by the lack of analytical techniques sensitive enough to detect low levels of residual carbohydrates in the complex matrix of wine (Fleet, 1985).

Glycerol may be degraded into bitter acrolein (Amerine, 1954; Radler, 1975; Whiting, 1975). Davis et al (1986a) reported that glycerol degradation coincided with the growth of lactobacilli and pediococci in wine of pH 3.7. Complete disappearance of glycerol was observed in ciders and perries in which LAB were not suppressed (Whiting, 1975). There has been no report of the degradation of erythritol by LAB either in semi-synthetic media, undefined media, or in wine or alcoholic beverages and in foods. Neither are there reports on the metabolism of pentitols and hexitols apart from by LAB. Mannitol accumulation due to the reduction of fructose by heterofermenters may cause wine spoilage (Amerine et al, 1980).

There have been no reports concerning the utilization of mannitol by LAB in wine. The mannitol metabolism by LAB in wine would be of practical

significance since mannitol can be formed from fructose by L. oenos. This may lead to a succession of LAB population, e.g., growth of L. plantarum.

Several researchers observed an increase in levels of glucose and fructose. Costello et al (1985) noted that sugar level, determined as glucose and fructose, increased from 0.06 to 0.23 g/L in both inoculated and uninoculated wines over 100 days. The same phenomenon was also noticed by Davis et al (1986a,b). They explained that the increased glucose and fructose levels were not related to bacterial growth, because the same phenomenon happened in uninoculated wines in which no growth occurred. The enzymatic and chemical hydrolysis of wine constituents, such as anthocyanins, phenolic glycosides, oligosaccharides and polysaccharides, might account for the increased concentrations of glucose and fructose. The same authors also believe that carbohydrate metabolism of wine LAB is quite complex. They concluded that glucose, fructose, myo-inositol, glycerol ribose, and xylose were utilized during the growth of pediococci and lactobacilli, but the growth of L. oenos was not accompanied by the specific utilization of any hexose or pentose sugars. These studies were carried out using commercial wines, where MLF was conducted by natural mixed wine LAB. Therefore, it is difficult to correlate the specific utilization of any carbohydrate with the species of wine LAB. Significantly, these studies showed the successive growth of wine LAB after MLF conducted by L. oenos, which implies that MLF does not render wine microbiologically stable as previously thought.

These findings suggest that wine is not a good medium for this type of research since it is so complex and undefined. The metabolism of wine

carbohydrates by LAB is quite complicated and demands more detailed investigations, using a defined medium which simulates the wine environment.

The beer LAB can be grouped as: 1) heterofermentative rods such as L. pastorianus, L. brevis, L. buchneri and L. fermentum; 2) the homofermentative coccus Pediococcus cerevisiae (Rainbow, 1975). Leuconostoc spp have not been reported to occur in beer. According to Rainbow (1975; 1981), these beer spoilage lactobacilli produce acid from glucose, fructose, maltose and (except L. brevis) sucrose and L-arabinose. The heterofermenters such as L. brevis also convert fructose to mannitol. An unusual feature of the carbohydrate metabolism of beer lactobacilli is their preferential use of maltose. The initial steps of metabolism of maltose involve phosphorylation to a free glucose and a β-glucose-1-phosphate mediated by maltose phosphorylase. Experimental evidences indicated that these brewery lactobacilli possessed neither detectable maltase, nor maltose kinase activity and lacked or possessed only weak glucokinase activity. The maltose phosphorylase system may have been developed in response to prolonged culture in the presence of maltose in beer (Wood and Rainbow, 1961). Perhaps a similar development of specialized carbohydrate metabolism may have occurred in wine LAB for the important disaccharide, the trehalose. Trehalose is a storage carbohydrate in yeasts, which is released from yeast cells upon autolysis. This suggests an interesting and significant area for study.

There is much less information on the metabolism of beer pediococci than on that of beer lactobacilli. Acid is produced by Pediococcus cerevisiae (now damnosus) from glucose, fructose, mannose, maltose and sometimes

from galactose and salicin, but not from pentoses, lactose, sucrose, α-methyl glucoside, mannitol, sorbitol nor starch (Rainbow, 1981).

The formation of dextran from sucrose by Leuconostoc mensenteroides may occur in ciders and in fruit wines (Whiting, 1975) and causes trouble in the sugar industry (Tilbury, 1975).

Beech (1972) reviewed English cidermaking and the malolactic fermentation in cider. The lactic acid bacteria from cider not only attack malic acid, but also break down citric, quinic, shikimic, caffeic, chlorogenic acids and other acids that occur in cider. They also produce lactic acid from sugars, and in addition, mannitol and acetate. Heterofermentative lactobacilli convert fructose to mannitol, lactate, acetate and CO_2.

Overall, the carbohydrate metabolism of wine LAB is poorly understood, in particular in the wine environment. This contrasts markedly with the large body of information for lactic acid bacteria from dairy products. Carbohydrate metabolism will exert profound influences on wine quality because various compounds result from carbohydrate metabolism. In addition, these carbohydrates act as energy and carbon for bacterial growth. In the future, it may well be that malolactic fermentation can be favourably controlled via regulation of carbohydrate metabolism of LAB. A more detailed understanding of the metabolism of lactic acid bacteria may allow control of the growth of particular species and may also result in a higher quality product through regulation of particular metabolic pathways. Obviously, more studies are required.

Chapter Three
Analyses of Carbohydrates, Organic acids and Amino Acids

3.1 The analysis of aldoses, polyols and disaccharides in wine using capillary gas-liquid chromatography

3.1.1 Introduction

Carbohydrates such as sugars, polyols and disaccharides have a considerable influence on the microbiological stability of wine because these compounds can be utilized by yeasts and bacteria as energy and sources (Lafon-Lafourcade, 1983; Wibowo et al, 1985; Davis et al, 1986a,b). In addition, polyols are also considered important in terms of sensory property, and adulteration of wines (Amerine et al, 1980) either directly or through the formation of acids and off-flavours by microorganisms.

The classical analytical methods for carbohydrates are laborious in nature and may not be sensitive enough to determine the low residual concentrations in wine (many compounds being present at levels below 50 mg/L (Esau and Amerine, 1966; Ribereau et al, 1972)). The current methods can be categorized into four groups: (1) physical; (2) chemical;(3) enzymatic and (4) chromatographic (Southgate, 1976; Amerine and Ough, 1980; Kearsley, 1985). Physical methods usually determine some overall feature of the carbohydrates, such as total soluble solids, but can not give any indication of the individual carbohydrates. Chemical methods may determine more specific features, such as reducing sugars or ketoses, but are still unable, in most cases, to determine more than one carbohydrate by one test. Enzymatic methods may determine specific

sugars, for example, glucose, fructose and sucrose. However, the reagents are costly, the techniques somewhat laborious and the methods are only available for a limited range of carbohydrates. Furthermore, the specifity depends on the purity of the enzyme preparation. Although thin-layer and paper chromatography are very useful methods for qualitative analysis, they are not commonly employed for quantitative purposes, because the quantitative methods are susceptible to large errors. High performance liquid chromatography (HPLC) and gas-liquid chromatography (GLC) have been applied to the analysis of food carbohydrates (West et al, 1977; Bittner et al, 1980; Folks, 1980; De Smedt et al, 1981; Li et al, 1983; Frayne, 1986) and comparisons between HPLC and GLC have been made by MacRae (1985) and Folkes (1985). In general, HPLC can determine only a limited number of individual carbohydrates, which are present at relatively high concentrations, owing to the poor sensitivity of refractive index detection. Although sensitivity may be increased by forming UV-absorbing derivatives, this complicates the analysis. In dry wines, the concentration of residual carbohydrates is normally less than 0.2% when measured as total reducing sugars. GLC offers a better sensitivity for determining individual carbohydrates which are present in low concentrations.

The use of GLC for carbohydrate analysis usually involves formation of derivatives to increase the volatility of the compounds. Details of the most commonly employed derivatives have been reviewed by Folkes (1985). Most derivatives give rise to more than one product which will produce multiple and/or overlapping peaks because of the possible formation of α- and β-anomers and of pyranose and furanose rings, and of anti- and syn- isomers of oximes (Varma et al, 1973; Mawhinney et al, 1980). The

aldononitrile acetates and alditol acetates produce only single peaks on chromatograms and their use in GLC analysis has benefits in accurate quantitation.

Carbohydrates have been analyzed by GLC using various derivatives, such as trimethyl silyl (TMS) ethers (Sweeley et al, 1963). There is little information on the use of acetates, aldononitrile acetates or alditol acetates in wine carbohydrate analysis apart from the reports of Davis et al (1986a,b) who used a packed column to separate aldoses as aldononitrile acetates and polyols as acetates. A further extension of the use of acetates and aldononitrile acetates to analyze a range of carbohydrates from glycerol to disaccharides in wine and related beverages by capillary GLC has been investigated as part of the project.

3.1.2 Experimental

3.1.2.1 Gas-liquid chromatography

All the results were obtained using a Carlo Erba GC 6000 Vega Series 2 capillary gas chromatograph (Italy). A fused silica capillary column, length 30 m, internal diameter 0.32 mm, was used. The liquid phase (film thickness 0.25 μm) was DB-5 (equivalent to phenyl methyl silicone) (J & W Scientific, Calif.). A FID system was employed. A split injector was used at a ratio of 10 : 1. The injector and detector temperatures were 260°C and 325°C, respectively. The oven was temperature-programmed from 100 to 300°C at 4°C/min, then held isothermally at 300°C for 10 minutes. Other conditions were: carrier gas helium, 2 ml/min; makeup gas nitrogen, 28 ml/min; air, 300 ml/min; and hydrogen, 30 ml/min. Both D-glucoheptose and phenyl β-D-glucoside were used as internal standards and were referred to as IS1 and IS2, respectively. The integration was

performed using a personal computer equipped with DAPA software (Data Acquisition Plotting Analysis) (version 4.52, DAPA Scientific Software, Western Australia.).

3.1.2.2 Sample preparation

The samples were dried for derivatization by a procedure based on the method by Southgate (1976) and Davis et al (1986a,b). The pretreatment process removes substances which may interfere with the resolution of aldoses, polyols and disaccharides by GLC. Wine (1 ml) and 1 ml of D-glucoheptose solution (250 mg/L) (IS1) were mixed in a 10 ml syringe which was attached to a C_{18} Sep-Pak cartridge (Waters Associates) and the mixture was eluted with water (three washings) into a small beaker containing mixed anion (acetate form, converted from OH-form, Amberlite, IRA-400 OH, 16-50 mesh, Sigma) resin, cation (H-form, Dowex, 50W-X8, 20-50 mesh, BDH) resin and 1 ml of phenyl β-D-glucoside solution (500 mg/L) (IS2). The ion-exchange was used to remove other anions (e.g., acids) and cations (e.g., metal ions) which may interfere with the derivatization and resolution. The ion-exchange time was 20 minutes. The beaker was stirred frequently to attain full exchange. The wine extract was decanted into another beaker and the pH was adjusted to 8. After pH adjustment, 95% alcohol was added so that the final concentration of alcohol was 50%. The alcoholic solution was then stored at 4°C overnight to precipitate polysaccharides and proteins. The supernatant fluid was filtered through a Millipore 0.45 μm cellulose acetate membrane into a 10 ml vial and dried under a stream of air at 60°C. The dried sample was then stored in a vacuum dessicator over phosphorus pentoxide.

3.1.2.3 Sample derivatization

The dried neutral extract was derivatized as described by Varma et al (1973) using hydroxylamine hydrochloride in pyridine (25mg/L) followed by acetic anhydride. In this procedure, aldoses are converted to aldononitrile acetates, and polyols and disaccharides to acetates. The derivatized solution was evaporated to dryness under a stream of nitrogen at 40°C. The residue was dissolved in 200 µl of chloroform and 1 µl was injected into the gas chromatograph.

3.1.2.4. Calibration, quantification and identification

Carbohydrate standard solutions were prepared at concentrations normally found in wine. The standard solution was diluted by 80, 60, 40, and 20% in order to obtain calibration curves for each carbohydrate. One ml of each solution and 1 ml each of IS1 and IS2 solution were added to a 10 ml vial and dried under a stream of air at 60°C. The dried residue was derivatized as described above.

The quantification was made by comparison with the two internal standards in terms of area ratio or height ratio, depending on the performance of the individual compound. The area or height ratio was plotted against the concentration of the compound and linear regression performed to obtain the calibration line and the response factor. Carbohydrates were identified by comparison of the retention times of unknown compounds with those of the standards.

3.1.2.5 Recovery test

An assessment of the percentage recovery of spiked carbohydrates from wine sample was done by spiking wine samples with a standard carbohydrate mixture. The standard carbohydrate mixture was the same as

that used for calibration but without any dilution. The unspiked, spiked and standard samples were prepared and derivatized as described above. The percentage recovery was obtained by dividing the area or height ratio difference between the spiked and unspiked samples by the area or height ratio of the standard as follows:

$$\frac{\text{(spiked) - (unspiked)}}{\text{(standard)}} \times 100\% = \text{recovery \%}.$$

All the samples were prepared in triplicate and each sample was injected in duplicate.

3.1.2.6 Reliability of the method

The reliability was expressed in terms of reproducibility and instrument stability. Five identical wine samples were prepared, derivatized, then injected into the gas chromatograph to determine the reproducibility. The instrument stability was acquired by injecting one sample eight times over a period of several weeks. Both reproducibility and instrument stability were expressed as the percentage coefficient of variation (CV%).

3.2 Determination of fructose by resorcinol method (ketoses)

3.2.1 Background

Since GLC methods are unsuitable for analysis of ketoses, a simple but accurate method, based on the resorcinol reaction, was adopted for this experiment. This method is well-suited to analysis of the model wine, since fructose is the only ketose present. The procedure described here is that of Kulka (1955).

3.2.2 Principles

Resorcinol reacts with sugars in strong acid to give coloured complexes and, although all hexoses react, the colour yield with ketoses is much greater than that with aldoses, so that the method can be used to give values for the ketohexoses (Southgate, 1986). In the model wine, however, fructose was the sole ketohexose added. Therefore, there was no interference from other ketoses.

3.2.3 Procedure

Reagents

Resorcinol. 0.5% (w/v; AnalaR) resorcinol in absolute ethanol. This reagent is reasonably stable if kept in the dark.

HCl/ferric ammonium sulphate. Conc. HCl containing 0.216 g/ferric ammonium sulphate per litre.

Determination

A 2 ml aliquot of sample (containing 5-50 µg/ml fructose) was pipetted into a stoppered tube and 3 ml of the resorcinol reagent was added, followed by 3 ml of the HCl/ferric ammonium sulphate solution. The mixture was heated for 40 min in a water-bath at $80^0C \pm 0.5^0C$, then cooled in ice-water for 1.5 min and the absorbance at 480 nm was measured using a Varian Series 634 spectrophotometer . A reagent blank containing 2 ml of water instead of ketose solution was included with each batch of analysis.

This method showed excellent linearity between concentration and absorbance at 480nm over fructose concentrations from 0 to 60 mg/L. Regression analysis of concentration versus absorbance gave a R^2 value of greater than 98%. The reproducibility was excellent (CV less than 5%).

3.3 Analysis of organic acids by HPLC

3.3.1 Background

The conventional methods of organic acids such as chemical and enzymatic assays generally involve the conversion of acids into other intermediates and normally only one organic acid can be analyzed by one test or assay. Therefore, these methods are inefficient. With the rapid development of instruments, organic acids now can be conveniently analyzed, with minimal sample preparation, by high-performance liquid chromatography (HPLC) using reverse phase columns (Schneider et al, 1987).

3.3.2 Sample preparation

Organic acid standards were prepared in distilled water. Model wine samples were also prepared in distilled water as follows: one ml of sample was mixed with 3 ml of distilled water in a beaker; the mixture was then passed through a C_{18} Sep-Pak, which was prewetted with 2 ml of methanol followed by two washings with distilled water; the first 2 ml of eluent were discarded and the last 2 ml of eluent were collected for injection into the HPLC system in order to avoid any dilution of the samples.

Wine samples were prepared in 0.01 M K_2HPO_4 (pH 8.0). One ml of wine sample was mixed with 3 ml of K_2HPO_4. The mixture was then filtered through a 0.2 μm membrane (Millipore, MA) followed by passing through a C_{18} Sep-Pak (Waters Associates). The first 2 ml of the eluent was discarded to avoid dilution inside the Sep-Pak, then the next 2 ml was collected as a sample at the same concentration as before passage through the Sep-Pak (modified from Anonymous, 1982).

3.3.3 HPLC system

A Waters Associates HPLC system was used to separate the organic acids. The system consisted of a Waters 600 Multi-solvent Delivery System, a Valco Instruments (Houston, TX) 6 port Sample Injector, a Lambda-Max 481 LC spectrophotometer and a Waters 740 Data Module. The two HPLC columns (in series) used were a Brownlee Labs$^{(TM)}$ Polypore$^{(R)}$ H cation exchange, ion exclusion MPLC$^{(R)}$ cartridge column (Applied Biosystem, Santa Clara, CA), comprising a 3 x 0.46 cm guard column and a 22 x 0.46 cm analytical column, and a C_{18}-bonded silica column (packed with Bio-Sil^{R} ODS-10), 25 x 0.40 cm (Bio-Rad Laboratories,CA). The Polypore H guard and analytical column preceded the C_{18}-bonded silica column in all experiments. The sample volume was 5 ul and the columns were eluted isocratically with 0.01 M H_3PO_4 at a flowrate of 0.25 ml/min. Peaks were detected by absorbance at 210 nm. Quantitation was made by peak height using external standards, because internal standards were not available. The temperature of the columns was regulated by immersing in a thermostatically-controlled water bath at 25^0C.

3.3.4 Performance of HPLC system

3.3.4.1 Separation of organic acid standards and organic acids in sample

The dual column system was able to separate the main organic acids in the sequence: tartaric, malic, lactic, citric, acetic and succinic acid. In model wine samples, some unknown compound(s) interfered with succinic acid. Therefore, succinic acid could not be quantified.

3.3.4.2 Reproducibility and recovery

This method gave the coefficients of variation in the range of 1.8 to 6.3% and the recovery ranged from 96 to 110%.

3.4 Analysis of amino acids by an amino acid analyzer

3.4.1 Background

Amino acids at analytical levels must be derivatized for detection (Hare et al, 1985). Various derivatization processes have been developed for different instruments. Gas-liquid chromatography involves the utilization of a variety of alcohols for esterification, including methanol, propan-1-ol, isopropyl alcohol, butan-1-ol and isobutyl alcohol (Engel and Hare, 1985). HPLC requires ninhydrin, dansyl chloride, fluorescamine or ortho-phthalaldehyde (OPA) for either pre-column or post-column derivatization (Perrett, 1985). Ion-exchange chromatography with post-column reaction, using ninhydrin or o-phthalaldehyde, is a popular method for amino acid analyses (Hare et al, 1985). For this project, the ion-exchange chromatographic method was adopted for amino acid analyses.

3.4.2 Sample preparation

Samples (2 ml) were filtered through 0.45 μm membranes (Millipore, MA) before injection into amino acid analyzer.

3.4.3 The amino acid analyzer system and its performance

The analyses were carried out by Department of Chemistry/Biochemistry, Massey University, using an automatic Amino Acid Analyzer (AAA, 119 BL) (Beckman, USA). The Analyzer separated amino acids by ion-exchange and consisted of an automatic sampler, a column of sulphonated polystyrene resins and spectrophotometric detection (colorimeter). Post-column

derivatization using ninhydrin was employed. The columns were eluted with buffers comprising a) 0.2 M, pH3.5 sodium citrate; b) 0.4 M, pH4.12 sodium citrate and c) 1 M, pH 6.4 sodium citrate. The accuracy of this method was $\pm$5%.

Chapter Four
Formulation of Model Wine and Experimental

4.1 Introduction

4.1.1 General characteristics of wine

Wine is a harsh medium for the growth and survival of lactic acid bacteria. Wine has a low pH value of usually 3 to 4 and a high alcohol concentration of 8 to 15% (v/v). Sulphur dioxide is added to conserve wine at concentrations of 50 to 100 mg/L (Amerine and Ough, 1980). A range of amino acids is present in wine, such as proline, arginine, glutamine, alanine and threonine at levels of from a few mg/L to several hundred mg/L and amino acids are known to be of significance in the growth of lactic acid bacteria. Dry wines normally contain low concentrations of residual sugars, ranging from 1 to 3 g/L when total carbohydrates are measured as reducing sugars (Melamed, 1962). The components and concentrations of carbohydrates and organic acids in wine are reviewed in Chapter 2. White wines often have low concentrations of sulphite-binding substances such as phenolic compounds, and therefore, have a high free SO_2 concentration that is inhibitory to LAB (Goswell, 1986).

Apart from the adverse factors (low pH, alcohol and SO_2) mentioned above, wine is also a chemically undefined medium, which makes it unsuitable as a growth medium for research purpose. Wine contains phenolic compounds such as anthocyanins (Singleton and Esau, 1969) and colloids such as polysaccharides, pectins, proteins, polyphenols and non-identified substances (Villettaz, 1981). The compositions of these

phenolic compounds and polysaccharides are largely unknown. Hydrolysis of these substances will alter the composition of the wine. Furthermore, components released into wine from yeast cells upon autolysis are also poorly defined.

4.1.2 Nutritional requirements of lactic acid bacteria

Lactic acid bacteria are nutritionally fastidious and they require a whole range of nutrients to support their growth. The requirements for growth include carbon source, nitrogen source, minerals, vitamins, bases and other growth factors. The nitrogen source must be organic; either amino acids or peptides. However, lactic acid bacteria cannot synthesize all of the required amino acids from other amino acids (Lafon-Lafourcade, 1983) and those amino acids which cannot be synthesized need to be supplied extrogenously using yeast extract, peptone, etc.

4.2 Origin and maintenance of cultures

The cultures used for this research project were from Dr Craig Davis' personal culture collection. These cultures include Leuconostoc oenos 122, Leuconostoc oenos 252, Lactobacillus plantarum 49 and Pediococcus parvulus 93. They were isolated and identified from Australian red wines.

The cultures were maintained by the method of Pilone (1979) and Duitschaever and Buteau (1986). Instead of using porcelain unglazed berl saddles, pumice stone granules were employed as the support for drying the bacteria. Briefly, this method involves the use of a sterile dehydration tube containing silica gel at the bottom, glass wool in the

middle and pumice stone granules at the top. An actively-growing culture in tomato juice broth was applied over the granules and the tube was sealed with a screw cap, then wrapped with parafilm. The dehydration tubes were stored in a refrigerator at 4^0C.

4.3 Composition and concentration of the model wine

This model wine was formulated on the basis of reported concentrations of wine components (Amerine et al, 1980). The concentrations of individual constituents used in these experiments are given in Tables 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6.

This synthetic medium simulates the composition of normal dry wines and is chemically defined and nutritionally complete.

Table 4.1 Carbohydrate concentrations in the model wine

Carbohydrate	Class	Concentration (mg/L)
Glycerol	Triol	5000
Erythritol	Tetritol	100
Threitol	Tetritol	50
Ribose	Pentose	100
Rhamnose	6-deoxy hexose	50
Lyxose	Pentose	30
Arabinose	Pentose	200
Fucose	6-deoxy hexose	10
Xylose	Pentose	100
Ribitol	Pentitol	100
Arabitol	Pentitol	200
Xylitol	Pentitol	100
Mannose	Hexose	100
Glucose	Hexose	200
Galactose	Hexose	200
Fructose	Hexose	200
Myo-inositol	Cyclitol	500
Mannitol	Hexitol	200
Glucitol	Hexitol	100
Galactitol	Hexitol	50
Maltose	Disaccharide	20
Lactose	Disaccharide	20
Sucrose	Disaccharide	100
Cellobiose	Disaccharide	20
Melibiose	Disaccharide	20
Trehalose.dihydrate	Disaccharide	500
Gentiobiose	Disaccharide	20

Table 4.2 Concentrations of organic acids in the model wine

Organic acid	Concentration (g/L)
Tartaric	5
L-(+) malic[a]	5
Citric	0.5
Lactic[a]	3
Gallic	0.05
Ascorbic	0.005

[a] Presence or absence depends on the treatment applied.

Table 4.3 Mineral concentrations in the model wine

Mineral	Concentration (mg/L)[a]
NaCl	526.5
KI	0.4
KF	1.5
$MgSO_4.7H_2O$	1540.0
$MnSO_4.4H_2O$	812.0
$FeSO_4.7H_2O$	100.0
$CaCl_2$	277.5
K_2HPO_4	1045.2
NH_4Cl	15.0
H_3BO_3	20.0
$CuSO_4$	0.3
$Al_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$	11.0

[a] The figures in mg/L are derived from mM/L, which was derived from mg/L of elements as documented by Amerine et al (1980).

Table 4.4 Concentrations of amino acids in the model wine

Amino acid	Concentration (mg/L)
Alanine	75
DL-α-aminobutyric acid	40
Arginine (free base)	300
Aspartic acid	50
Asparagine	40
Cystine	50
Glutamic acid (free acid)	200
Glutamine	30
Glycine	20
Histidine	20
Isoleucine	30
Leucine	30
Lysine.HCl	40
Methionine	25
Phenylalanine	20
Proline	200
Serine	40
Threonine	100
Tryptophane	10
Tyrosine	20
Valine	20
Cysteine (free base)	10
Citrulline	10
Hydroxyproline	5
DL-ornithine.HCl	5

Table 4.5 Vitamin concentrations in the model wine

Vitamin	Concentration (mg/L)
Thiamine.HCl	0.5
Riboflavin	0.7
Pyridoxine.HCl	1.5
Pantothenic acid calcium salt	5.0
Nicotinic acid (free)	4.5
Biotin	0.05
P-aminobenzoic acid (free)	0.05
Choline chloride	2.0
Folic acid (free base)	0.05
Cyanocobalamine	0.07

Table 4.6 Concentrations of purines and pyrimidines in the model wine

Purines and pyrimidines	Concentration (mg/L)
Adenine sulphate	10.0
Guanine.HCl	10.0
Thymine	10.0
Uracil	10.0
Xanthine	5.0
Cytosine	10.0
Orotic acid, monosodium salt	20.0

Table 4.7 Concentrations of other constituents

Constituents	Concentration
Tween 80 (dispersing agent)	0.1 g/L
Ethanol	10% (v/v)

4.4 Preparation of stock solutions

Stock solutions (10-fold concentration) for each category of components as shown above were made up and frozen at -18^{0}C. A 10-fold stock solution of minerals could not be made up, due to chemical precipitation. Therefore, the minerals were weighed and added separately to the model wine. Malic and lactic acids were not included in the stock solution of organic acids, as their presence depended on the treatments. Heating (near boiling) and the addition of HCl were needed to help dissolution of the amino acids and the bases.

4.5 Experimental design

This research project was designed to investigate the physiology and metabolism of LAB at different pH values and in the presence or absence of malic acid, so that information could be derived about the effects of pH and malolactic fermentation on the metabolic pattern of LAB.

It is known that Leuconostoc oenos can grow at pH 3.0 (Bousbouras and Kunkee, 1971; Fleet, 1985) so the two pH levels chosen for strains of this species were 3.2 and 4.0. Since wines below pH 3.5 generally do not support the growth of Lactobacillus spp and Pediococcus spp (Wibowo et al, 1985), the pH values chosen were 3.5 and 4.0. The experimental design of this project is shown in Table 4.8.

Table 4.8 Treatments of the model wine

Bacteria	pH	Malic acid	Lactic acid
All bacteria	4	+	-
All bacteria	4	-	+
L. plantarum 49 and	3.5	+	-
P. parvulus 93	3.5	-	+
L. oenos 122 and	3.2	+	-
252	3.2	-	+

4.6 Preparation of the preculture medium and the model wine

Firstly, frozen stock solutions were thawed by warm water. Then aliquots of each stock solution were mixed as required in a 2 L measuring cylinder. The minerals were dissolved in water and then added to the mixture. Malic or lactic acids were added afterwards in accordance with the experimental design. Distilled water was used to make up to the volume required where a 10 x dilution of stock solutions could be obtained.

The pH of this freshly made up model wine was then adjusted in the case of Leuconostoc oenos 122 and 252 to 3.2 with KOH pellets, or 3.5 in the case of Lactobacillus plantarum 49 and Pediococcus parvulus 93. After pH adjustment, the model wine was stored at 4^0C for 2 days to precipitate potassium bitartrate and any other possible materials. This cold treatment assured that the final composition of the model wines at all pH levels would not vary significantly when readjusted to higher levels (see below).

After chill-stabilization, cysteine was added to the model wine as a reducing agent at 0.5g/L. The pre-reduced model wine was then divided

into two batches. One batch was re-adjusted to pH4 and the other re-adjusted to pH3.2/3.5. After these pH adjustments, the model wine was filter-sterilized by passing through a sterile filtration apparatus with a 0.45μm membrane (Millipore, MA). The membrane-filtered model wine was dispensed into sterile 200 ml medical flat bottles. There were 3 replicates for every treatment.

The preculture medium was identical to the model wine, except that no ethanol was added. The preculture medium was prepared in the same way as the model wine. Leuconostoc oenos strains 122 and 252 were precultured at pH 3.2. Lactobacillus plantarum 49 and Pediococcus parvulus 93 were precultured at pH 3.5. The pre-culture medium was dispensed into sterile 100 ml medical flat bottles.

4.7 Culturing procedure and sampling regime

Three pumice stone granules were taken aseptically from the dehydration tube and placed in tomato juice broth (TJB, see Appendix for recipe) and incubated at 30^0C for seven days (Leuconostoc oenos strains 122 and 252 and Pediococcus parvulus 93), and four days (Lactobacillus plantarum 49). The cultures from TJB were also streaked onto tomato juice agar (TJA) plates to check purity. These plates were incubated under the same conditions as for TJB. Pure colonies of each organism were inoculated into TJB and incubated at 30^0C. A sample (1 ml) from each TJB culture was then subcultured in the appropriate preculture medium prepared as in Section 4.6 and incubated at 22^0C until the maximum growth was achieved (judged by turbidity changes) after ten days for L. oenos strains 122 and 252, three days for L. plantarum 49 and seventeen days for P. parvulus 93. These broths were then further subcultured in the

same preculture media and incubated under identical conditions until the maximum growth was attained. Two drops (approximately 0.1 ml) of the final subculture were inoculated into the model wine and incubated under the same conditions as the preculture media. Uninoculated controls at pH 3.2, 3.5 and 4 were also prepared to assess changes, such as hydrolysis of disaccharides during incubation.

Three samples were taken during storage of each trial: the first, immediately after addition of the preculture; the second, at the end of the log phase (as judged by turbidity); and thirdly, after several weeks of stationary phase. Bottles were shaken moderately before sampling and the sample (15 ml) was taken from the middle of the bottle and transferred into a sterile 15 ml bottle. Samples were stored at -18^{0}C.

4.8 Measurement of cell number and pH

The cell number changes were determined by the conventional plating procedure. This method also served as a check on the purity of the organisms. After appropriate dilution in 0.1% peptone water, 0.1 ml of solution was spread in duplicate onto the surface of TJA plate. The plates were incubated at 30^{0}C for seven days for the cocci and four days for the rods.

The pH was measured using a pH meter (Orion Research, Model 501 digital ionalyzer) which was calibrated using pH 4.0 and 7.0 buffers. Five ml of the samples were used for pH measurement and then discarded.

Carbohydrates were determined by capillary gas-liquid chromatography and organic acids were analysed by HPLC. Amino acids were assayed by an

automatic amino acid analyzer. Details of these analyses were described in Chapter 3.

4.9 Growth of lactic acid bacteria in the model wine with single carbon/energy sources

Since information cannot be derived from the model wine about whether the individual carbohydrate will support the growth of LAB or not, because of the presence of a mixture of carbohydrates, it was decided to investigate the effects of single carbon/energy sources on the growth of LAB. Because of time limitations, only some of the carbohydrates used in the model wine were tested. The carbohydrates chosen for this experiment were: glycerol, arabinose, arabitol, glucose, fructose, myo-inositol, mannitol and trehalose. So far it has not been shown that malic acid can be used as a sole energy source for malolactic bacteria. Pilone and Kunkee (1972) obtained only slight growth of L. oenos ML 34 grown on malic acid only. Therefore, attempts were made to see if malic and citric acids could serve as single energy sources. Arginine was also tested to see if it could serve as a single energy source for LAB.

The following treatments were prepared in a basal medium (Table 4.9). The composition of the basal medium contained bases, vitamins, amino acids, organic acids, minerals and Tween 80. Stock solutions of the basal medium were the same as those of model wine in respect to amino acids, bases and vitamins. All the concentrations were identical to the model wine. Experiments were conducted at pH 4 for all the LAB.

Table 4.9 Treatments of the model wine with single carbon/energy sources

Compound	Treatments										
	1	2	3	4	5	6	7	8	9	10	11
Malic acid	+	-	-	+	+	+	+	+	+	+	+
Citric acid	-	+	-	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+
Glycerol	-	-	-	+	-	-	-	-	-	-	-
Arabinose	-	-	-	-	+	-	-	-	-	-	-
Arabitol	-	-	-	-	-	+	-	-	-	-	-
Glucose	-	-	-	-	-	-	+	-	-	-	-
Fructose	-	-	-	-	-	-	-	+	-	-	-
Myo-inositol	-	-	-	-	-	-	-	-	+	-	-
Mannitol	-	-	-	-	-	-	-	-	-	+	-
Trehalose	-	-	-	-	-	-	-	-	-	-	+

In all cases, tartaric (5 g/L) and lactic acid (3 g/L) were incorporated into the media. The media were filter sterilized through 0.45 μm membranes. The sterile media were dispensed into 100 ml medical flat bottles. The culturing procedure was similar to that of the model wine, i.e., TJA -> TJB -> Preculturing I -> Preculturing II -> model wine. The preculture media and model wine were identical, except that the preculture media contained no ethanol. One drop of culture was used for all inoculations. Growth was judged by turbidity changes.

4.10 Growth and metabolism of lactic acid bacteria in a red wine

Although the model wine is chemically similar to real wine, they are not identical, since wine is a highly complex medium. Wine may contain some extra growth factors for lactic acid bacteria. Consequently, findings from the model wine may not be applied to real wines without testing the comparability and compatibility of the model and real wines. The aim of

this experiment was to study the growth and metabolism of lactic acid bacteria in real wine, so that information gained from the model and real wine could be correlated.

4.10.1 General characteristics of the red wine

This red wine was vinified from the Merlot grape in 1989 (Hawkes Bay) without undergoing MLF. The sulphur dioxide content was zero (determined by aspiration method, Amerine and Ough, 1980) and ethanol concentration was 11% v/v (measured by ebulliometry). It contained 2g/L of malic acid and detectable amounts of citric acid, which could not be quantified. The initial carbohydrate concentrations are described in Chapter 8.

4.10.2 Preparation of preculture media

The preculture was made up based on half-strength grape juice (Greenways, purchased from a local supermarket) in water, containing 5 g/L yeast extract (Hayman and Monk, 1982; Wibowo et al, 1988). The pH was adjusted to 3.2 with 1 mM HCl. The medium was dispensed into 100 ml medical flat bottles and was autoclaved at 121^0C for 15 minutes.

It is difficult to judge visually the growth state of L. oenos 122 in the red grape juice medium and therefore, it was uncertain whether sufficient cells had been inoculated into the wine in some experiments. Because of this, another medium, apple juice (UHT Aseptic, Fresh-up) with a natural pH value of 3.3, was also used for preculturing, so that the growth of this organism could be judged visually and enough cells inoculated.

4.10.3 Preparation of the red wine

Upon receipt of this red wine, it was passed through a coarse pad filter followed by filter sterilization through a 0.45 μm membrane (Millipore, MA) and then stored at 4^0C until required.

Before inoculation, the composition of the wine was adjusted as follows: L-malic acid was supplemented to achieve a final concentration of 4 g/L. Citric acid was added at 0.5 g/L. The wine was divided into two batches and the pH was adjusted to 3.4 and 4.0 respectively. Following pH adjustment, the wine was filter sterilized, using a 0.45 μm membrane (Millipore, MA) and dispensed into 300 ml medical flat bottles.

4.10.4 Culturing procedures and sampling plan

Pure colonies from TJA were inoculated into TJB and incubated at 30^0C for seven days. Then one ml of the TJB culture was inoculated into the grape juice preculture media and incubated at 22^0C until the maximum growth was accomplished (judged by pH measurement and microscopic examination). After this stage of preculturing, one ml of the solution was inoculated into the same preculture media and incubated under the identical conditions until the maximum growth was achieved. Finally, four drops of solution from the second-stage of preculture media were inoculated into the wine. Precultures using grape juice were inoculated into the wine of pH 4 and precultures using apple juice were inoculated into the wine of pH 3.4. The reasons for using apple juice as a preculturing medium is described in Section 4.10.2. The inoculated wine was incubated under the same conditions as for preculturing.

The sampling regime was essentially the same as for the model wine, except that more frequent samplings were required, since it was more

difficult to judge the growth visually. Samples were taken at 17 days, 20 days, 26 days, and 31 days for the treatment at pH 3.4; 18 days, 25 days, 32 days and 39 days for the treatment at pH 3.8. Samples were stored at -18^{0}C until analysed.

4.11 Growth and metabolism of lactic acid bacteria in the modified MRS medium

To date most published work on the growth and metabolism of lactic acid bacteria have been accomplished using semi-synthetic media, such as MRS and media containing tomato juice, yeast extract, tryptone and peptone at pH 4.5 to pH 6. These media are highly enriched compared with wine. Therefore, in the present study, the growth and metabolism of lactic acid bacteria were compared under these distinct conditions. Owing to time limitations, only L. plantarum 49 was tested.

In this experiment, L. plantarum 49 was cultured in the modified MRS medium (see Appendix 2 for composition). The medium was formulated based on the original MRS formula. 'Lab-Lemco' powder (beef extract) and dextrose (20.0 g/L) were omitted. The carbohydrate concentrations in the modified MRS medium were 10 x higher than those in the model wine, except for glycerol, glucose, fructose and trehalose. The medium was adjusted to pH 6 and were autoclaved at 115^{0}C for 20 minutes. Precultures from TJB were directly inoculated into the medium. Samples were taken at 1 day and 3 days and stored at -18^{0}C until analysed.

Chapter Five Results and Discussion
Capillary Gas-Liquid Chromatography of Carbohydrates in Wines and Model Wines

5.1 Retention times of standard carbohydrates

The retention times of standard carbohydrate derivatives are presented in Table 5.1.

Table 5.1 Retention times (n=7) of aldose, polyol and disaccharide derivatives when separated by capillary gas-liquid chromatography[a]

Compound	Class of Compound	Mean Retention Time (min)	SD[b]	CV(%)[c]
Glycerol	Triol	11.09	0.06	0.50
Erythritol	Tetritol	19.02	0.05	0.26
Threitol	Tetritol	19.51	0.05	0.24
Ribose	Pentose	21.47	0.05	0.22
Rhamnose	6-deoxyhexose	21.53	0.05	0.21
Lyxose	Pentose	21.85	0.05	0.22
Arabinose	Pentose	22.05	0.06	0.27
Fucose	6-deoxyhexose	22.35	0.05	0.21
Xylose	Pentose	22.47	0.05	0.21
Ribitol	Pentitol	26.22	0.05	0.19
Arabitol	Pentitol	26.51	0.05	0.19
Xylitol	Pentitol	26.99	0.05	0.19
Mannose	Hexose	28.52	0.05	0.18
Glucose	Hexose	28.88	0.06	0.21
Galactose	Hexose	29.48	0.06	0.19
Myo-inositol	Cyclitol	32.45	0.09	0.29
Mannitol	Hexitol	32.69	0.06	0.19
Glucitol	Hexitol	32.92	0.06	0.19
Galactitol	Hexitol	33.09	0.06	0.18
D-Glucoheptose	Heptose	34.69	0.05	0.16
Phenyl β-D-Glucoside	Phenyl glucoside	40.55	0.06	0.14
Salicin	Phenyl glucoside	47.96	0.05	0.11
Arbutin	Phenyl glucoside	48.26	0.08	0.16
Maltose	Disaccharide	52.31	0.07	0.14
Lactose	Disaccharide	52.69	0.07	0.13
Sucrose	Disaccharide	53.10	0.08	0.15
Cellobiose	Disaccharide	53.41	0.08	0.14
Melibiose	Disaccharide	53.52	0.08	0.14
Trehalose	Disaccharide	53.82	0.07	0.13
Gentiobiose	Disaccharide	55.19	0.05	0.16

[a] Samples were separated using conditions described in the Experimental Section (Chapter 3).
[b] SD, standard deviation.
[c] CV (%), percentage coefficient of variation.

The mean retention times of the carbohydrate standards were produced by injecting one sample on seven occasions over a period of several weeks (Table 5.1). There were only very small variations in retention times among the seven injections, indicating the reliability of the method. A typical chromatogram of a standard carbohydrate mixture is given in Figure 5.1a.

This method resolves 30 carbohydrates in 55 minutes; this compares favourably with the separation achieved by Szafranek et al (1973), who used a similar polarity capillary column to separate the same types of derivatives. Under the current conditions, xylose and fucose (6-deoxygalactose), which were reported to be inseparable by Szafranek et al (1973), were separated. However, rhamnose and ribose almost co-eluted. Nevertheless, they could be quantified using height ratio. Preliminary experiments showed that raffinose and melezitose did not appear consistently on chromatograms. These high molecular weight trisaccharides may have been poorly derivatized or were unstable at 300^{o}C, which was the temperature required to elute them from the column in a reasonable period of time. Although consistent quantitative results were not obtained for gentiobiose, the rest of the disaccharides were separated and quantified consistently, and calibration curves could be produced using either area ratio or height ratio. At the temperatures needed for elution of di- and tri-saccharide acetates, thermal degradation effects could occur, in particular, loss of water to form anhydro derivatives with concomitant conformational inversion of the sugar rings (Birch, 1973). This may account for the instability of some disaccharides and trisaccharides. The polyols, pentoses and hexoses showed excellent stability and consistency in recorder response.

5.2 Calibration and quantification

The plots of area ratio versus concentration of carbohydrate gave R-squared values greater than 98% for most of the aldoses, polyols and

disaccharides. In general, area ratio gave better linearity than height ratio except for lactose, maltose, cellobiose and melibiose, for which better linearity was obtained using height ratio. The two internal standards showed similar performance in terms of linearity. However, preliminary trials indicated that phenyl β-D-glucoside, salicin and arbutin were absorbed by the non-polar C_{18} Sep-Pak cartridge, presumably because of the polarity of these phenolic glucosides. In consequence, phenyl β-D-glucoside was added to the sample after elution from the C_{18} Sep-Pak cartridge.

5.3 Recovery

Most of the spiked carbohydrates were recovered at 86 to 110% from wine samples. Glycerol, glucose and myo-inositol were recovered at percentages outside this range, perhaps because they were present at high concentrations in the wine used for the recovery test. Some interference and co-elution between ribose and rhamnose would explain the higher variations in recoveries for these sugars. Through assessment of the calibration curves, it was decided to use internal standard 1 for quantitation using area ratio for all compounds except for ribose, rhamnose, maltose, lactose, cellobiose and melibiose, where height ratio was used. However, area ratio generally gave better recovery than height ratio for most of the aldoses and polyols. The low recovery of erythritol was attributed to interference from unknown sources. The recovery of salicin and arbutin was not assessed as these compounds were absorbed by the C_{18} Sep-Pak.

5.4 Reliability of the method

The coefficient of variation (CV) of reproducibility and instrument stability was in the range of 2.4 to 8.3% and 2.5 to 9.3% for most of

the carbohydrates. There were relatively larger variations for those carbohydrates which were present in very high or very low concentrations, e.g., glycerol and some pentoses, respectively. However, for carbohydrates present in high concentrations, such as glycerol, this error could be avoided by dilution before sample cleanup. Variations for ribose and rhamnose were because of proximity of peaks. The variation for erythritol was due to interference of the erythritol peak by unknowns.

5.5 Determination of carbohydrates in wine and apple juice

Samples analyzed by this method were: white wine I (sweet, 1986), white wine II (medium-dry, 1986), Cabernet Sauvignon wine (1985), Pinot Noir wine (1983), kiwifruit wine (1988) and apple juice. The two white wines were from China and the remaining samples were from New Zealand.

Representative chromatograms of some samples are shown in Figure 5.1 and 5.2. Chromatograms of similar resolution were achieved for the other samples. Quantitative data for the carbohydrates are presented in Table 5.2.

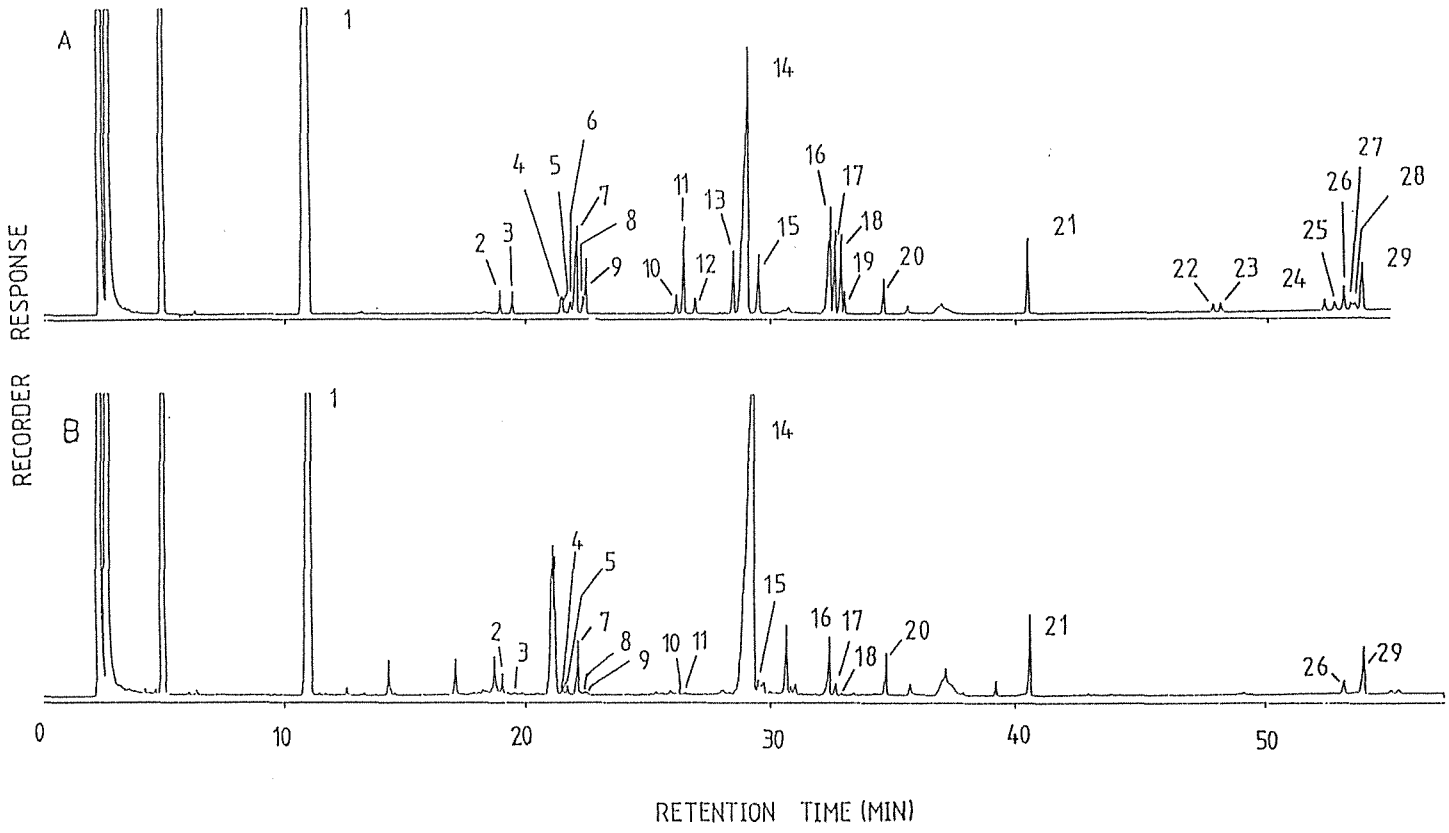

Figure 5.1
Chromatograms of carbohydrate derivatives from (A) standard solution and (B) medium-dry white wine. Identification of peaks: (1) glycerol, (2) erythritol, (3) threitol, (4) ribose, (5) rhamnose, (6) lyxose, (7) arabinose, (8) fucose, (9) xylose, (10) ribitol, (11) arabitol, (12) xylitol, (13) mannose, (14) glucose, (15) galactose, (16) myo-inositol, (17) mannitol, (18) glucitol, (19) glucoheptose (internal standard), (21) phenyl β-D-glucoside, (22) salicin, (23) arbutin, (24) maltose, (25) lactose, (26) sucrose, (27) cellobiose, (28) melibiose and (29) trehalose.

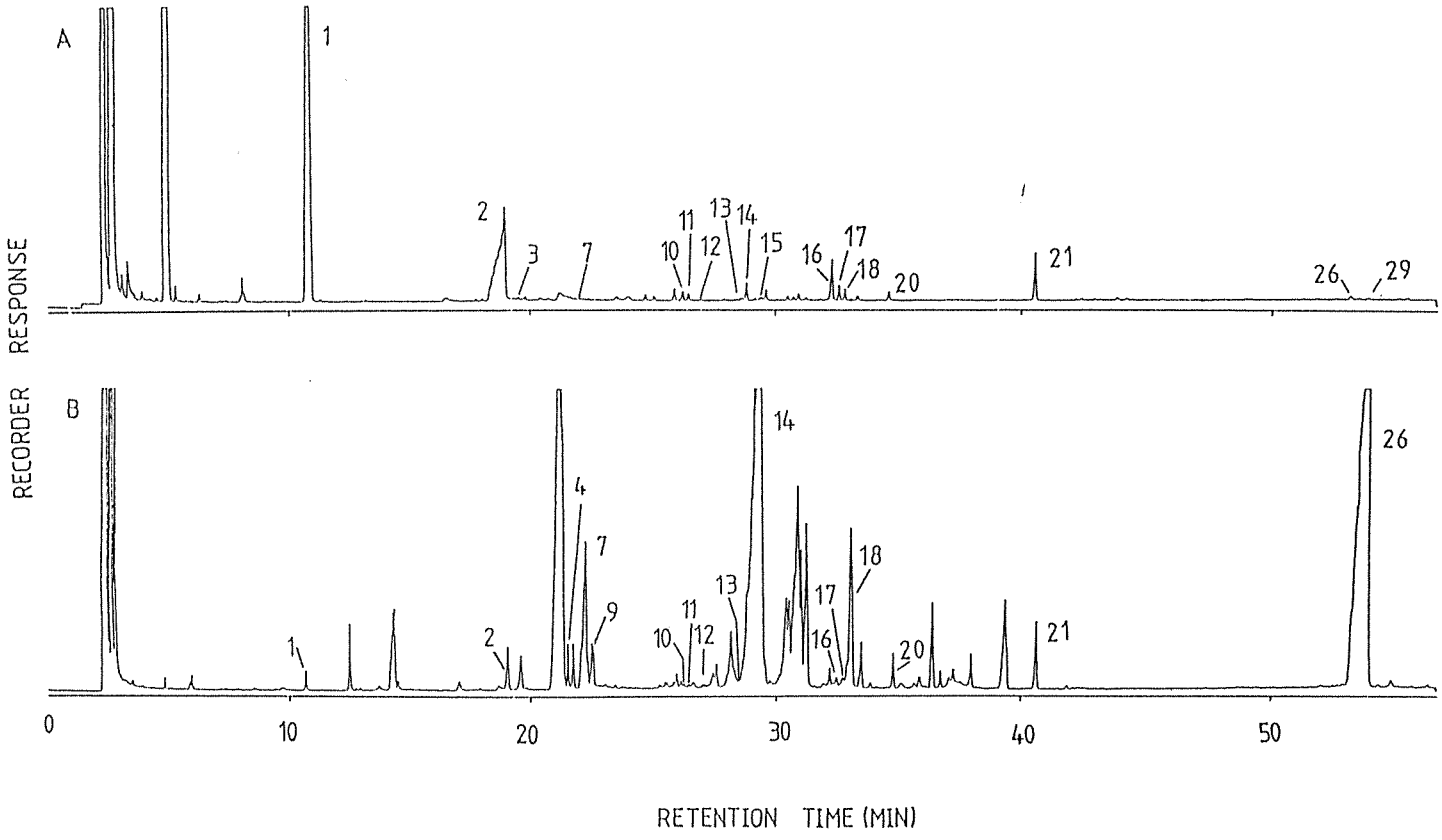

Figure 5.2
Chromatograms of carbohydrate derivatives from (A) Pinot Noir wine and (B) apple juice. Identification of peaks as in Figure 5.1.

Table 5.2 Concentrations of carbohydrates in wine and apple juice determined using capillary gas-liquid chromatography

Compound	Concentration [a]						
	white wineI (sweet)	white wineII (medium-dry)	Cabernet Sauvignon wine	Pinot Noir wine	Kiwifruit wine	Apple juice	Literature[b] values of wine
Glycerol	9.79	6.86	17.78	15.36	10.34	trace	3-15
Erythritol	214	59	I[c]	I	44	245	10-20
Threitol	25	7	19	17	3	ND[d]	ND
Ribose	321	24	71	ND	52	108	40
Rhamnose	341	23	76	ND	46	ND	200-400
Arabinose	2050	510	10	37	50	2710	500-1000
Fucose	313	13	ND	ND	13	ND	5
Xylose	1078	25	11	ND	68	505	20
Ribitol	23	3	106	140	39	9	ND
Arabitol	18	8	108	83	50	10	10-30
Xylitol	37	ND	15	ND	29	6	35-135
Mannose	I	I	35	73	395	7	40
Glucose	10.17	9.69	0.15	0.40	0.49	16.9	0.5-0.1
Galactose	I	80	140	940	530	ND	130
Inositol	250	320	740	730	1090	70	220-730
Mannitol	116	53	493	194	65	224	10-40
Glucitol	237	8	4	138	22	1560	0-50
Galactitol	ND	ND	9	ND	25	ND	ND
Sucrose	0.06	0.12	0.02	0.11	0.04	16.82	0-0.2
Trehalose	550	420	8	37	50	ND	0-500

a All concentrations are mg/L, except for glycerol, glucose, and sucrose which are g/L.
b Literature values are from Melamed (1962), Ribereau-Gayon et al (1972), Bertrand et al (1975), Amerine et al (1980).
c I, interference does not allow quantitation.
d ND, not detectable.

The great sensitivity and resolution of this method is well illustrated by these chromatograms. Many compounds, as yet unidentified, are resolved in the chromatograms. This GLC method is able to analyze a broad range of carbohydrates in wines and other alcoholic and non-alcoholic beverages, using a single injection to separate from simple straight chain polyols (e.g., glycerol, tetritols, pentitols and hexitols) through to aldoses, glycosides and disaccharides. Preliminary trials showed that amino sugars such as N-acetyl glucosamine could also be separated from other carbohydrates by the same derivatization

procedure. Amino sugars are also derivatized as aldononitrile acetates. However, aldononitrile acetates of amino sugars exhibit erratic chromatographic properties in addition to having appreciable water solubility, thus making the derivative cleanup procedure difficult (Mawhinney et al, 1980). The derivatization procedure used in this experiment is such that both aldoses and polyols can be determined in one analysis. This is not the case with the use of alditol acetate, as the reduction step required to avoid separation of anomers in aldoses (Selvendran et al, 1979; Bittner et al, 1980; Adam, 1981) is omitted. On the other hand, the reduction method may also lead to the formation of the same alditol from two hexoses and two pentoses, for instance, D-glucose and D-gulose both yield D-glucitol; D-arabinose and D-lyxose both yield D-arabitol (Biermann, 1989); in particular D-glucose and D-fructose both give glucitol (sorbitol) (Birch, 1973). The reduction method also requires the removal of all of the boron if pyridine is used as an acetylation catalyst (Biermann, 1989). Although there have been several reports on the use of alditol acetates and TMS ethers for analysis of carbohydrates in wine, other beverages and foods (Heatherbell, 1975; Selvendran et al, 1979; Heatherbell et al, 1980; de Smedt et al, 1981; Li et al, 1983), the author believes that the method employed in this experiment offers the benefits of ease of derivatization, availability and inexpensiveness of reagents and stability of derivatives. It is worthwhile noting that the method can quantify carbohydrates which may occur at concentrations from only a few milligrams per liter up to grams per liter. Glycerol clearly lies outside this range as it is present at concentrations of up to 10 to 15 g/L and so errors have been introduced into its determination. A separate assay of diluted sample would alleviate this problem.

A number of carbohydrates have been determined, including glycerol, pentose, xylitol, mannose, glucose, inositol, trehalose and sucrose, in dry wines at concentrations consistent with the published data (Melamed, 1962; Ribereau-Gayon et al, 1972; Bertrand et al, 1975; Amerine et al, 1980). Mannitol was found at levels higher than the literature values while rhamnose was found at lower concentrations. Other carbohydrates found in the wine samples were erythritol, threitol, fucose, ribitol, arabitol, galactose, glucitol and galactitol at various levels. The presence of threitol, ribitol and galactitol has not been reported before. However, analysis using GLC-Mass Spectrometry is required to confirm the identities of these compounds. Glycerol, glucose, galactose, inositol, mannitol, sucrose and trehalose are the major carbohydrates in dry wines. Pentoses (ribose, arabinose and xylose) and methyl pentoses (fucose and rhamnose) were present in large concentrations in the sweet wine. Washuttl et al (1973) studied the polyols in various foods. They found only glucitol (sorbitol) in red and white wine and apple juice at concentrations of 14.5, 8 and 203 mg/100g, respectively. In addition to glucitol, Makinen and Soderling (1980) found xylitol in apple and two wine samples. Apart from xylitol and glucitol, other polyols were found in this experiment, including tetritols, pentitols and hexitols, as shown in Table 5.2. Trehalose together with smaller amounts of sucrose was the predominant disaccharide in wine samples analyzed. This trend has been reported by others (Bertrand et al, 1975; Olano, 1983). The high concentrations of glucose and sucrose in apple juice are consistent with the information of Moyer and Aitken (1971). The apple juice also contains a high concentration of arabinose and glucitol (sorbitol). Several other sugars and polyols were found in the apple juice sample as

well (Table 5.2). The data of this experiment indicate that kiwifruit wine contains a wider range of carbohydrates than previously found. Heatherbell et al (1980) found only glucose and fructose in kiwifruit wine. However, our analysis has shown that kiwifruit wine contains carbohydrates ranging from glycerol, tetritols, pentoses, ribitol, hexoses, hexitols and disaccharides (trehalose), though hexoses are the main sugars found.

5.6 Summary

In summary, a variety of carbohydrates (from glycerol to disaccharides) in wine and related beverages can be analysed in 55 minutes, using capillary gas-liquid chromatography of acetates and aldononitrile acetates. Sample preparation is carried out on only 1 ml of wine or juice and involves simple cleanup procedures. A large number of samples can be prepared at one time to shorten the sample preparation time. The derivatives are formed using inexpensive, easily obtainable reagents. The method is sensitive, reproducible and provides good recovery of carbohydrates. It offers the advantage that most derivatives give single peaks. In addition, the great resolution and sensitivity of this method has revealed the presence of unidentified carbohydrates in wines. Further research using GLC-Mass Spectrometry is required for identification of these compounds. Acetates and aldononitrile acetates are particularly suited to this analysis, as they have a predictable electron impact fragmentation pattern (Lonngren and Svensson, 1974).

Chapter Six Results and Discussion
Growth and Metabolism of *Leuconostoc oenos* 122 and 252 *Lactobacillus plantarum* 49 and *Pediococcus parvulus* 93 in the Model Wine System

6.1 Changes in cell concentration and pH during growth

The cell concentration, expressed as colony-formimg units (cfu/ml), and the corresponding pH changes of the medium are shown in Tables 6.1, 6.2, 6.3 and 6.4, respectively.

The end of the log phase was visually assessed by observing the turbidity changes of the medium in order not to disturb the environment in the model wine system or introduce any contaminating microorganisms. The second sample was taken as closely as possible to that time corresponding to the maximum growth (defined as the end of log phase). The third sample was taken from one week to several weeks later, dependent on the strain, to ensure that the cells were well into the stationary phase (defined as the end of stationary phase).

The coding system used is described as follows:

pH 4.0 +malic acid -lactic acid = 4 M;

pH 4.0 -malic acid +lactic acid = 4 L;

pH 3.5 +malic acid -lactic acid = 3.5 M;

pH 3.5 -malic acid +lactic acid = 3.5 L;

pH 3.2 +malic acid -lactic acid = 3.2 M;

pH 3.2 -malic acid +lactic acid = 3.2 L;

where + = presence, - = absence.

Table 6.1 The cell concentration changes of Leuconostoc oenos 122 inoculated into the model wine and the corresponding pH changes of the medium

Treatment	Initial		End of log phase			End of stationary phase		
	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH
4 M	5.35×10^4	4.0	17	2.00×10^7	4.4	42	4.95×10^5	4.6
4 L	5.58×10^4	4.0	27	3.60×10^6	4.0	56	2.76×10^6	4.1
3.2 M	5.05×10^4	3.2	21	1.32×10^7	3.3	50	8.68×10^5	3.5
3.2 L	4.30×10^4	3.2	50	1.06×10^3	3.2	84	3.80×10^2	3.25

[a] The definitions of "end of log phase" and "end of stationary phase" have been given earlier in this chapter.

As shown in Table 6.1, malic acid stimulated the growth rate and cell population of L. oenos 122 at pH 4 and allowed its growth at pH 3.2, since 4 M reached 2 x 10^7 in 17 days which was quicker than 4 L (which produced a lower count). However, growth was inhibited by the absence of malic acid at pH 3.2. Assessment of the growth rate and cell concentrations of treatments 4 L and 3.2 M showed that better growth occurred in treatment 3.2 M than in 4 L.

From Table 6.1, it can also be seen that the pH increased significantly in treatments 4 M and 3.2 M, the effect being greater in 4 M. The pH of 4 L increased only slightly.

Table 6.2 The cell concentration changes of Leuconostoc oenos 252 inoculated into the model wine and the corresponding pH changes of the medium

Treatment	Initial		End of log phase			End of stationary phase		
	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH
4.0 M	$<10^3$	4.1	32	3.45×10^7	4.4	42	2.97×10^6	4.5
4.0 L	$<10^3$	4.1	49	8.54×10^7	4.0	63	8.12×10^6	4.0
3.2 M	$<10^3$	3.3	132	5.00×10^6	3.4	146	5.80×10^4	3.5
3.2 L	$<10^3$	3.3	___	_____	___[a]	146	40	3.3

[a] No sampling due to failure of growth.

As shown by Table 6.2, when Leuconostoc oenos 252 was inoculated into the model wine, similar trends to those of L. oenos 122 (Table 6.1) occurred. Yet it seemed that in the case of L. oenos 252 the presence of malic acid was more stimulatory to the growth rate, rather than the cell concentration, since this strain reached its maximal growth faster in 4 M than in 4 L, but the cell concentration achieved by the end of log phase was similar.

Increases in pH of treatments 4 M and 3.2 M were also similar to those of L. oenos 122, but the pH of 4 L decreased slightly.

Table 6.3 The cell concentration changes of Lactobacillus plantarum 49 inoculated into the model wine and the corresponding pH changes of the medium

Treatment	Initial		End of log phase			End of stationary phase		
	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH
4.0 M	1.68×10^5	4.01	7	1.78×10^8	4.12	14	9.57×10^5	4.08
4.0 L	1.11×10^5	3.99	10	1.15×10^8	3.75	17	1.43×10^6	3.74
3.5 M	1.17×10^5	3.55	—	—	—[a]	46	1.18×10^2	3.45
3.5 L	1.33×10^5	3.48	—	—	—[a]	46	0	3.41

[a] No sampling due to failure of growth.

As shown in Table 6.3, L. plantarum 49 grew well in 4 M and 4 L, but died out in 3.5 M and 3.5 L. The stimulatory effect of of malic acid on growth not evident at pH 4, as treatment 4 L showed a parallel increase in cell concentration and the time required to achieve the maximal growth was similar.

As also indicated in Table 6.3, strain 49 raised the pH only slightly during growth in the presence of malic acid. The pH of treatment 4 L fell significantly.

Table 6.4 The cell concentration changes of Pediococcus parvulus 93 inoculated into the model wine and the corresponding pH changes of the medium

Treatment	Initial		End of log phase			End of stationary phase		
	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH	Time (days)	Cell number (cfu/ml)	pH
4.0 M	2.43×10^5	3.97	20	2.98×10^7	4.25	34	9.50×10^4	4.28
4.0 L	2.65×10^5	3.97	28	8.86×10^6	3.85	41	3.30×10^3	3.91
3.5 M	2.81×10^5	3.46		——— [a]		63	0	3.50
3.5 L	3.03×10^5	3.54		——— [a]		63	6.60×10^2	3.54

[a] No sampling due to failure of growth.

From Table 6.4, it can be seen that, compared with treatment 4 L, malic acid showed no marked stimulation on the cell concentration of P. parvulus 93 in treatment 4 M. However, this strain achieved its maximal growth faster in 4 M than in 4 L. As with L. plantarum 49, the growth of P. parvulus 93 was inhibited in treatments 3.5 M and 3.5 L.

Table 6.4 also indicates that the decarboxylation of malic acid moderately increased the pH, while the pH of 4 L dropped slightly.

6.2 Carbohydrate utilization and degradation of organic acids

The results of carbohydrate analysis are presented in Tables 6.5, 6.6, 6.7 and 6.8. Some disaccharides, including maltose, lactose. melibiose, cellobiose and gentiobiose, were present at very low concentrations and were therefore impossible to quantitate accurately by the GLC method developed in Chapter 3.

The percentage change is calculated as :

$$\% \text{ degradation} = \frac{(\text{initial concentration}) - (\text{residual concentration}) \times 100}{}$$

(initial concentration)

Table 6.5 Percentage changes in some carbohydrates and organic acids during storage of the model wine inoculated with Leuconostoc oenos 122[a]

Compound	End of log phase			End of stationary phase		
	4 M	4 L	3.2 M	4 M	4 L	3.2 M
Ribose	-47	-6	0	-47	-62	-20
Rhamnose	-56	-16	-6	-69	-81	-6
Arabinose	-13	-4	-3	-26	-41	-6
Mannose	-22	0	0	-38	-81	-9
Glucose	-98	-2	-11	-100	-48	-44
Fructose	-36	-6	-2	-52	-15	-26
Galactose	-55	0	-3	-70	-48	-35
Mannitol	+51	0	0	+91	+31	+6
Sucrose	-17	-24	-30	-33	-35	-34
Trehalose	-72	-18	-22	-98	-39	-22
Malic acid	-93	-[b]	-33	-99	-[b]	-60
Citric acid	-98	-43	-53	-99	-85	-91

[a] + = increase, - = decrease or absence.
[b] Not added.

From Table 6.5, it can be seen that sugar utilization measured at the end of log phase was much reduced in the 4 L sample, compared with 4 M. This corresponded with the lower cell population produced (Table 6.1). However, by the end of stationary phase, more ribose, rhamnose, arabinose and mannose had been utilized, while the use of glucose, fructose, galactose and trehalose remained less than in 4 M. Mannitol production was observed in both 4 M and 4 L samples, but was lower in 4 L. The effect of malic acid on sugar metabolism was thus clearly evident.

Table 6.5 also shows that by the end of log phase at pH 3.2 in the presence of malic acid, the consumption of most sugars was less than

that at pH 4, though a portion of malic and citric acids were degraded. The utilization of sugars continued during the stationary phase; two thirds of the malic acid and most of the citric acid were degraded by the end of stationary phase. The production of mannitol at 3.2 M was practically insignificant. This experiment therefore shows the impact of pH on sugar metabolism and the degradation of malic and citric acids in this strain of Leuconostoc oenos.

There were no significant changes in any treatment in other carbohydrates including glycerol, erythritol, threitol, lyxose, fucose, xylose, myo-inositol, glucitol and galactitol (data not shown).

No significant changes to concentrations of carbohydrates occurred during storage of any control model wines (uninoculated), with the exception of sucrose concentration, which decreased by approximately 34% presumably through acid hydrolysis (data not presented).

Table 6.6 Percentage changes in some carbohydrates and organic acids during storage of the model wine inoculated with Leuconostoc oenos 252[a]

Compound	End of log phase			End of stationary phase		
	4 M	4 L	3.2 M	4 M	4 L	3.2 M
Ribose	-92	-30	-32	-85	-90	-42
Rhamnose	-76	-25	0	-59	-53	0
Arabinose	-4	-49	-9	-8	-86	-2
Mannose	-9	-5	-24	-66	-66	-26
Glucose	-100	-65	-88	-100	-100	-100
Fructose	-24	-13	-22	-57	-51	-26
Galactose	-36	-16	-28	-72	-48	-32
Mannitol	+33	+19	+9	+133	+62	+29
Sucrose	0	-13	-49	0	-5	-74
Trehalose	-41	-52	-49	-95	-96	-8
Malic acid	-83	-[b]	-100	-97	-[b]	-100
Citric acid	-100	-100	-100	-100	-100	-100

[a] + = increase, - = decrease.
[b] Not added.

Table 6.6 shows changes in some carbohydrates and organic acids caused by the growth of L. oenos 252. As can be seen, the trends were similar to those observed with L. oenos 122 (Table 6.5). Nonetheless, there are some differences: arabinose was hardly attacked by strain 252 in 4 M, compared with 4 L; the utilization of mannose, glucose, fructose and trehalose in strain 252 was practically unaffected by malic acid at the end of the stationary phase; whereas the catabolism of glucose by strain 252 was not influenced by pH value; the production of mannitol by strain 252 in 3.2 M was observed. It seemed that this strain degraded malic and citric acids more efficiently than strain 122, especially at low pH values.

Table 6.7 Percentage changes in some carbohydrates and organic acids during growth of L. plantarum 49 in the model wine at pH 4[a]

Compound	End of log phase		End of stationary phase	
	4 M	4 L	4 M	4 L
Ribose	-74	-82	-87	-95
Rhamnose	-100	-100	-100	-100
Arabinose	-71	-74	-100	-100
Mannose	-100	-74	-100	-100
Glucose	-100	-100	-100	-100
Fructose	-95	-95	-97	-97
Galactose	-97	-93	-100	-100
Mannitol	-100	-100	-100	-100
Sucrose	-100	-100	-100	-100
Trehalose	-80	-78	-100	-97
Malic acid	-72	___[b]	-85	___[b]
Citric aci	-66	-70	-70	-85

[a] - = decrease.
[b] Not added.

As seen in Table 6.7, L. plantarum 49 fermented a wider variety of carbohydrates than strains of L. oenos and these carbohydrates were almost entirely degraded by strain 49. For instance, mannitol and sucrose were completely catabolised by this bacterium. Other carbohydrates were also nearly totally degraded. However, malic and citric acids were not entirely catabolised. There were no significant differences in carbohydrate utilization between 4 M and 4 L in this bacterium.

There were also no major changes in other carbohydrates, including xylose and polyols. Lyxose and fucose were not detected in the initial sample, perhaps due to their low concentrations.

Table 6.8 Percentage changes in some carbohydrates and malic acid during growth of Pediococcus parvulus 93 in the model wine at pH 4[a]

Compound	End of log phase		End of stationary phase	
	4 M	4 L	4 M	4 L
Mannose	-100	-100	-100	-100
Glucose	-100	-100	-100	-100
Trehalose	-100	-100	-100	-100
Malic acid	-86	____[b]	-100	____[b]

[a] - = decrease.
[b] Not added.

As shown in Table 6.8, this bacterium attacked only mannose, glucose and trehalose and moreover, these sugars were degraded thoroughly. Other carbohydrates, including pentoses, other hexoses, sucrose and polyols were not utilised. Citric acid was not catabolised by this bacterium. The effect of malolactic fermentation on substrate utilization cannot be discerned, since the degradation of mannose, glucose and trehalose was complete both in the presence and absence of malic acid.

6.3 Comparisons of the theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation

Lactic and acetic acids are derived from the degradation of malic and citric acids and from the fermentation of carbohydrates. The quantities of lactic and acetic acids derived from the degradation of malic and citric acids are calculated according to the following equations:

1 malic acid --> 1 lactic acid + 1 CO_2

(assuming malic acid is entirely converted to lactic acid)

1 citric acid --> 1 acetic acid + 1 lactic acid + 1 CO_2

(assumimg no acetoin, diacetyl or other compounds are formed).

Fermentation balances of carbohydrates are calculated from measured carbohydrate consumption in terms of theoretical yields of lactic and acetic acids produced. The computation of theoretical yields of lactic acid and acetic acid is based on the conventional homo- and heterofermentation of carbohydrates, as follows (Moat, 1979):

homofermentation of hexoses:

1 hexose --> 2 lactic acid + 2 ATP;

Heterofermentation of hexoses (assuming no ethanol formed):

1 hexose --> 1 lactic acid + 1 acetic acid + 2 ATP + 1 CO_2;

3 fructose --> 2 mannitol + 1 lactic acid + 1 acetic acid + 2 ATP + 1 CO_2;

fermentation of pentoses:

1 pentose --> 1 lactic acid + 1 acetic acid + 2 ATP.

Rhamnose is a methyl pentose (6-deoxy hexose) and regarded as a pentose for calculation purposes, since its metabolic pathway is not clear. Disaccharides are treated according to their monosaccharide molecules.

To calculate the actual yields of lactic and acetic acids from carbohydrate fermentation, corrections must be applied to account for the breakdown of malic and citric acids:

AY of LA = (total LA) - (LA from MA) - (LA from CA);

AY of AA = (total AA) - (AA from CA);

where AY = actual yield, LA = lactic acid, AA = acetic acid, MA = malic acid and CA = citric acid.

Changes in the concentrations of tartaric acid are not presented, because of complications resulting from crystallization of potassium bitartrate. However, this should not introduce serious errors, as tartaric acid is not utilized by LAB under normal conditions found in wines (Pilone, 1989; pers. comm.).

The comparisons are presented in Tables 6.9, 6.10, 6.11 and 6.12.

Table 6.9 A comparison of the theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation by L. oenos 122

	Concentration (mg/L)											
	End of log phase						End of stationary phase					
	4 M		4 L		3.2 M		4 M		4 L		3.2 M	
	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA[a]
TY[b] from pentoses	88	58	19	13	0	0	113	76	146	100	15	10
TYHT[d]	466	311	82	54	88	33	593	396	321	214	176	98
Total TY	554	369	101	67	88	33	706	472	467	314	191	108
AY[c]	-80	96	58	111	-135	185	101	349	621	293	486	170

[a] LA = lactic acid, AA = acetic acid.
[b] TY = theoretical yield, calculated according to the equations given in section 6.3.
[c] AY = actual yield, calculated according to the equations given in section 6.3.
[d] TYHT = theoretical yield from fermentation of hexoses and trehalose.

As shown in Table 6.9, large differences between the theoretical and actual yields, in particular of lactic acid during the log phase in 4 M and 3.2 M samples, exist. The negative figure means that the measured total yield of lactic acid was smaller than the theoretical yield of lactic acid resulting from the degradation of malic and citric acids.

However, the theoretical and actual yields of lactic and acetic acids of 4 L samples were reasonably similar at the end of stationary phase, especially acetic acid, taking into account the analytical precision and undetected carbohydrates present at low concentrations.

Table 6.10 A comparison between theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation by L. oenos 252

	Concentration (mg/L)											
	End of log phase						End of stationary phase					
	4 M		4 L		3.2 M		4 M		4 L		3.2 M	
	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA[a]
TY[b] from pentoses	82	55	98	65	20	13	80	54	192	129	26	18
TYHT[d]	303	203	409	272	157	103	575	382	781	520	185	123
Total TY	385	258	507	337	177	116	655	436	973	649	211	141
AY[c]	3134	193	881	165	1519	3	3464	230	1768	314	2264	68

[a] LA = lactic acid, AA = acetic acid.
[b] TY = theoretical yield, calculated according to the equations given in section 6.3.
[c] AY = actual yield, calculated according to the equations given in section 6.3.
[d] TYHT = theoretical yield from fermentation of hexoses and trehalose.

As indicated in Table 6.10, extra amounts of lactic acid (more than expected) were produced by strain 252 in treatments 4 M and 3.2 M, in striking contrast with strain 122 (Table 6.9). There are also some differences between the theoretical and actual yields of lactic acid in 4 L. The theoretical yields of lactic acid by the end of log phase and stationary phase of 4 L were recalculated, using the homofermentative pathways. Values of 916 and 1754 mg/L were obtained, respectively, which agreed excellently with the actual yields. This suggests that strain 252

may employ the homofermentative pathways as the predominant fermentation routes at low pH values. An examination of Table 6.10 also showed that the actual yields of acetic acid were lower than the theoretical yields in all treatments. This further indicated that homofermentation could act as the predominant fermentation pathways at low pH values in this strain.

Table 6.11 A comparison between theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation by L. plantarum 49

	Concentration (mg/L)							
	End of log phase				End of stationary phase			
	4 M		4 L		4 M		4 L	
	LA	AA	LA	AA	LA	AA	LA	AA[a]
TY[b] from pentoses	133	88	142	95	174	115	181	120
TYHD[d]	1679	0	1662	0	1861	0	1844	0
Total TY	1812	88	1804	95	2035	115	2025	120
AY[c]	4230	305	1487	117	4584	625	2003	224

[a] LA = lactic acid; AA = acetic acid.
[b] TY = theoretical yield, calculated according to the equations given in section 6.3.
[c] AY = actual yield, calculated according to the equations given in section 6.3.
[d] TYHD = theoretical yield from fermentation of hexoses and trehalose.

As shown in Table 6.11, as with L. oenos 252, there were large disparities between the theoretical and actual yields of acetic acid and particularly lactic acid in treatment 4 M. However, the theoretical and actual yields of lactic acid agreed excellently in 4 L, especially at the end of stationary phase.

Table 6.12 A comparison between the theoretical and actual yields of lactic acid from carbohydrate fermentation at pH 4 by P. parvulus 93

	Concentration (mg/L)			
	End of log phase		End of stationary phase	
	Lactic acid		Lactic acid	
	4 M	4 L	4 M	4 L
TY[a]	869	1020	869	1020
AY[b]	2572	1203	2697	1233

[a] TY = theoretical yield.
[b] AY = actual yield.

As already shown by L. oenos 252 and L. plantarum 49, Table 6.12 shows that extra amounts of lactic acid were also produced by P. parvulus 93 in treatment 4 M. The theoretical and actual yields of lactic acid agreed adequately well in treatment 4 L. Acetic acid was not detected.

6.4 Changes in amino acid concentrations

The results of amino acid analysis are presented in Tables 6.13, 6.14 6.15 and 6.16. The ammonia content was estimated by the relative peak height. Only samples taken before and after MLF were assayed owing to the high cost of analyses, but meaningful results could still be gained. Cysteine was added as a reducing agent to produce a pre-reduced condition. Its loss might be due to oxidation and was not further discussed. The following amino acids listed in the formula (Chapter 4) could not be assayed because of the limitations of the Amino Acid Analyzer: asparagine, glutamine, DL-α-aminobutyric acid, tryptophane, citrulline and hydroxyproline.

Table 6.13 Changes in amino acid concentrations during growth of L. oenos 122 in the model wine

Compound	Amino acid concentration (mg/L)					
	Initial			End of stationary phase		
	4 M	4 L	3.2 M	4 M	4 L	3.2 M
Aspartic acid	61	58	54	54	49	55
Threonine	109	107	100	102	98	99
Serine	54	55	52	45	49	48
Glutamic acid	204	204	190	178	176	175
Proline	195	195	182	184	177	176
Cysteine	174	223	103	113	142	91
Glycine	10	10	10	10	10	10
Alanine	82	82	76	74	73	76
Valine	24	21	20	19	19	20
Methionine	26	26	25	19	23	21
Isoleucine	33	32	30	28	28	29
Leucine	30	30	28	23	25	26
Tyrosine	21	21	19	17	17	17
Phenylalanine	22	22	21	18	19	20
Histidine	21	19	18	17	17	18
Lysine	18	21	20	18	21	20
Arginine	330	329	306	1	177	293
Ornithine	16	19	18	236	86	18
Ammonia	5	5	5	17	10	5

As indicated in Table 6.13, appreciable decreases in arginine concentration and increases in ornithine and ammonia levels occurred in treatments 4 M and 4 L, but did not occur in treatment 3.2 M. The influence of pH on arginine metabolism was manifest. Concentrations of other amino acids declined only slightly. No significant changes in amino acid concentrations occurred in the uninoculated model wine controls.

Table 6.14 Changes in amino acid concentrations during growth of L. oenos 252 in the model wine

Compound	Amino acid concentration (mg/L)					
	Initial			End of stationary phase		
	4 M	4 L	3.2 M	4 M	4 L	3.2 M
Aspartic acid	28	46	51	28	36	44
Threonine	107	102	104	99	81	88
Serine	52	50	49	46	41	42
Glutamic acid	203	192	194	199	159	167
Proline	179	174	172	176	143	153
Cysteine	170	232	133	138	225	148
Glycine	9	9	9	12	8	9
Alanine	75	71	68	73	60	64
Valine	20	19	20	19	16	20
Methionine	24	23	21	20	18	20
Isoleucine	31	30	30	29	23	27
Leucine	29	27	28	23	21	23
Tyrosine	14	12	15	9	9	13
Phenylalanine	20	19	19	16	14	16
Histidine	19	18	19	19	16	17
Lysine	32	29	31	28	22	28
Ornithine	11	15	15	231	201	16
Arginine	327	291	297	34	5	274
Ammonia	5	5	5	29	25	5

Table 6.14 presents changes in amino acid concentrations during the growth of strain 252 in the model wine. It can be seen that strain 252 metabolised amino acids in a way similar to that of strain 122 (Table 6.13). However, the presence of malic acid did not affect the catabolism of arginine by strain 252 and this corresponds with the similar cell population achieved in treatments 4 M and 4 L. The effect of pH on arginine degradation and ornithine production was also demonstrated.

Table 6.15 Changes in amino acid concentrations during growth of L. plantarum 49 in the model wine

Compound	Amino acid concentration (mg/L)			
	Initial		End of stationary phase	
	4 M	4 L	4 M	4 L
Aspartic acid	34	35	25	55
Threonine	119	116	86	87
Serine	57	54	47	46
Glutamic acid	209	203	179	194
Proline	247	232	168	164
Cysteine	248	264	197	149
Glycine	10	10	8	9
Alanine	75	72	57	62
Valine	20	21	15	15
Methionine	24	22	20	21
Isoleucine	31	29	22	24
Leucine	28	27	19	22
Tyrosine	16	21	2	7
Phenylalanine	21	20	2	9
Histidine	19	18	16	17
Lysine	21	20	21	19
Arginine	311	299	288	290
Ornithine	19	18	19	17

As indicated in Table 6.15, tyrosine and phenylalanine were almost totally degraded in treatment 4 M and were catabolised by more than 50% in treatment 4 L. Concentrations of other amino acids decreased slightly. Noteworthy was the decline in the proline level. The decrease in arginine concentration was not considered significant. The influence of malolactic fermentation on amino acid metabolism was therefore not clear, except perhaps for tyrosine and phenylalanine.

Table 6.16 Changes in amino acid concentrations during growth of P. parvulus 93 in the model wine

Compound	Amino acid concentration (mg/L)			
	Initial		End of stationary phase	
	4 M	4 L	4 M	4 L
Aspartic acid	23	24	33	33
Threonine	74	70	64	66
Serine	39	40	36	38
Glutamic acid	147	145	130	138
Proline	137	132	127	129
Cysteine	153	171	121	201
Glycine	14	15	12	13
Alanine	57	57	54	56
Valine	15	15	12	13
Methionine	19	18	15	17
Isoleucine	23	23	17	19
Leucine	24	24	18	20
Tyrosine	15	15	11	14
Phenylalanine	14	14	10	14
Histidine	14	14	13	13
Ornithine	9	9	9	8
Lysine	22	22	16	19
Arginine	228	226	219	217

Table 6.16 indicates that concentrations of all amino acids did not vary greatly in treatments 4 M and 4 L, except in the case of cysteine, which was a reducing agent.

6.5 Growth of L. oenos 122 and 252, L. plantarum 49 and P. parvulus 93 in the model wine with single carbon/energy sources

Cells of these bacteria were inoculated into model wines containing single carbon/energy sources. The purposes and procedures of these experiments have been given in Chapter 4.

The following notations were used to describe the status of growth:

- = no growth;

+ = weak/slight growth;

++ = medium growth;

+++ = heavy growth;

++++ = very heavy growth.

Growth was judged visually by turbidity changes. Tables 6.17 and 6.18 show their growth status.

Table 6.17 Growth of L. oenos 122 and 252 in the model wine (4 M) with single carbon/energy sources

Carbon/energy source	Preculturing I		Preculturing II		Model wine	
	122	252	122	252	122	252
Malic acid	-	-				
Citric acid[a]	-	-				
Arginine[a]	-	+++		-		
Glycerol	-	-				
Arabinose	+++	+++	+++	+++	++	++
Arabitol	-	-				
Glucose	+++	+++	++	++	++	++
Fructose	++	++	+	+	-	-
Myo-inositol	-	-				
Mannitol	-	-				
Trehalose	+++	+++	++	++	++	++

[a] without malic acid, the others with malic acid.

From Table 6.17 it can be seen that neither could use malic acid, citric acid or arginine as the sole source of carbon and energy. However, these substances were degraded in the complete model wine system. Although fructose reduction was noticed in the complete model wine, it could not be used as a sole carbon/energy source by either strain. Strains 122 and 252 failed to grow on glycerol, mannitol, arabitol with malic acid, but they grew well on glucose and trehalose with malic acid, and in the complete model wine, in which malic acid was degraded. These results

suggest that the degradation of malic acid, citric acid and arginine by these strains probably requires, or may be induced by the presence of other factors, such as fermentable sugars in the complete model wine.

Table 6.18 Growth of L. plantarum 49 and P. parvulus 93 in the model wine (4 M) with single carbon /energy sources

Carbon/ energy source	Preculturing I		Preculturing II		Model wine	
	49	93	49	93	49	93
Malic acid	-	-				
Citric acid[a]	-	-				
Arginine[a]	-	-				
Glycerol	+	-	-			
Arabinose	++	-	++		+	
Arabitol	++	-	-			
Glucose	++++	++	+++	++	++	+
Fructose	++	-	++		++	
Myo-inositol	-	-				
Mannitol	++++	-	+++		+++	
Trehalose	++++	+++	++++	++	++	+

[a] without malic acid, the others with malic acid.

It can be seen from Table 6.18 that, like strains 122 and 252, neither grew on malic acid, citric acid or arginine, nor grew on glycerol, arabitol and myo-inositol as sole carbon/energy sources. However, both strains grew on glucose and trehalose as sole carbon/energy sources. L. plantarum 49 grew vigorously on mannitol as a sole carbon/energy source. P. parvulus 93 did not grow on fructose. These results agree with those of strains 122 and 252 (Table 6.17) and support the information gained from the complete model wine.

6.6 Discussion of results

6.6.1 Effects of malolactic fermentation on bacterial growth and the corresponding pH changes

This study demonstrates that the presence of malic acid had a stimulatory effect on the growth rate and cell concentration of L. oenos 122 (Table 6.1) and the effect was more striking at low pH values. However, this stimulation was not the result of the pH increases resulting from the decarboxylation of malic acid, since a higher cell population and faster growth rate were attained in treatment 3.2 M than in 4 L. This finding agrees with the report of Pilone and Kunkee (1976). The stimulatory effect of malic acid on growth of strain 252 differed slightly from that of strain 122, since a higher cell concentration of strain 252 was achieved in 4 L than in 4 M (Table 6.2). This may be attributed to the low inoculum of strain 252. Nonetheless, strain 252 grew faster in 4 M than in 4 L. The inability of strains 122 and 252 to grow in the absence of malic acid at low pH values suggests that wine may be stabilized after MLF by lowering pH to a level dependent on the wine type.

This study also shows that ethanol had a determinative effect on the growth of L. plantarum 49 and P. parvulus 93, as demonstrated in treatments 3.5 M and 3.5 L (Tables 6.3 and 6.4). These two bacteria were precultured in 3.5 M without ethanol (Chapter 4) and good growth was achieved. The inhibitory effects of 10% ethanol on these bacteria were thus clearly shown. The study therefore suggests that a wine of pH 3.5 or below may prevent the growth of spoilage bacteria like strains of lactobacilli and pediococci. It also suggests that strains of lactobacilli and pediococci may not be appropriate for conducting malolactic fermentation in wine, since they can not grow at pH 3.5 with 10% ethanol. Moreover, induction of MLF before the completion of alcoholic fermentation may permit growth of lactobacilli and pediococci,

since the ethanol level may not be high enough and/or pH may not be low enough to inhibit them. The use of L. plantarum 49 to carry out malolactic fermentation in wines of higher pH values may suffer from the risk of pH reduction and spoilage, since large amounts of carbohydrates can be utilised by this strain (Table 6.7).

Generally, malolactic fermentation raises the pH, as a result of the decarboxylation of malic acid. However, changes in pH are also influenced by other factors, such as fermentation of carbohydrates and catabolism of arginine. The fermentation of carbohydrates tends to lower pH, as shown in L. plantarum 49 (Table 6.3), while arginine degradation may extend to deamination which increases pH as ammonia is released. In this study, it was found that the increases in pH were larger in treatment 4 M than 3.2 M in the case of L. oenos 122 and 252 (Table 6.1 and 6.2). A similar phenomenon has been reported by Bousbouras and Kunkee (1971), who regarded it as resulting from the buffering capacity of the major organic acids present in wine. Taking into consideration the fermentation of carbohydrates, the degradation of arginine, and pH changes in all the treatments, as a generalization it can be stated that ammonia released from arginine catabolism contributed significantly to the pH increases in treatment 4 M. This conclusion is strongly supported by the increases in pH as a result of complete decarboxylation of malic acid by P. parvulus 93, which did not degrade arginine (Table 6.4 and Table 6.16).

6.6.2 Growth substrates

Generally, this work confirms that sugars are the main growth substrates for L. oenos 122 and 252. The particular sugars involved vary, depending

on the pH and the presence or absence of malic acid. Glucose and trehalose were preferred in treatment 4 M. Not all sugars were fermentable. For example, fucose, lyxose and xylose were not fermented. Polyols were not fermented. The decrease in sucrose concentration was not caused by bacterial growth, but through acid hydrolysis.

Malic acid, citric acid and arginine can not act as sole carbon/energy sources. Their breakdown in the complete model wine system may require, or induced by the presence of a fermentable substrate. This is supported by the fact that the presence of malic acid together with an unfermentable substrate (e.g., glycerol or arabitol) as the sole carbon/energy source did not permit the growth of the bacteria.

A decline in fructose concentration and gain in mannitol content were noted (Tables 6.5 and 6.6). But fructose may not be used a direct energy/carbon source. It is known that fructose can be reduced to mannitol by acting as a hydrogen acceptor and this is characteristic of heterofermentative lactic acid bacteria (Kunkee, 1967; Sharp, 1981). The reduction of fructose to mannitol is accompanied by a fall in levels of ethanol and formation of excess acetic acid (Eltz and Vandemark, 1960), since acetyl phosphate is converted to acetic acid, instead of ethanol, with the generation of one mole of ATP. Although the ATP is energetically important to the bacteria, this conversion may exert an adverse impact on wine quality, owing to the excess acetic acid (volatile acidity) produced.

As with strains 122 and 252 of L. oenos, the current study also show that sugars were the main growth substrates for L. plantarum 49 and P.

parvulus 93. Polyols did not serve as growth substrates, except in the case of mannitol, which acted as a growth substrate for L. plantarum 49. The two bacteria (strains 49 and 93) differed vastly in the pattern of carbohydrate utilization: strain 49 fermented a wide range of carbohydrates while strain 93 attacked only mannose, glucose and trehalose (Tables 6.7 and 6.8).

Results using the model wine containing single carbon/energy sources correlated well with the findings from the complete model wine in all the strains tested.

6.6.3 Effects of malolactic fermentation on substrate utilization

The present study shows that malolactic fermentation had a marked influence on substrate utilization by leuconostocs. In the main, malolactic fermentation resulted in an increased utilization of sugars (Tables 6.5 and 6.6), particularly in the case of strain 122. This could be due to the higher cell concentration achieved in the presence of malic acid. The utilization of glucose and trehalose by strain 252 was not affected by malic acid.

Unlike L. oenos 122 and 252, the present findings did not indicate the effects of malolactic fermentation on the utilization of carbohydrates by L. plantarum 49 and P. parvulus 93, since the types and quantities of carbohydrates utilized were very similar or identical both in the presence and absence of malic acid (Tables 6.7 and 6.8).

6.6.4 Effects of pH on substrate utilization and degradation of malic and citric acids

In general, the pH had a highly significant impact on substrate utilization. At pH values below 3.5, sugars were scarcely attacked, except for glucose, which was the main substrate. However, pH did not affect glucose utilization by strain 252.

The degradation of malic and citric acids by strain 122 was influenced greatly by pH. Upon reaching the maximum growth, only a portion of malic and citric acids had been degraded, while hardly any sugars were utilised. This suggests that the degradation of malic and citric acids required the presence of fermentable sugars, but these sugars may not necessarily be utilized or only small amounts of sugars may be required. Moreover, the degradation of malic acid by strain 122 was incomplete at low pH. This may result in poor deacidification and microbiological instability. On the other hand, the degradation of malic and citric acids by strain 252 was unrelated to pH, which is useful for conducting malolactic fermentation at low pH values.

6.6.5 Formation of biogenic amines and arginine catabolism

The present study indicates that L. oenos 122 and 252 did not greatly reduce the concentrations of amino acids (including histidine, tyrosine and phenylalanine), except for arginine. This implies that neither strain would be a potential amine former (histamine, tyramine and phenylethylamine), which is oenologically desirable because of the medical significance of these amines as reviewed in Chapter 2 (section 2.2.3).

The role of arginine degradation can be regarded as ATP generation, with the formation of ornithine and ammonia, as discussed in the Literature

Review (section 2.2.3). The ammonia released may have contributed to the pH rises, as discussed in section 6.6.1. This work shows that the catabolism of arginine is affected by pH. Arginine was not catabolised at pH values below 3.5, thereby, it will not play any role in the bacteria at low pH values. The significance of this finding is that the role and effect of arginine catabolism on wine quality can be ignored in practice, since malolactic fermentation is commercially conducted in wines of pH below 3.5 (Eggenberger, 1988).

This study shows that L. plantarum 49 can be a potential producer of tyramine and phenylethylamine, because it markedly decreased the concentrations of tyrosine and phenylalanine (Table 6.15). It is highly possible that tyrosine and phenylalanine are decarboxylated into the corresponding amines, tyramine and phenylethylamine, respectively. In this regard, this bacterium is not appropriate as a starter culture. However, more studies are required to determine the presence and concentrations of these amines in finished wines, and to analyse the potential decarboxylases involved. P. parvulus 93 is not a potential former of amines, since it did not appreciably catabolise any amino acids (Table 6.16).

This work also shows that arginine was not degraded by either L. plantarum 49 and P. parvulus 93. This suggests that arginine catabolism would not play any role in the two bacteria, in contrast with strains of L. oenos. It was also noted that L. plantarum 49 reduced the proline concentration significantly. Yet the metabolism of proline and its significance in wine LAB are unknown.

6.6.6 The energetics of malolactic fermentation

Although the phenomenon of malolactic fermentation has been known for many years, the reasons for its occurrence are still unclear. This study may throw some light on the role and energetics of MLF.

Results of this work show that negative amounts of lactic acid were obtained in L. oenos 122, while L. oenos 252, L. plantarum 49 and P. parvulus 93 produced significantly higher amounts of lactic acid than expected. Obviously their metabolic mechanisms differed. Therefore, discussions on these strains will be treated separately.

6.6.6.1 The energetics of Leuconostoc oenos 122

The negative actual yield of lactic acid can be accounted for by the theory of pyruvate cleavage (Figure 6.1).

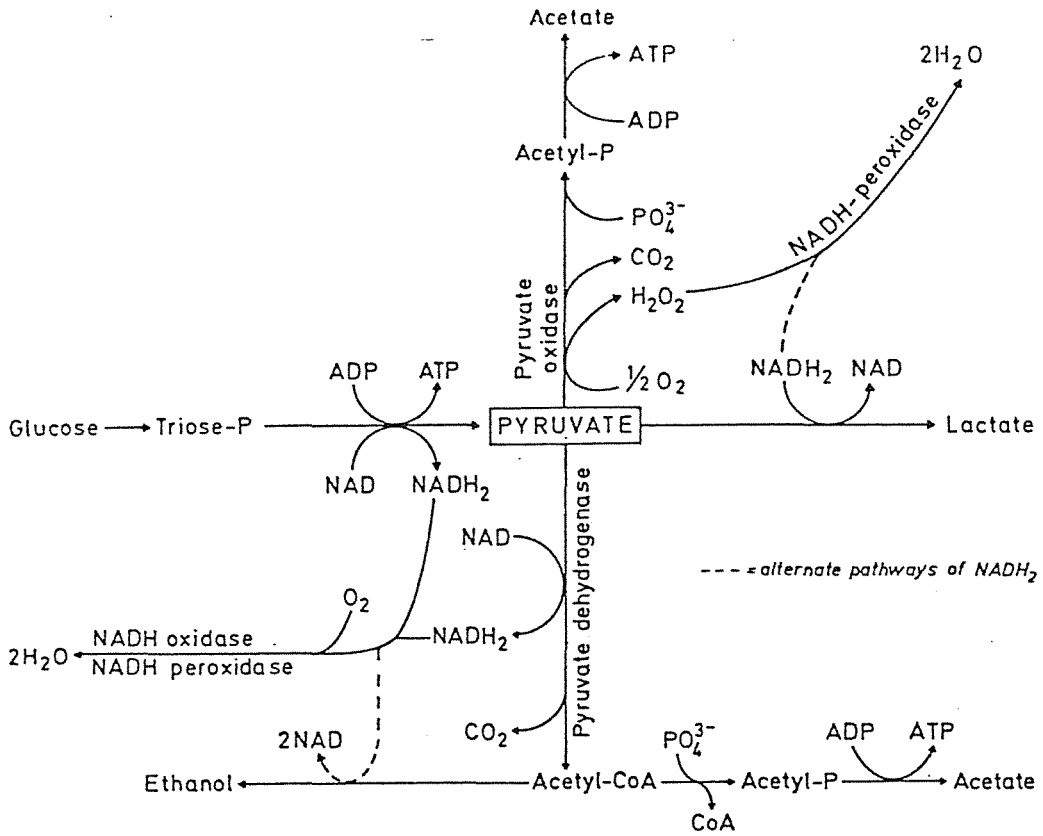

Figure 6.1 Hypothetical scheme of pyruvate dissimilation (after Kandler, 1983).

It is known that cleavage of pyruvic acid by oxidation or phosphoroclastic reaction can produce extra ATP in certain microorganisms (Gunsalus and Shuster, 1961; Kunkee, 1967; Sokatch, 1969; Kandler, 1983). Pyruvic acid is oxidized to acetic acid and carbon dioxide via acetyl phosphate. The oxidation of pyruvic acid is an exergonic reaction and thus, can serve to increase the yield of ATP from hexose during fermentation. The phosphoroclastic reaction is a decomposition of pyruvic acid to acetyl phosphate and either formic acid, or hydrogen and carbon dioxide through inorganic phosphate. It is highly possible that pyruvic acid resulting from the degradation of malic and/or citric acids and/or carbohydrate fermentation, instead of being reduced to lactic acid, was cleaved during MLF to increase yields of ATP. If this hypothesis were verified experimentally, i.e., via analysis of enzymes and intermediates involved, the biological importance for MLF in strains of this type can be elucidated. It is recognized that some free pyruvic acid is evolved from malic and citric acid degradation (Morenzoni, 1974; Kunkee; 1975). This theory of cleavage of pyruvic acid can satisfactorily explain the negative actual yield of lactic acid and establish the energetics of malolactic fermentation. However, this theory needs to be supported experimentally by analysis of enzymes and intermediates to show what the cleavage route of pyruvic acid is, and the fate of acetyl phosphate resulting from cleavage of pyruvic acid. The diversion of acetyl phosphate to ethanol by regulation of the metabolism of the perspective strains is oenologically desirable.

According to Lafon-Lafourcade et al (1980), the formation of acetic acid during fermentation of sugars by lactic acid bacteria is specifically

linked to the physiological state of the bacterial populations; it is low during the cell multiplication phase, during which malic and citric acids are possibly degraded; the presence of malic acid in wine ensures limited acetic acid formation. The formation of acetic acid is complicated by several probable factors: 1) a change from heterofermentation to homofermentation, which reduces acetic acid formation; 2) the degradation of pyruvic acid resulting from the catabolism of malic and citric acids, which promotes acetic acid formation; 3) the reduction of acetyl phosphate to ethanol and 4) acetyl phosphate could have been incorporated into the synthesis of cell materials such as lipids, as with Streptococcus diacetilactis (Harvey and Collins, 1963). Consequently, it is difficult to make any generalizations about the acetic acid formation.

6.6.6.2 The energetics of L. oenos 252, L. plantarum 49 and P. parvulus 93

The pyruvic acid cleavage theory is obviously unable to explain the extra lactic acid generated by these strains. However, the chemiosmotic energy coupling theory (Renault et al, 1988; Cox and Henick-Kling, 1989) is apparently able to explain the biological function of the extra lactic acid. The chemiosmotic theory, as proposed by Mitchell (1966, 1972), postulates that metabolic energy (ATP) could be generated by reversal of the ATPase reaction via translocation of protons through the cell membrane. Mitcels et al (1979) extended the chemiosmotic hypothesis by proposing that carrier-mediated efflux of metabolic end products (e.g., lactic acid) can lead to the generation of an electrochemical proton gradient across the bacterial cytoplasmic membrane. Assuming that this postulate holds true for these bacteria, the question is, where did

the extra lactic acid come from? The consistent occurrence of the extra lactic acid excludes the possibility of analytical errors. The finding of the extra lactic acid produced by these bacteria is supported by the adequate agreement between the theoretical and actual yields of lactic acid in the absence of malic acid (Tables 6.10, 6.11 and 6.12). Fermentation of undetected disaccharides still can not explain the extra lactic acid, since these disaccharides were present at very low concentrations (Chapter 4). Homofermentation of hexoses cannot account for the extra lactic acid, because calculations indicate that L. oenos 252 might even use the homofermentative pathways as the predominant fermentation routes at pH 4 in the absence of malic acid and strains 49 and 93 are both homofermentative. How did malic acid relate to the extra lactic acid? Would the presence of malic acid induce the biosynthesis of lactic acid from CO_2 or other carbon sources? These questions remain to be answered.

Chapter Seven Results and Discussion
Growth and Metabolism of Lactobacillus plantarum 49 in the Modified MRS Medium

L. plantarum 49 was the only bacterium tested for growth and metabolism in the modified MRS medium, owing to the limited time. The intention was to obtain an indication of how a nutritionally-rich medium of higher pH value would affect the growth and metabolism of lactic acid bacteria by comparing growth and metabolism in this medium with that in a purely synthetic medium of lower pH value, such as the model wine system described in Chapter 4.

7.1 Cell concentration and pH changes

The cell concentration (cfu/ml) of this bacterium and the corresponding pH changes of the medium are presented in Table 7.1.

Table 7.1 The cell concentration changes of L. plantarum 49 inoculated into the modified MRS medium and the corresponding pH changes of the medium

	Initial	Day 1	Day 2
Cell concentration	1.42×10^6	1.80×10^9	2.07×10^9
pH	5.91	4.08	3.57

The abundant growth of this bacterium in the modified MRS medium correlated with the remarkable decline in pH value (Table 7.1). The major reduction of pH occurred after day 1.

7.2 Carbohydrate utilization by L. plantarum 49 in the modified MRS medium

Carbohydrates were assayed using the GLC method described in Chapter 3. However, 400 ul chloroform was added to the derivatives (instead of 200 ul) to dilute the carbohydrate derivatives before injection. Internal standard 2 (phenyl-β-D-glucoside) (see Chapter 3) was used to calculate the carbohydrate concentrations, because the peak of the internal standard 1 (D-glucoheptose) was too small to be accurate after dilution.

Table 7.2 Changes in carbohydrate concentrations during growth of Lactobacillus plantarum 49 in the modified MRS medium[a]

	Carbohydrate concentration (mg/L)[b]				
Compound	Initial	Day 1	Changes%	Day 3	Changes %
Ribose	833	739	-11	160	-81
Rhamnose	615	454	-26	191	-67
Lyxose	261	235	-10	112	-57
Arabinose	2600	2211	-15	763	-71
Fucose	82	86	0	18	-78
Xylose	1048	963	-8	663	-37
Mannose	1145	853	-26	0	-100
Glucose	6010	364	-94	0	-100
Fructose	6600	4070	-38	870	-87
Galactose	2270	1818	-20	1150	-49
Mannitol	2118	1088	-49	825	-61
Sucrose	1290	1318	+2	1371	+6
Trehalose	7698	6338	-18	5096	-34

[a] + = increase; - = decrease.
[b] All the concentrations were calculated using internal standard 2 (phenyl-β-D-glucoside) (see Chapter 3).

Table 7.2 shows the utilization of carbohydrates by L. plantarum 49 in the modified MRS medium. As can be seen, the majority of the sugars were used. However, polyols, except for mannitol, were not attacked (data not shown). Xylose, which was not utilised by this strain in the model wine at pH 4 with or without malic acid, was attacked in the modified MRS medium. Trehalose, which was preferred by this strain in the same model wine, was degraded moderately. Sucrose, which was completely degraded by this strain in the same model wine, was not catabolised. The small increase in sucrose concentration was within the allowable ranges of analytical precision. This strain showed a preferential utilization of

glucose and mannitol during the early stage of growth (after day 1) and degraded mannose and fructose in preference over other sugars during the later stage of growth and maintenance (after day 3). The degradation of most carbohydrates occurred mainly between day 1 and day 3 with the exception of glucose and mannitol.

7.3 A comparison of the theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation by L. plantarum 49 in the modified MRS medium

The approach to calculating the theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation has been described in section 6.3. The comparison is presented in Table 7.3

Table 7.3 A comparison between the theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation in the modified MRS medium by L. plantarum 49

	Organic acid concentration (g/L)			
	Day 1		Day 3	
Compound	Lactic	acetic	Lactic	acetic
TY[a] from pentoses	0.444	0.297	2.094	1.397
TY from hexoses and disaccharides	11.381	____[c]	18.037	____[c]
Total TY	11.825	0.297	20.131	1.397
AY[b]	10.182	0.579	24.172	1.880

[a] TY = theoretical yield, calculated in accordance with the equations given in section 6.3.
[b] AY = actual yield, calculated using the equations given in section 6.3.
[c] theoretically absent.

As can be seen in Table 7.3, the theoretical and actual yields of lactic acid after day 1 agree closely. This is in accordance with the homofermentative property of the strain. The agreement between the theoretical and actual yields of lactic acid after day 3 is considered adequate, taking into account the undefined nature of the modified MRS

medium, which may also explain the higher than expected acetic acid concentration.

7.4 Discussion of results

This experiment demonstrates the influence of a rich medium on the utilization of carbohydrates in P. plantarum 49. As discussed in section 6.2, this strain did not use xylose at pH 4 in the model wine, but used it in the modified MRS medium; this strain degraded sucrose completely at pH 4 in the model wine but did not attack sucrose in the modified MRS medium; this strain preferred trehalose at pH 4 in the model wine but did not prefer it in the rich medium. These results confirm that information obtained from a rich medium cannot be applied directly to a harsh medium such as wines of low pH. This can also justify the use of the model wine system to study the physiology and metabolism of lactic acid bacteria isolated from wines.

The differences in the utilization of carbohydrates (particularly sucrose and xylose) by this strain were quite significant and could be caused by high pH and/or rich nutrients of the modified MRS medium. But it is impossible to show from these experiments which factor is more important, since both factors differ from those of the model wine system. Probably a combination of both factors affect the utilization of carbohydrates in this strain.

The good agreement between the theoretical and actual yields of lactic acid resulting from carbohydrate fermentation in the modified MRS medium and in the model wine without malic acid (section 6.3) indicates that this strain uses the established homofermentative pathway in the absence

of malic acid. However, the homofermentative pathway cannot account for the extra lactic acid produced by this strain in the model wine with malic acid (section 6.3). This suggests that other metabolic pathways may function in this strain in the presence of malic acid, which may also be applied to L. oenos 252 and P. parvulus 93.

Chapter Eight Results and Discussion
Growth and Metabolism of *Leuconostoc oenos* 122 in a Red Wine at Different pH Values

Previous results (Chapters 6) showed a good correlation between the complete model wine and the model wine with single carbon/energy sources. This chapter presents results using a real wine, so that the correlation between the model wine and the real wine and hence, the suitability of the model wine as a culture medium to study the physiology and metabolism of lactic acid bacteria could be assessed. Only *L. oenos* 122 was tested because of the limited time.

8.1 Cell population and pH changes

The cell population (cfu/ml) changes of *L. oenos* 122 grown in the wine (Merlot, 1989, Hawkes Bay) at pH 3.42 and 3.80 and the corresponding pH changes of the wine are presented in Figure 8.1.

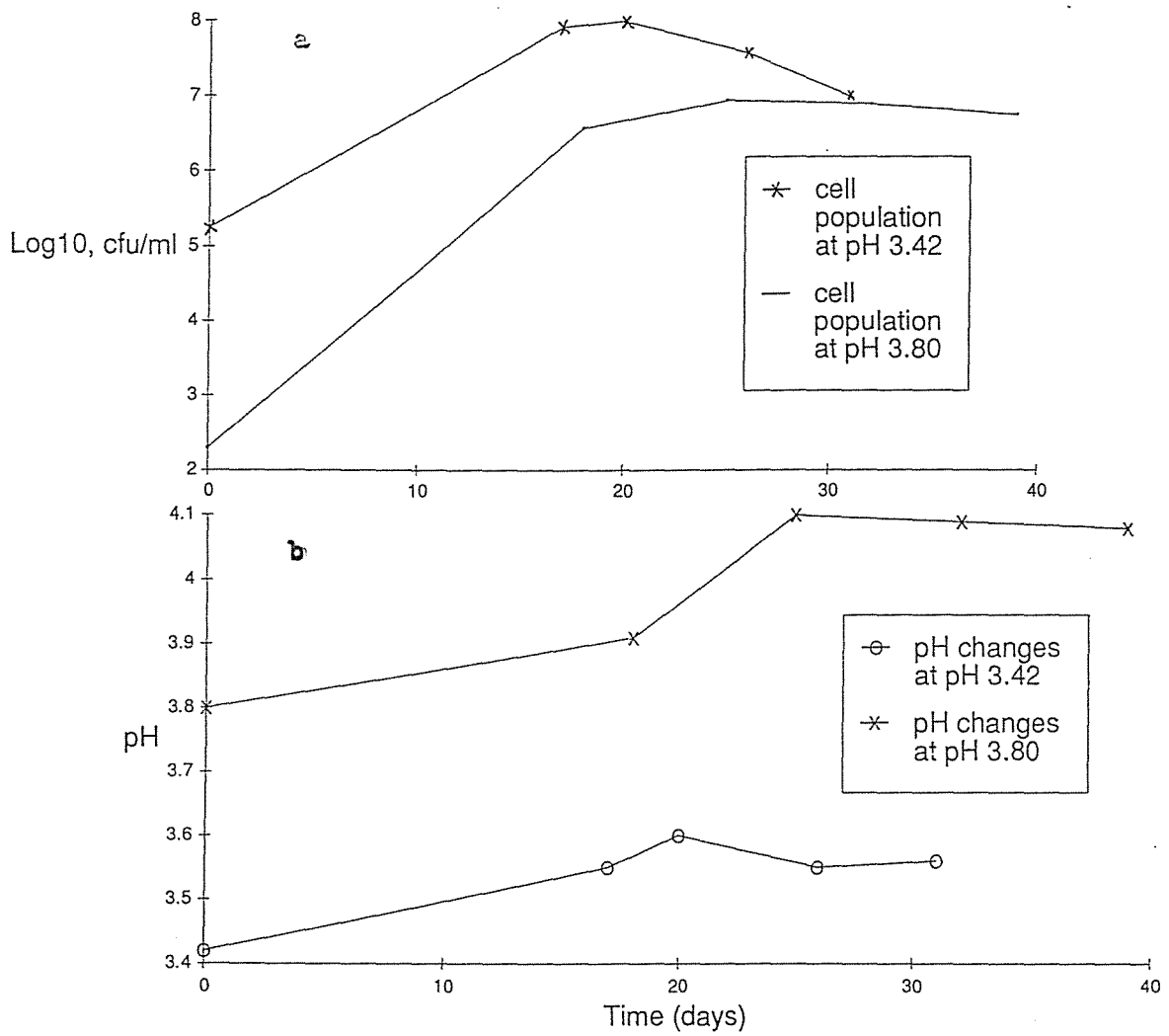

Figure 8.1 The cell population changes of L. oenos 122 and the corresponding pH changes of the wine at pH 3.42 and 3.80

It can be seen from this plot (Figure 8.1a) that the maximum cell population achieved at pH 3.42 (9.73×10^7 cfu/ml) in 20 days was approximately 10 times higher than that at pH 3.80 (8.63×10^6 cfu/ml) in 25 days. However, because the initial level of pH 3.80 was only 200 cfu/ml, growth in this sample was actually more extensive. The growth pattern in the wine also varied at different pH values. After the maximum growth had been reached, the cell population fell more rapidly at pH 3.42 than at pH 3.80.

As shown in Figure 8.1b, in both samples, the pH rose and the maximum rate of change occurred soon after the maximum cell population was attained. There was a slight decrease in pH thereafter.

8.2 Changes in carbohydrate concentrations during growth of L. oenos 122 in wine

GLC analyses indicated that this red wine contained a variety of carbohydrates as follows: glycerol, erythritol, ribose, rhamnose, arabinose, ribitol, arabitol, fructose, galactose, myo-inositol, mannitol, glucitol, sucrose and trehalose. Xylose and mannose were not detected. Consistent results could not be obtained for glycerol and erythritol, possibly because the high level of glycerol caused its incomplete derivatization, and unknown compounds sometimes interfered with the analysis of erythritol.

8.2.1 Carbohydrate changes at pH 3.42

Changes in carbohydrate concentrations of the wine at pH 3.42 are presented in Figure 8.2.

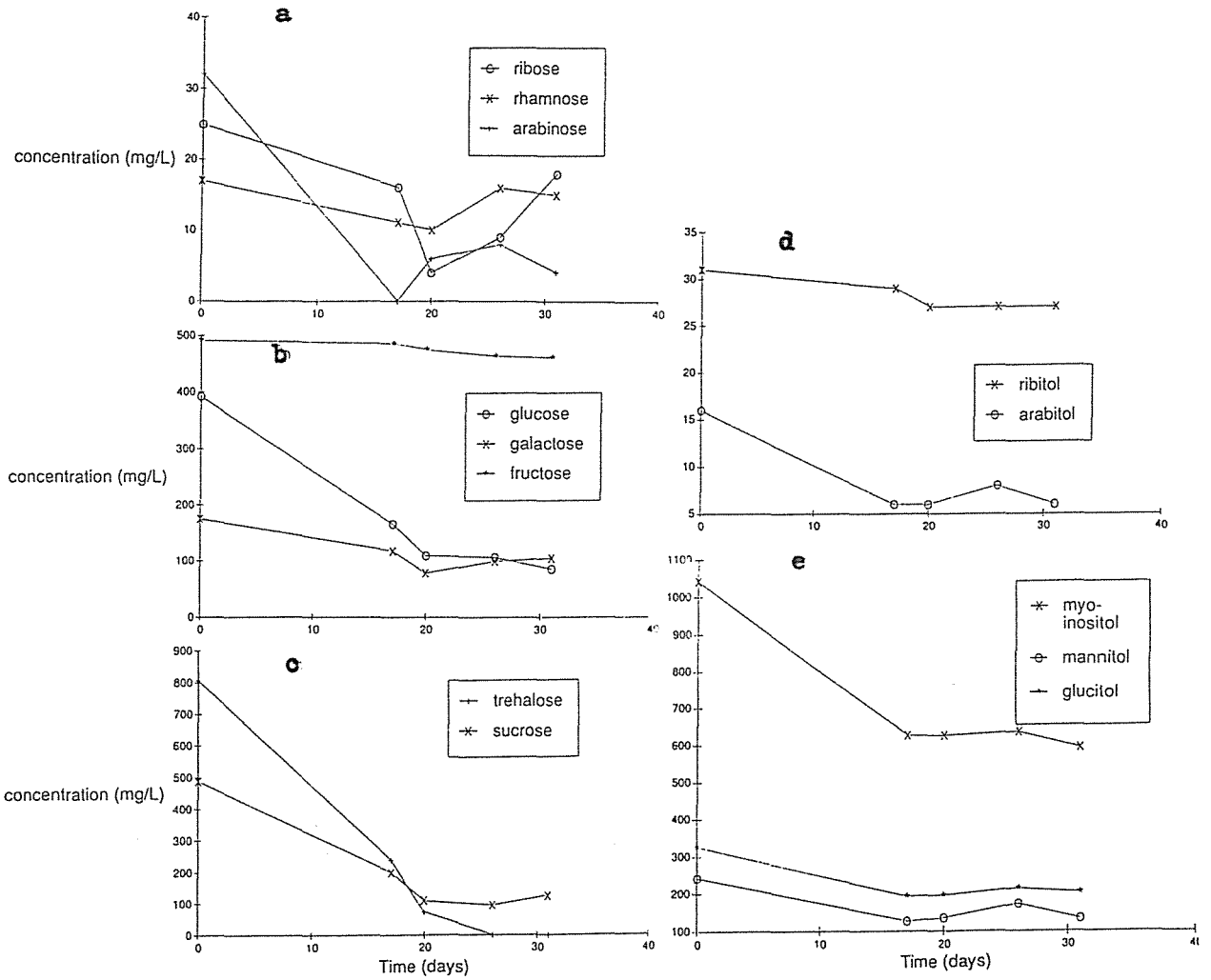

Figure 8.2 Changes in carbohydrate concentrations during growth of L. oenos 122 in wine at pH 3.42 (Scales are different.)

Figure 8.2a shows that ribose and rhamnose concentrations declined quickly after the maximum cell population was achieved (17 to 20 days). Arabinose was undetectable after 17 days. Then, these sugars increased slightly in concentrations, possibly due to the hydrolysis of polymers such as arabans and other pentosans. The glucose level (Figure 8.2b) fell from 400 mg/L to about 100 mg/L, and the majority of this reduction occurring during growth (before 17 to 20 days). There was a slight fall in glucose level after the growth phase. The galactose concentration decreased during the growth period, but increased slightly thereafter. The fructose content dropped only very slightly throughout the incubation. Most of the trehalose (Figure 8.2c) was catabolized in 20 days, coinciding with the maximum cell population achieved during that period. At 26 days, trehalose was completely degraded. However, the sucrose level also fell appreciably from approximately 500 mg/L to about 100 mg/L. A reduction in sucrose concentration also took place in the wine at pH 2.92, though there was no occurrence of bacterial growth.

The ribitol level (Figure 8.2d) stayed basically unaltered within the precision of the assay. There was a marked variation for arabitol, of which the concentration was about 15 mg/L. The concentrations of myo-inositol, mannitol and glucitol (Figure 8.2e) were reduced by the 17^{th} day and after that, myo-inositol remained almost constant. The mannitol level rose slightly, which concurred with the slight decline in the fructose level. Glucitol content stayed nearly unchanged.

8.2.2 Carbohydrate changes at pH 3.80

Changes in carbohydrate concentrations of the wine at pH 3.80 are presented in Figure 8.3.

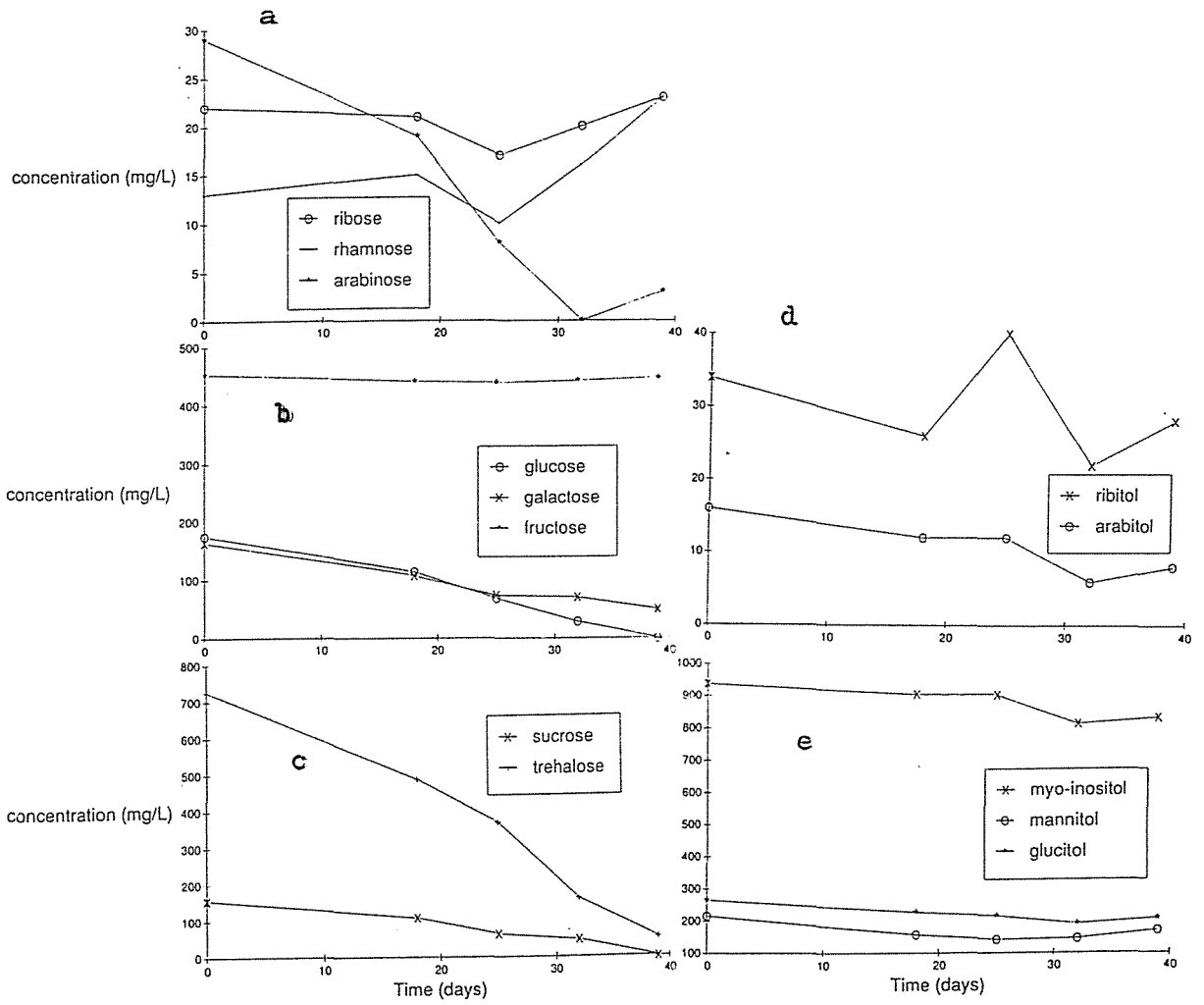

Figure 8.3 Changes in carbohydrate concentrations during growth of L. oenos 122 in wine at pH 3.80 (Scales are different.)

As with the samples at pH 3.42, the concentrations of ribose and rhamnose at pH 3.80 decreased, as the cell population peaked in 25 days and then increased (Figure 8.3a). The arabinose level fell as at pH 3.42. It can be seen from Figure 8.3b that the glucose level declined continuously and completely from about 190 mg/L to 0 mg/L in 39 days. The galactose level fell, the major change occurring during the growth phase. The fructose concentration remained fundamentally unchanged. The trehalose level fell from 730 mg/L to 50 mg/L in 39 days (Figure 8.3c). This has occurred throughout the incubation period. The sucrose concentration also decreased from the initial 150 mg/L to be undetectable in 39 days.

The concentrations of the pentitols fluctuated appreciably (Fig 8.3d). However, because of the low concentrations present initially, some of the fluctuation could be explained by analytical errors. Slight decreases were recorded for myo-inositol, glucitol and mannitol.

8.3 Changes in organic acid concentrations during growth of L. oenos 122 in the wine at pH 3.42 and pH 3.80

The general trends of changes in organic acid concentrations at pH 3.42 and pH 3.80 are presented in Figure 8.4.

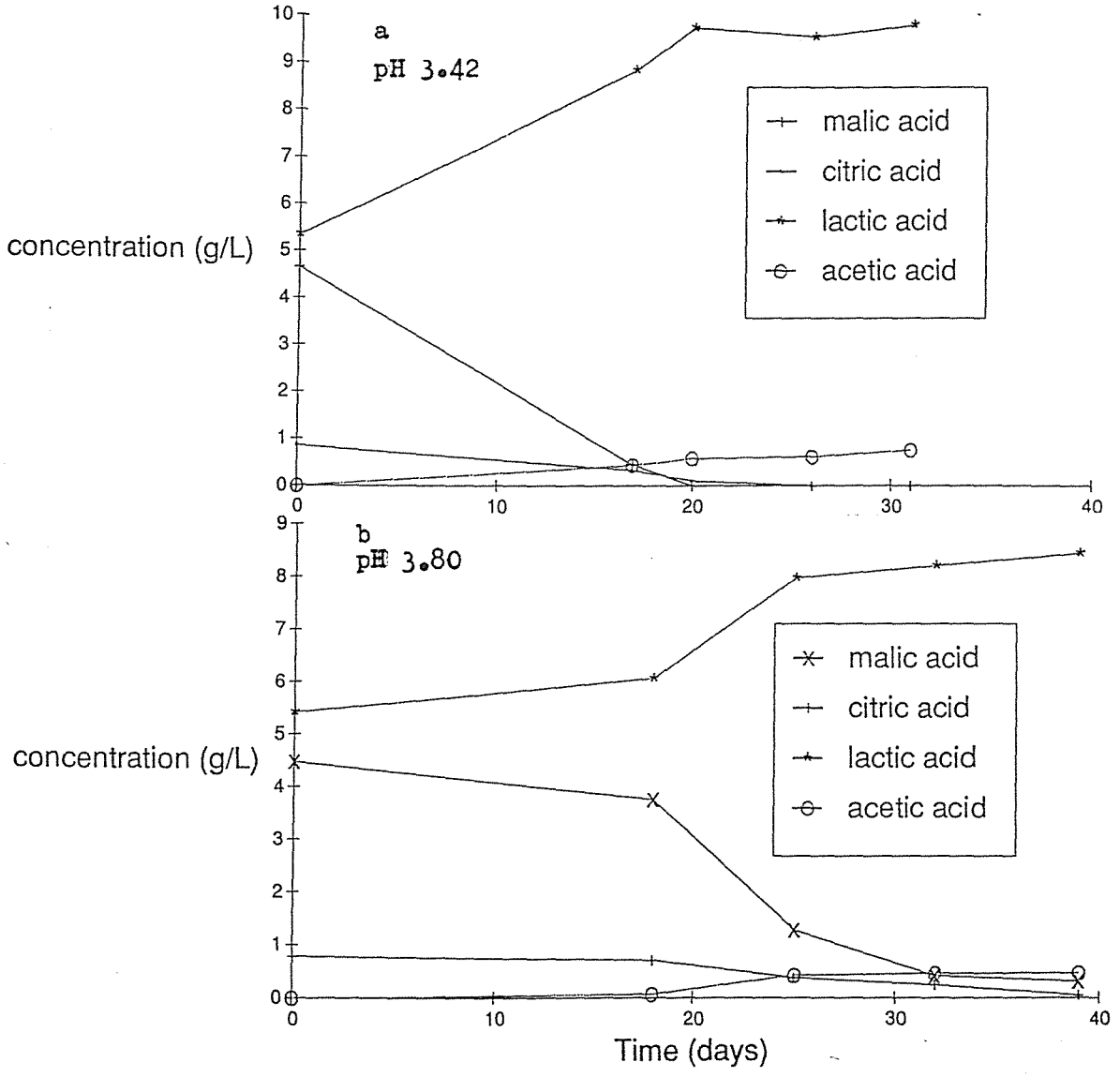

Figure 8.4 Changes in organic acid concentrations during growth of L. oenos 122 in wine at pH 3.42 and pH 3.80

As shown by Figure 8.4a, malic acid was totally degraded at pH 3.42 in 20 days, coninciding with the development of the population. As the malic acid was consumed, lactic acid was produced until growth ceased. Citric acid was degraded during growth and it was undetectable at 26 days. The acetic acid level rose steadily during growth (in 20 days), but continued to rise after growth ceased.

Figure 8.4b shows similar trends in organic acid concentrations at pH 3.80. Malic and citric acids were catabolised and lactic and acetic acids were formed. However, malic and citric acid degradation were incomplete at pH 3.80. The acetic acid concentration increased mainly after growth ceased, but reached a level similar to that found at pH 3.42. The major change in lactic acid content occurred during growth, but continued at reduced rate thereafter.

Table 8.1 presents a comparison of the theoretical and actual yields of lactic and acetic acids produced from carbohydrate fermentation by this bacterium.

Table 8.1 A comparison of the theoretical and actual yields of lactic and acetic acids from carbohydrate fermentation during growth of L. oenos 122 in wine at pH 3.42 and pH 3.80

	Organic acid concentration (mg/L)															
	pH 3.42								pH 3.80							
Days	17		20		26		31		18		25		32		39	
	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA	LA	AA[a]
TY[b]	461	307	596	367	586	391	595	398	191	126	301	199	435	290	518	345
AY[c]	414	198	912	326	677	348	921	425	114	137	473	232	120	235	-119	187

[a] LA = Lactic Acid; AA = Acetic Acid.
[b] TY = theoretical yield, calculated according to the equations described in section 6.3.
[c] AY = actual yield, calculated according to the equations given in section 6.3.

In the treatment at pH 3.80, from 25 days onwards, the actual yield of lactic acid decreased consistently and a negative yield was obtained after 39 days. This is in line with the findings from the model wine inoculated with the same strain (Chapter 6). However, the comparison between the theoretical and actual yields of lactic acid in the treatment at pH 3.42 could not produce consistent results and this does not agree with the findings from the model wine (Chapter 6). Reasons for this disagreement will be discussed later in this chapter.

8.4 Changes in amino acid concentrations during growth of L. oenos 122 in the wine at pH 3.42 and 3.80

Results of amino acid assays of wine at pH 3.42 and 3.80 during growth of L. oenos 122 are presented in Table 8.2.

Table 8.2 Changes in amino acid concentrations during growth of Leuconostoc oenos 122 in wine at pH 3.42 and pH 3.80

	Amino acid concentration (mg/L)					
	pH 3.42			pH 3.80		
Time (days)	0	17	31	0	25	39
Aspartic acid	15	15	15	7	10	11
Threonine	18	13	12	14	11	10
Serine	12	13	13	10	11	11
Glutamic acid	27	27	26	22	23	23
Proline	1314	1177	1136	1110	1119	1101
Glycine	13	13	13	11	12	13
Alanine	31	31	30	29	30	29
Valine	12	9	8	7	8	12
Methionine	2	3	3	2	2	3
Isoleucine	3	3	3	2	3	3
Leucine	8	8	8	7	8	8
Tyrosine	17	16	16	16	16	15
Phenylalanine (?)[a]	33	38	37	36	36	34
Histidine	9	9	9	8	9	9
Ornithine	15	15	57	13	26	76
Lysine	8	9	9	8	9	7
Arginine	76	70	15	67	59	12

[a] Identity in doubt.

It can be seen from Table 8.2 that at both pH values concerted trends emerged for arginine concentration, which decreased significantly and for ornithine concentration, which increased markedly. These changes occurred mainly after the maximum cell population was achieved (after 17 days for pH 3.42 and after 25 days for pH 3.80). It was also noted that the levels of tyrosine and histidine did not vary appreciably. The concentrations of other amino acids varied only very slightly.

8.5 Discussion of results

8.5.1 Growth of lactic acid bacteria in wine in relation to pH changes

This study showed that the final pH value of the wine of pH 3.42 was 3.60. However, pH 3.5 has been reported to be the growth limit of lactocbacilli and pediococci (Wibowo et al, 1985). This implies that these bacteria may develop after MLF, which has been shown by Davis et al, (1986 a,b). This would likely cause spoilage in commercial situations, since the wine environment is not easily controlled. Induction of MLF at pH 3.4 or above may therefore not be a viable practice for the wine industry, unless close monitoring is imposed.

8.5.2 Substrate utilization

The current work confirmed that L. oenos 122 utilized glucose and trehalose as the major growth substrates during growth in wine. This supports the findings from the model wine (Chapter 6) and furthermore, justifies the suitability of the model wine as a culture medium for research purposes. In particular, trehalose may be of special significance, because it was present in high amount (up to 800 mg/L) and it is a storage carbohydrate in yeasts and therefore, it will be released upon autolysis of yeast cells. Galactose, ribose and rhamnose may be used as growth substrates, especially when glucose and trehalose are depleted. The decrease in sucrose concentration also occurred in the uninoculated control (data not shown), suggesting that this decrease was not the result of bacterial growth but of acid hydrolysis.

Apparent fluctuations in the pentitol concentrations may be artefacts resulting from the low concentrations and the analytical precision. Combining the results from the model wine and the model wine with single carbon/energy sources, it appears unlikely that slight decreases in hexitol concentrations were caused by bacterial growth. It is difficult to explain these small decreases in hexitol concentrations.

The hydrolysis of polysaccharides in the red wine may have complicated the analysis of carbohydrates. For example, the increases in pentose concentrations at certain stages may have been the result of this hydrolysis. The stability of the fructose concentration perhaps was also due to this type of hydrolysis. However, there was no increase in the mannitol level. In other words, the correlation between fructose reduction and mannitol production was not clearly demonstrated in the red wine, as in the model wine (Chapter 6). This demonstrates the advantage of using a totally defined medium (the model wine system).

8.5.3 Changes in amino acid concentrations

The changes in amino acid concentrations were similar to those in the model wine system (Chapter 6). This confirms the applicability to actual wines of results obtained with the model wine.

8.5.4 The comparison between the theoretical and actual yields of organic acids

Calculations based on the real wine were not completely comparable with those using the model wine. This is partly because wine is a largely undefined medium. Some fermentable carbohydrates may be generated via chemical and/or enzymatic hydrolysis of polysaccharides such as pentosans, mannans, glucosans and phenolic compounds during incubation. These generated carbohydrates may then be fermented and contribute to the actual yields of organic acids. Fermentation balances are therefore difficult to calculate from known fermentation pathways. This demonstrates the unsuitability of wine as a culture medium for research purposes.

Chapter Nine
General Discussion

9.1 Development of the capillary GLC system for analysis of carbohydrates in wine and its application to the model wine system

The determination of carbohydrates in wine using capillary gas-liquid chromatography has been discussed at length in Chapter 5. Briefly, this method can determine both qualitatively and quantitatively a range of carbohydrates (including monosaccharides, disaccharides and polyols) in one analysis. This method requires only inexpensive and easily available reagents and the analytical precision is adequate. This procedure should also find applications in analysis of carbohydrates in other beverages, food and non-food samples with minor modifications. The slightly longer sample cleanup time can be offset by preparing more samples at one time. The analysis of carbohydrates in the model wine supported the accuracy of this analytical system, since the analysis results agreed with the concentrations of carbohydrates in the formula (Chapter 4).

9.2 Development of the model wine

9.2.1 Benefits of using the model wine system

This model wine is a purely synthetic, defined medium. Therefore, the drawbacks of undefined media (e.g., wine, Chapter 8), such as generation of carbohydrates through hydrolysis of polysaccharides and interference from unknown sources, can be avoided. Fermentation balances can also be calculated using a defined medium and this is crucial in the study of the physiology and metabolism of lactic acid bacteria and other microorganisms.

9.2.2 Suitability of this system as an indicator of metabolism of lactic acid bacteria in wine

Results from both the model wine (Chapter 6) and the red wine (Chapter 8) inoculated with L. oenos 122 showed that sugars, especially glucose and trehalose, were the main substrates used by this strain. In other words, there was a good correlation between the model wine and the red wine data. This justifies this model wine system as an indicator of bacterial metabolism in wine. However, as shown below in section 9.3.1, some differences remain between the behaviour of lactic acid bacteria in the model wine system and real wines.

9.3 Growth and metabolism of lactic acid bacteria in the model wine system

9.3.1 Effect of pH and ethanol on growth

The effect of pH on the growth of leuconostocs differed from the effect on lactobacilli and pediococci. The reported pH limit of growth for leuconostocs in wine is 3.0 (Bousbouras and Kunkee, 1971; and Fleet, 1985). This study stresses that both pH and the stimulatory effect of malic acid must be taken into account when considering control of bacterial growth, since leuconostocs grew at pH 3.2 with malic acid but did not grow at the same pH without malic acid. Also, the reported pH limit of growth for lactobacilli and pediococci in wine is 3.5 (Wibowo at al, 1985). But neither L. plantarum 49 nor P. parvulus 93 grew at pH 3.5 either with or without malic acid. This disagreement could be attributable to the differences in composition between the model wine and wine. It should be noted that both strain 49 and strain 93 were precultured in the same model wine at pH 3.5 but without 10% ethanol.

Good growth was achieved. This demonstrates the inhibitory effect of ethanol on the growth of the two bacteria.

9.3.2 Effect of malic acid on growth

This work also demonstrated that the stimulatory effect of malic acid on growth varied with the species or the strain of lactic acid bacteria. For example, the effect of malic acid on the growth of strains of L. oenos was more obvious than on L. plantarum 49 and P. parvulus 93. The presence of malic acid allowed growth, but its absence retarded growth of leuconostocs at pH 3.2. The better growth of leuconostocs in treatment 3.2 M than 4 L confirmed the more striking stimulatory effect of malic acid at low pH reported by Pilone and Kunkee (1976). There has been no published work regarding stimulation of growth of lactobacilli and pediococci by malic acid. The present study indicated that malic acid had a slight effect on the growth rate of L. plantarum 49 and P. parvulus 93. However, malic acid failed to support the growth of these organisms at pH 3.5.

9.3.3 Growth substrates for wine lactic acid bacteria

This project provides confirmation of the potential growth substrates of wine LAB. Combining results from growth on multiple and single carbon sources and the modified MRS medium (Chapters 6 and 7) and from inoculation of a red wine (Chapter 8), it can be stated that generally sugars (and not polyols, except mannitol) are the main growth substrates under conditions similar to those of wine. These sugars include ribose, rhamnose, arabinose, mannose, glucose, fructose, galactose, sucrose and trehalose. Wine LAB vary in their ability to utilize these growth substrates. For example, sucrose and mannitol, which were not fermented

by L. oenos 122 and 252 and P. parvulus 93, were degraded completely by L. plantarum 49. However, P. parvulus 93 only attacked mannose, glucose and trehalose. Leuconostocs probably used fructose as a hydrogen acceptor, not as a carbon/energy source, with the formation of mannitol (Chapter 6). The formation of mannitol is of practical importance, since mannitol is a preferred growth substrate for L. plantarum 49. Therefore, successive growth of L. oenos and L. plantarum during MLF may occur. Universally, glucose and trehalose were preferred over other sugars by the wine LAB tested. The utilization and preference of trehalose under wine conditions are reported here for the first time, and would be particularly meaningful, since trehalose is liberated from autolysis of yeasts. Further discussion on trehalose metabolism in relation to yeast autolysis will be presented later in this chapter. Effects of malic acid and pH on the utilization of carbohydrates by wine LAB will also be discussed in subsequent sections.

9.3.4 Effects of malic acid on carbohydrate metabolism of wine lactic acid bacteria

There are few reports regarding the influence of malic acid on the carbohydrate metabolism of wine LAB. However, this study shows that malic acid may have a profound impact on their metabolism, which varies with the species and strain of the LAB. Previous work (Pilone and Kunkee, 1972) using L. oenos ML 34 showed that malic acid could not act as a sole energy source, but that malolactic fermentation in the presence of sugars stimulated the utilization of carbon sources. The present investigation demonstrated L. plantarum 49 and P. parvulus 93 also could not use malic acid as an sole energy source. No reports are available concerning the stimulatory effects of malic acid on the growth

of Lactobacillus spp and Pediococcus spp. Results presented in Chapter 6 indicate that the presence of malic acid seemingly did not alter carbohydrate utilization in L. plantarum 49 and P. parvulus 93. However, results in Chapter 6 imply that malic acid did alter carbohydrate utilization by either strain of L. oenos. Consequently, the following discussions will concentrate on the effect of malic acid on the carbohydrate metabolism of L. oenos. Comparisons of carbohydrate utilization of L. oenos 122 and 252 are given in Tables 9.1 and 9.2.

Table 9.1 A comparison of carbohydrate utilization by L. oenos 122 at pH 4 in the presence and absence of malic acid (% decrease)

	With malic acid		Without malic acid	
Phase	log	stationary	log	stationary
cfu/ml	2.00×10^7	4.95×10^5	3.60×10^6	2.76×10^6
Ribose	47	47	6	62
Rhamnose	58	69	16	81
Arabinose	13	26	4	41
Mannose	22	38	0	81
Glucose	98	100	2	48
Galactose	55	70	0	48
Fructose	36	52	6	15
Trehalose	72	98	18	39

As can be seen from Table 9.1, the presence of malic acid led to an increased utilization of glucose, galactose, fructose and trehalose, both during and after growth coincidental with an increased cell population. More pentoses and mannose were used during growth in the presence of malic acid than without malic acid. However, it was also noted that more pentoses and mannose were utilized after the growth phase in the absence of malic acid; this could be attributable to the higher viable cell numbers after growth in the absence of malic acid. It

is difficult to explain the low utilization of carbohydrates during the growth phase in the absence of malic acid.

Table 9.2 A comparison of carbohydrate utilization by L. oenos 252 at pH 4 in the presence and absence of malic acid (% decrease)

	With malic acid		Without malic acid	
Phase	log	stationary	log	stationary
cfu/ml	3.45×10^7	2.97×10^6	8.54×10^7	8.12×10^6
Ribose	92	85	30	90
Rhamnose	76	59	25	53
Arabinose	4	8	49	86
Mannose	13	66	6	66
Glucose	100	100	65	100
Galactose	36	72	16	48
Fructose	24	57	13	51
Trehalose	41	95	52	96

As shown in Table 9.2, the presence of malic acid markedly affected the utilization of arabinose in this organism. When malic acid was present, hardly any arabinose was attacked, whereas 86% of arabinose was used when malic acid was absent. The presence of malic acid also resulted in increased utilization of ribose, rhamnose, mannose, glucose, galactose and fructose during growth. However, there were no profound differences in the overall utilization of these sugars after growth apart from galactose, which was catabolised by 72% in the presence of malic acid, compared with 48% without malic acid. It should be noted that a higher cell population was achieved, but in a relatively longer period of time in the absence of malic acid. It is uncertain why arabinose utilization was reduced during MLF and why trehalose utilization was not affected by MLF.

Overall, these results substantially complement the findings of Pilone and Kunkee (1972), and further expand the knowledge of malolactic

fermentation both qualitatively and quantitatively.

9.3.5 Effects of pH on carbohydrate metabolism of wine lactic acid bacteria

Since L. plantarum 49 and P. parvulus 93 did not grow at pH 3.5 either with or without malic acid, the following discussion is restricted to L. oenos 122 and 252.

This project confirmed that pH had a pronounced effect on the metabolism of LAB and played an important role in control of the nature of the degradable substrate.

Kunkee (1967) reviewed the relationship between pH and MLF. It was stated that pH thresholds for fermentation of malic acid differ from those for the fermentation of sugars and the formation of volatile acidity. It has been proposed that the best organisms for MLF in wine would be those which had the greatest differences between pH thresholds, so that MLF could be obtained with a minimum of other kinds of metabolism. Pilone and Kunkee (1976) observed more striking stimulation by malic acid at low pH values (below pH 4). Goswell (1986) classed wine bacteria involved in de-acidification on the basis of their ability to ferment malic acid in preference to sugars at low pH values as follows:

the homofermentative Pediococcus cerevisiae, which do not ferment malic acid in preference to sugars at low pH and should be classed as undesirable;

the heterofermentative L. oenos, which can attack malic acid at a pH of 3.0, but only attack sugars at pH values of 3.4 and above, making them particularly desirable as agents for carrying out the MLF in wine;

the homofermentative rods such as L. plantarum and L. casei, which also tend to attack malic acid at a lower pH value than sugar, and therefore, may be classed as desirable organisms (although not all can attack malic acid at pH values below 3.3);

and finally, the heterofermentative rods, such as L. brevis, L. hilgardii, L. fructivorans, and L. desidiosus, which do not seem to attack malic acid at a pH lower than they can metabolise sugars, and to that extent, should be regarded as undesirable.

Findings from this project support the classifications of wine LAB by Goswell (1986) in the case of L. oenos 122 and 252, and P. parvulus 93, but not in the case of L. plantarum 49, which did not grow at pH 3.5 either with or without malic acid.

Comparisons of carbohydrate utilization at different pH values in the model wine system and in the red wine are presented in Tables 9.3, 9.4 and 9.5.

Table 9.3 A comparison of carbohydrate utilization and degradation of malic and citric acids by L. oenos 122 at pH 4 and 3.2 (% decrease)

	pH 4		pH 3.2	
Phase	log	stationary	log	stationary
cfu/ml	2.00×10^7	4.95×10^5	1.32×10^7	8.68×10^5
Actual pH	4.40	4.62	3.31	3.47
Ribose	47	47	0	20
Rhamnose	58	69	0	0
Arabinose	13	26	0	0
Mannose	22	38	0	0
Glucose	98	100	11	44
Galactose	55	70	3	35
Trehalose	72	98	22	22
Malic acid	93	99	33	60
Citric acid	98	99	53	91

The impact of pH on carbohydrate utilization of L. oenos 122 is clear-cut, as shown in Table 9.3. Hardly any sugars were attacked at pH 3.2 to 3.31 during growth. Rhamnose, arabinose and mannose were not metabolised at all at pH 3.2 to 3.47. Corresponding to this weak fermentation of sugars was the low efficiency of malic acid degradation at low pH values. In view of the fact that similar cell populations were attained at both pH values, it may be speculated that the small amount of malic acid degraded (33%, 1.6 g/L) at pH below 3.31 must have yielded sufficient energy to meet the requirements of this organism. Perhaps this strain benefitted from the cleavage of potential pyruvic acid resulting from the degradation of malic and/or citric acids (section 6.6.6.1) in preference to sugars at low pH values (<3.5), supporting the classifications of Goswell (1986). However, the inefficient degradation of malic acid by this strain at low pH values must be regarded as undesirable as far as the bacteriological stability and deacidification of wine are concerned. Alternatively, could this low efficiency of malic acid degradation be improved by inoculating with a large inoculum?

Table 9.4 A comparison of carbohydrate utilization and degradation of malic and citric acids by L. oenos 252 at pH 4 and 3.2 (% decrease)

	pH 4		pH 3.2	
Phase	log	stationary	log	stationary
cfu/ml	3.45×10^7	2.97×10^6	5.00×10^6	5.80×10^4
Actual pH	4.40	4.50	3.42	3.48
Ribose	92	85	32	42
Rhamnose	76	59	0	0
Arabinose	4	8	0	0
Mannose	13	66	24	36
Glucose	100	100	88	100
Galactose	36	72	28	32
Fructose	24	57	22	26
Trehalose	44	95	6	8
Malic acid	83	97	100	100
Citric acid	100	100	100	100

The influence of pH on carbohydrate utilization by L. oenos 252, as with L. oenos 122, is definitely demonstrated in Table 9.4. Rhamnose and arabinose were not attacked at all at pH values below 3.5. Also trehalose was not catabolised considering comparison with the inoculated control. The utilization of ribose, mannose, galactose and fructose were also significantly reduced at pH values below 3.5, but it appeared that glucose catabolism was not affected by pH. Furthermore, this organism degraded malic and citric acid highly efficiently at low pH. However, this strain required 132 days to reach the maximum cell population, probably owing to the extremely low inoculum. The discrepancies in the efficiency of malic acid degradation between L. oenos 122 and 252 may be relevant to the differences in the actual yields of lactic acid between the two strains (Chapter 6).

Table 9.5 A comparison of carbohydrate utilization and degradation of malic and citric acids by L. oenos 122 at pH 3.80 and 3.42 in a red wine (% decrease)

	pH 3.80		pH 3.42	
Phase	log	stationary	log	stationary
cfu/ml	8.63×10^6	5.65×10^6	9.37×10^7	9.68×10^6
Actual pH	4.10	4.08	3.60	3.56
Arabinose	72	90	81	88
Glucose	61	100	72	79
Galactose	55	70	55	40
Trehalose	49	93	91	100
Malic acid	72	93	100	100
Citric acid	52	93	88	100

As shown by Table 9.5, it is difficult to make any generalizations about carbohydrate utilization by L. oenos 122 at the pH values shown. This could be due to the undefined nature of the wine, thus indicating the value of using the defined medium (model wine). Nonetheless, more glucose and galactose were utilized at pH 3.80 than at pH 3.42 by the end of the stationary phase. The lowered decrease of galactose at pH 3.42 at the end of stationary phase (40%) than during log phase (55%) could be due to the generation of galactose from unknown polysaccharide sources in the red wine. But more trehalose was used at the lower pH, particularly during growth. Malic and citric acid were fermented in preference to sugars at low pH values (below 3.60). Also malic and citric acids were degraded more efficiently at pH 3.42.

Through assessment of Tables 9.3, 9.4 and 9.5, it was found that pH 3.5 could be a critical value for the metabolism of L. oenos. At pH below 3.5, much lower quantities of carbohydrates were catabolised (Tables 9.3 and 9.4), but at pH above 3.5, carbohydrate utilization was not affected

greatly by pH, suggesting that either induced or spontaneous malolactic fermentation should be conducted at pH 3.2-3.3 and the final pH should not exceed 3.5. However, the optimal pH for conducting MLF depends on the compromise between the efficiency of malic acid degradation and carbohydrate fermentation. The ideal pH should enable the achievement of a complete degradation of malic acid, but least utilization of carbohydrates. More work is required to search for such starter cultures.

9.3.6 Energetics, role and mechanism of malolactic fermentation in wine LAB

As discussed in the Literature Review (Chapter 2), the role, energetics and mechanism of malolactic fermentation have not been elucidated. The present study implies that energy generation could be the role of malolactic fermentation, and that at least two potential mechanisms may operate in the energetics of malolactic reaction, dependent on the species and strain of LAB. The two energy-yielding mechanisms include ATP generation through pyruvic acid cleavage (substrate level phosphorylation, unreal malolactic fermentation) such as indicated in L. oenos 122, and chemiosmotic ATP synthesis via production of extra lactic acid (non-substrate level phosphorylation, real malolactic fermentation) such as indicated in L. oenos 252, L. plantarum 49 and P. parvulus 93.

Stoichiometrically, a shortfall in lactic acid concentration during and after MLF was noted. It is believed that this is the first report of this phenomenon. This shortage suggests that instead of being reduced to lactic acid, pyruvic acid resulting from the catabolism of sugars, malic or citric acids could be dissimilated via other metabolic pathways.

Kandler (1983) reviewed the alternative fates of pyruvic acid. According to this review, in addition to formation of diacetyl, acetoin and butylene glycol, pyruvic acid can also be split to formic acid and acetic acid by pyruvic acid-formic acid lyase, via acetyl-CoA and acetyl-phosphate, with the formation of one mole of ATP per mole of pyruvic acid, as shown in the case of bifidobacteria (De Vries and Stouthamer, 1968) and Lb. casei (De Vries et al, 1970).

Two other mechanisms for converting pyruvic acid to acetic acid and CO_2 without formic acid production, each yielding 1 mole of ATP per mole of pyruvic acid, have been reported for lactic acid bacteria (Fig 9.1, which is the same as Fig 6.1). One requires lipoate and coenzyme A and involves acetyl-CoA and acetyl-phosphate as intermediates. This system has been found in S. faecalis (O'Kane and Gunsalus, 1948; Gunsalus et al, 1952). In the other mechanism, reported for Lactobacillus delbruckii (Hager et al, 1954) and Lactobacillus plantarum (Dirar and Collins, 1973; Gotz et al, 1980), lipoate and coenzyme A are not required and acetyl-phosphate is directly formed during oxidation. Thereby, an extra 2 moles ATP (homofermenters) and 1 mole ATP (heterofermenters) per mole of hexose could be gained (Fig 9.1), provided that all pyruvic acid is cleaved, in addition to the ATP formed under anaerobic conditions. Similarly, extra moles of ATP produced from pentoses and disaccharides via pyruvic acid dissimilation can be calculated. Likewise, one mole of ATP per mole of malic or citric acids can be gained through pyruvic acid cleavage. Apart from ATP, $NADH_2$ may also be generated if the pyruvic acid dehydrogenase pathway via acetyl-CoA is employed. Considering the quantities of malic acid in must and wine, the amount of energy (ATP) gained from malic acid degradation via pyruvic acid cleavage would be

significant to the growth of the strain. This is supported by the higher growth rate of L. oenos 122 achieved at pH 3.2, where only 33% malic acid (1.6g/L) was degraded, but hardly any carbohydrates were attacked. This suggests that pyruvic acid may have been produced from the degradation of malic and/ or citric acids and may have been cleaved to provide energy. Further work is required to determine the recovery of L-lactic acid from L-malic acid degradation, in order to confirm the source of pyruvic acid which may have been cleaved. The pyruvic acid cleavage theory could satisfactorily explain the role, energetics, and mechanism of unreal malolactic fermentation for organisms of this type (L. oenos 122). However, the details of pyruvic acid dissimilation (enzymes and intermediates) have not yet been fully elucidated, in particular in the case of wine LAB. Therefore, intensive studies, using radio-labelled carbon sources and enzyme studies, are required to find out which pathway wine LAB adopt in pyruvic acid dissimilation so that the mechanism of MLF of organisms of this type can be substantiated. Findings from L. oenos 122 (Chapter 6) suggest that pyruvic acid dissimilation is affected by pH: the lower the pH (3.2-4.5), the more pyruvic acid being cleaved. Alternatively, acetyl-CoA or acetyl-P derived from pyruvic acid degradation may be incorporated into the biosynthesis of cell materials such as lipid, as shown in Streptococcus diacetilactis (Harvey and Collins, 1963).

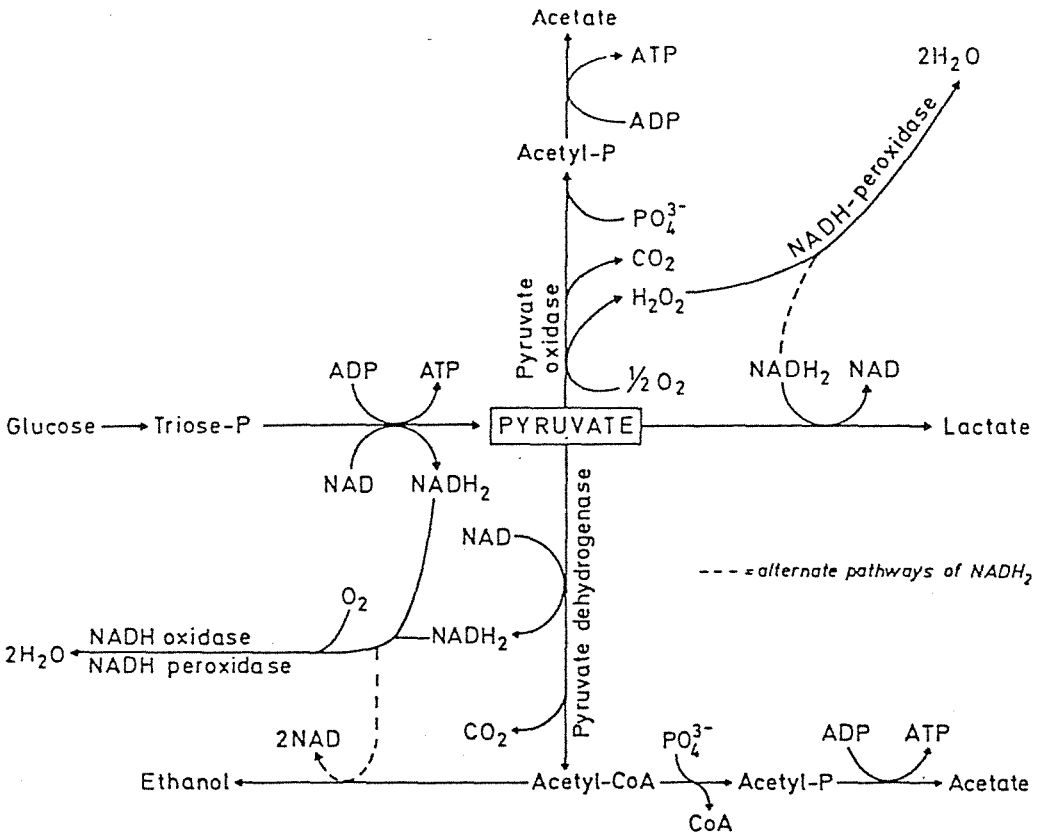

Figure 9.1 Hypothetic scheme of pyruvate dissimilation (after Kandler, 1983). This figure is the same as Figure 6.1.

According to Figure 9.1, acetyl-CoA and acetyl-P are the intermediates, and the end product is either acetic acid, accompanied by the formation of 1 mole of ATP, or ethanol without ATP being formed, but rather $NADH_2$ being used.

The relationship of aeration to cell biomass, growth rate, acetic acid and ethanol was studied by Stamer and Stoyla (1967) for Lactobacillus brevis and by Lucey and Condon (1986) for Leuconostoc. These researchers found that aeration brought about rapid growth rate, more biomass, and caused an increase in acetic acid production with a decrease in ethanol generation. These results also confirmed that oxygen and organic catalysts (e.g., fructose) play an active role as hydrogen/electron acceptors in energy metabolism (formation of ATP and acetic acid from acetyl-P) of heterofermentative lactic acid bacteria. Conversely, acetyl-P can be reduced to ethanol in the absence of oxygen and organic catalysts such as fructose, aldehyde. In the presence of $NADH_2$ or other hydrogen donors, acetyl-CoA or acetyl-P may be reduced to ethanol, which would be extremely beneficial to wine-making in terms of prevention of formation of volatile acidity (acetic acid). Further studies are required to understand the regulation of acetic acid/ethanol diversion, if an organism of this type is used to conduct deacidification.

By contrast with L. oenos 122, extra amounts of lactic acid were found during malolactic fermentation in the case of L. oenos 252, L. plantarum 49 and P. parvulus 93. A real net increase in D-lactic acid production has also been noticed by Pilone and Kunkee (1972) in the case of L. oenos ML 34, denoting that these wine LAB may possess the same mechanism of malolactic reaction. The function of the surplus lactic acid can

perhaps be accounted for on the basis of the chemiosmotic postulate if this hypothesis holds true experimentally for wine lactic acid bacteria.

The theory of chemiosmotic energy coupling has been described in Chapter 6. Konings and Otto (1983) believe that this theory is indeed applicable to lactic acid efflux in symport with protons in streptococci. In fact, experimental evidence for the generation of a proton motive force by lactic acid efflux has been supplied for S. cremoris (Brink and Konings, 1982; Brink et al, 1985). For organisms which perform homolactic fermentation of sugars, such as streptococci, the additional energy produced by lactic acid excretion can be as high as 50% (Brink and Konings, 1982). Malic acid decarboxylation also produces a lactic acid efflux and MLF may then help the cell to keep the proton motive force required for maintaining its intracelluar metabolite pool. Based on this mechanism, Renault et al (1988) attempted to explain the stimulation of growth observed during MLF by S. lactis, but failed to come up with any satisfactory answers. The latest work of Cox and Henick-Kling (1989) may throw some light on the applicability of the chemiosmotic energy coupling theory to malolatic fermentation. They demonstrated the generation of ATP during malolactic fermentation in whole cells of Leuconostoc oenos Lc5b1, but not in a cell extract, indicating that the intact cell membrane is essential to the energy-yielding mechanism. The chemiosmotic theory could satisfactorily explain the role of the excretion of metabolic end products and therefore, may account for the energetics of malolactic fermentation and the role of the extra lactic acid. However, the true biological function of malolactic fermentation would be the formation of extra lactic acid from other sources.

The unique discovery of extra lactic acid production by the relevant wine LAB pose the question: where did the extra lactic acid stem from? The metabolism of amino acids, vitamins, purines and pyrimidines can not balance the material mass of fermentation. One speculation involves carbon dioxide assimilation. CO_2 has been shown to stimulate the growth of L. oenos ML 34 (Stamer and Stoyla, 1970). The same authors stated that the exact role of carbon dioxide remained to be elucidated and it appeared unlikely that anaerobiosis was a requirement for growth, since hydrogen and nitrogen atmospheres were no more conducive to establishing good growth than an atmosphere of air. However, Kelly et al (1989) reported that anaerobic conditions facilitated the growth of L. oenos and this growth did not appear to be stimulated specifically by carbon dioxide. This could be due to the heterogeneity among strains of L. oenos. No reports have evolved, concerning the CO_2 assimilation by wine LAB. It is impossible to calculate the fermentation balance even if CO_2 assimilation is taken into account, since the pathway of lactic acid biosynthesis via CO_2 assimilation is unknown. Wood and Stjernholm (1960) reviewed the assimilation of carbon dioxide by heterotrophic bacteria. In this review, there was no mention of lactic acid biosynthesis via carbon dioxide by any bacteria, nor was the mention of carbon dioxide assimilation by any wine LAB.

Overall, the formation of extra lactic acid during MLF can be one of the most important biological functions of MLF, according to the chemiosmotic theory. The origin of the surplus lactic acid remains an intriguing puzzle. The resolution of this puzzle will be meaningful both theoretically and practically for the wine and related fruit wine industry.

9.3.7 Biogeneration of amines and arginine metabolism

Previous reviews (Chapter 2) imply that the biogenesis of amines in wine is still a matter of controversy. In other words, the role of wine LAB in formation of biogenic amines is vague. Results from this project may shed light on this issue.

It can be observed from Chapter 6 that L. oenos 122 and 252, and P. parvulus 93 did not greatly reduce the levels of tyrosine, histidine and phenylalanine, indicating weak or non-existent decarboxylation of these amino acids to their respective amines: tyramine, histamine and phenylethylamine. L. plantarum 49 did not noticeably reduce the content of histidine, but drastically decreased the levels of tyrosine and phenylalanine. It was highly probable that tyrosine and phenylalanine were decarboxylated to tyramine and phenylethylamine, respectively, suggesting that L. plantarum 49 may be a potential amine producer. The extent of tyrosine and phenylalanine degradation was more complete in the presence of malic acid (Chapter 6). Therefore, hygiene and sanitation are imperative during vinification and aging for controlling the formation of biogenic amines in wine.

These results are supported by the report of Buteau et al (1984) who showed that amines were not the products of L. oenos and P. cerevisiae. The findings of this study are also supported by Ough et al (1987) and Delfini (1989) who demonstrated the inability of wine LAB to produce significant amounts of histamine.

These results are of significance for selecting low amine-producing strains, a very important character when deciding which LAB to use as

MLF starters. These results also justify that L. oenos is the most desirable species for preparing starters to induce MLF in wine, and L. plantarum 49 is highly undesirable as a starter, at least in relation to the potential formation of biogenic amines.

According to Garvie (1986), one of the characteristics of the genus Leuconostoc is that arginine is not hydrolyzed. However, this has been questioned (Pilone, Pers. comm., 1989) and is contradictory to reports of Kuensch et al (1974), Garvie and Farrow (1980) and findings of this project.

As indicated in Chapter 6 and 8, both strains of L. oenos transformed arginine to ornithine and ammonia while L. plantarum 49 and P. parvulus 93 did not. These results are in accordance with those of Mayer et al (1973), Kuensch et al (1974) and Davis et al (1986a,b). There is a report that a strain of L. plantarum isolated from fish could convert arginine to ornithine and ammonia (Jonsson et al, 1983). None of these wine LAB (L. oenos 122 and 252) could use arginine as a sole energy source (Chapter 6). The transformation of arginine to ornithine has been demonstrated to yield energy in other organisms such as Streptococcus faecalis, Clostridium spp and Halobacterium salinarum (Bauchop and Elsden, 1960; Doelle, 1969; Sokatch, 1969). There have been no studies regarding ATP generation, enzymes and intermediates implicated in arginine metabolism of L. oenos. Evidently, this is an area worthy of further interest.

This project also demonstrated that arginine catabolism by L. oenos is affected by pH. Arginine was not attacked at a pH below 3.5, suggesting

that arginine may not facilitate the growth of L. oenos at low pH (<3.5).

There are no reports concerning the influence of ammonia released from arginine catabolism on pH rises. Appraisals of pH changes (Chapter 6) suggest that the large amounts of ammonia liberated from arginine breakdown might have contributed to the pH increases. At low pH (<3.5) at which there were no appreciable changes in arginine levels, malic acid degradation (33% by L. oenos 122 and 100% by L. oenos 252) raised the pH by increments of only 0.2-0.3. However, at higher pH (>4) at which both malic acid and arginine were catabolised, the pH increments reached up to 0.6, even though acids produced from carbohydrate fermentation would have counteracted the change. The sensory effect of arginine metabolism was not studied here. But according to Eggenberger (1988), arginine catabolism can have a very beneficial effect, since arginine tastes somewhat bitter and musty and its conversion can be regarded as a totally positive effect. Furthermore, ornithine can impart a stabilizing and yeast-retarding influence on the microorganisms in wine (Mayer et al, 1973; Eggenberger, 1988). More work is needed to study arginine metabolism in L. oenos and its effect on growth.

The suspected carcinogenic nature of ethyl carbamate (Mirvish, 1968) has triggered several recent studies investigating ethyl carbamate formation in wine (Ough et al, 1988a,b; Monteiro et al, 1989). It has been demonstrated that urea released from arginine metabolism by yeasts, a reaction catalyzed by arginase, is involved in the ethyl carbamate formation (Monteiro et al, 1989). This manifestation merits further study of arginine metabolism by Leuconostoc oenos and relevant wine LAB.

According to Abdelal (1979), the following major pathways exist for arginine catabolism by microorganisms: 1) the arginase pathway; 2) the arginine transamidinase pathway; 3) the arginine deiminase pathway; 4) the arginine decarboxylase pathway. Therefore, it becomes important to comprehend which pathway of arginine catabolism L. oenos or other wine LAB adopt, so that the relationship between ethyl carbamate formation and arginine catabolism by wine LAB can be elucidated. Ethyl carbamate can be formed in a spontaneous chemical (nonenzymic) reaction involving ethanol and a compound containing a carbamyl group (Ough et al, 1988a). With the arginine deiminase pathway (Abdelal, 1979), citrulline and carbamyl phosphate are two of the intermediates of arginine catabolism. It is possible that citrulline and carbamyl phosphate liberated from arginine catabolism by the arginine deiminase pathway may be implicated in ethyl carbamate formation if wine LAB employ this pathway. Another significance of arginine catabolism can be clearly seen here, in addition to the significance mentioned earlier. As discussed earlier, however, arginine catabolism is affected by pH. There was hardly any arginine catabolised at low pH (<3.5). Thereby, the formation of ethyl carbamate may be circumvented by conducting MLF at low pH values (3.2 to 3.3) and by preventing rise of pH above 3.5, if intermediates from arginine catabolism by wine LAB are involved in the formation of ethyl carbamate.

9.4 The relationship between yeast autolysis and malolactic fermentation

The encouragement of malolactic fermentation by delayed racking from the yeast lees has been advocated (Kunkee and Amerine, 1970; Kunkee and Goswell, 1977) because of the amino acids and other growth factors released from yeast autolysis (Kunkee and Amerine, 1970).

The findings of this project demonstrate that amino acids did not vary markedly after the growth of wine LAB, except in the case of arginine with L. oenos 122 and 252, and tyrosine and phenylalanine with L. plantarum 49. Trehalose was always preferentially utilized by all wine LAB. Undoubtedly amino acids are necessary for the growth of the nutritionally fastidious LAB, but it seems that trehalose may play a more crucial role than amino acids for LAB as a source of carbon/energy. This study shows that the role of yeast autolysis in encouraging malolactic fermentation should not be overemphasized in terms of simply release of amino acids, rather, it should be re-considered from the point of view of liberation of carbon/energy sources, i.e., carbohydrates being released from yeast autolysis. The following discussion will be centred on the relationship between carbohydrate release and yeast autolysis.

It is known that trehalose and glycogen are the storage carbohydrates (energy reserves) of yeasts (40% of the dry weight of yeast cells) (Sols et al, 1970). Trehalose is a disaccharide of two molecules of D-glucose, and glycogen consists of monomers of glucose (Manners, 1970). The yeast cell envelope also contains polysaccharides such as mannan, glucan, phosphomannan, phosphogalactan, galactomannan, pentosyl mannan and other heteropolysaccharides (Phaff, 1970). Trehalose is quantitatively meaningful as well, since it has been found to be the principal disaccharide present in wines at levels of up to 800 mg /L (Bertrand et al, 1975; Chapter 5; Chapter 8). It can be concluded that trehalose is one of the main growth substrates. Logically, these carbohydrates are released from yeast cells into wine along with amino

acids upon autolysis. The hydrolysis of polysaccharides may yield considerable amounts of monosaccharides and these monosaccharides may contribute to the carbon/energy sources for wine LAB.

The metabolic mechanism of trehalose utilization by wine LAB is unknown. Trehalase has been detected in yeast cells and other bacteria (Souza and Panek, 1968), but so far there have been no reports concerning the presence of trehalase in wine LAB. The preferential use of maltose by beer lactobacilli (Rainbow, 1975; 1981) and the presence of maltose phosphorylase in Lactobacillus brevis (Wood and Rainbow, 1961) have been reported. Similarly, a development of specialized carbohydrate metabolism such as trehalose catabolism by "trehalose phosphorylase" may have occurred in wine LAB. This area merits more research.

In summary, the role of yeast autolysis in encouraging malolactic fermentation in terms of simply release of amino acids is questioned. The role of amino acids from autolysis of yeast cells should not be overestimated. However, the role of carbohydrates (particularly trehalose) from yeast autolysis must be fully appreciated. A combination of both amino acids and carbohydrates (and other nutrients) released from yeast autolysis should be taken into account when considering the benefits of yeast autolysis in induction of malolactic fermentation, which also needs to be balanced against development of off-flavours.

9.5 Practical implications of this project

9.5.1 Selection of malolactic starter cultures

Modern winemaking technology has moved from the traditional spontaneous malolactic fermentation by the indigenous LAB towards controlled MLF by

inoculation with starter cultures (Beelman et al, 1977; 1980; Kunkee, 1984; Davis et al, 1985; Wibowo et al, 1985). One of the major problems associated with the induction of MLF is the selection of suitable starter cultures. Past work has shown that strains of L. oenos, such as L. oenos ML 34 (Pilone and Kunkee, 1972) and L. oenos PSU 1 (Beelman et al, 1977; 1980), are the most suitable starters for inoculation into wine.

The current selection criteria given by Kunkee (1984) include ease of growth, good viability on storage of the cultures, rapidity of their growth in wine and a resulting neutral effect on the flavour. Henick-Kling (1988) regarded as important the ability to grow well at pH values between 3.0 and 3.4, active degradation of malic acid, resistance to phage, and production of desirable flavours. The importance of pH as a selection parameter has also been recognized in relation to other types of metabolism, for instance, carbohydrate metabolism (Kunkee, 1967; Goswell, 1986), because the degradation of carbohydrates produces lactic acid ("lactic flavour") and acetic acid (volatile acidity).

This project provides fundamental but useful information on the selection criteria of malolactic starter cultures. L. plantarum 49 and P. parvulus 93 would not be appropriate as they did not even grow at pH 3.5. Recommendations of inducing MLF at low pH values (3.2-3.3) (Eggenberger, 1988) justify L. oenos 122 and 252 as being most suitable.

Some generalizations about the characteristics of the ideal starter cultures may arise from this project: 1) rapid growth at pH 3.2-3.3; 2) utilization of little or no carbohydrates at low pH (<3.5); 3) high

efficiency of malic acid degradation at low pH (3.2-3.3); 4) production of low levels of acetic acid and lactic acid and 5) no formation of amines. However, malic acid degrading activity at low pH (3.2-3.3) should be regarded as the most essential selection parameter.

Theoretically, bacteria, which possess the enzymes of pyruvic acid cleavage, such as L. oenos 122, should generate less lactic acid. But there exists a risk of producing more acetic acid in the case that acetyl-CoA or acetyl-phosphate formed from pyruvic acid breakdown is converted to acetic acid, instead of to ethanol (see Figure 9.1). The reduction of acetyl-CoA or acetyl-P to ethanol can be effected by exclusion of oxygen and organic catalysts (e.g., fructose), as discussed earlier. Furthermore, the efficiency of malic acid degradation by L. oenos 122 at pH below 3.5 is low, making this organism undesirable as a starter culture at low pH. The low efficiency of malic acid degradation below pH 3.5 could be due to the high energy yield of pyruvic acid cleaved, 1 mole of ATP per mole of pyruvic acid, as discussed in section 9.3.6. The low efficiency of malic acid degradation probably could be improved by inoculating with a large inoculum. On the other hand, the bacteria which form extra lactic acid during malolactic fermentation, for instance, L. oenos 252, may have a desirable high efficiency of malic acid degradation at low pH, but could suffer from the drawback of producing extra amounts of lactic acid.

Rapidity of growth in wine can be achieved by the adaptation procedure of successive transfer of the culture from the high pH media to low pH media (Henick-Kling, 1988) and by inoculating the wine with a large number of viable cells (greater than 10^6 cfu/ml), as recommended by Eggengerger (1988) and Wibowo et al (1988).

Overall, the foregoing discussions are based on the preliminary studies of two strains of L. oenos. Further biochemical and enzymatic studies are required to substantiate the pyruvic acid cleavage theory and chemiosmotic postulate, so that the selection of starters can be properly guided in the production of active and efficient starter cultures for malolactic fermentation in the wine industry.

9.5.2 Control of malolactic fermentation

On the basis of previous discussions, to induce a successful malolactic fermentation in wine, the initial pH must be relatively low, preferably between 3.2-3.3. Otherwise, other types of metabolism may occur, e.g., large amounts of carbohydrates can be fermented and amines formed by "wild" bacteria. The yeast lees should not be left too long in contact with wine since carbohydrates, especially trehalose, the preferred growth substrate, can be liberated from yeast cells. One of the major advantages of conducting MLF at pH 3.2-3.3 is that the final pH after MLF will not exceed 3.5, at which species such as L. plantarum 49 and P. parvulus 93 can not grow. This renders the wine comparatively bacteriologically stable.

The reduction in pyruvic acid content after MLF (Rankine, 1965; Fornachon and Lloyd, 1965) and the practice of addition of pyruvic acid by Fornachon to white table wines to produce rapid MLF (Rankine, 1965) can be explained on the basis of pyruvic acid cleavage theory. The encouragement of MLF in red and white wines by adding pyruvic acid is worthy of further research.

Another problem associated with induction of MLF is the timing of inoculation. Simultaneous alcoholic and malolactic fermentation (Beelman and Kunkee, 1985), inoculation during alcoholic fermentation (Gallander, 1979; Blackburn, 1984) and inoculation after alcoholic fermentation (Kunkee, 1974; Gallander, 1979) have all been advocated. The results of this project may justify the inoculation of the wine with starter cultures during alcoholic fermentation, since the pyruvic acid content is high during this period (Whiting and Coggins, 1960; Rankine, 1965). The pyruvic acid-binding effect of sulphur dioxide can reduce the level of pyruvic acid and thus, may exert a secondary inhibitory effect on wine LAB, in addition to the direct toxicity of molecular sulphur dioxide.

9.6 Further research

Future work has been described throughout this chapter where appropriate. Briefly, the following areas merit further study:

1) mechanisms of pyruvic acid cleavage;

2) mechanisms of production of extra lactic acid;

3) elucidation of arginine degradation;

4) detection of biogenic amines.

Chapter Ten
Summary and Conclusions

10.1 Capillary gas-liquid chromatography of carbohydrates in wine

A method for determination of wine carbohydrates by capillary gas-liquid chromatography has been developed, using polyacetate and aldononitrile polyacetate derivatives of polyols, aldoses and disaccharides. This method involves simple sample cleanup procedures that involve passage of a wine sample through a C_{18} Sep-Pak cartridge, followed by ion exchange and drying, before derivatization. A wide range of aldoses, polyols and disaccharides (30 compounds) were analysed in 55 minutes using a single injection. All the derivatives were well-separated, except for ribose and rhamnose, which almost co-eluted. In addition to polyols, aldoses and disaccharides, amino sugars could also be assayed by this method. Preliminary trials showed that N-acetyl glucosamine was well resolved. The method recovered spiked carbohydrates from 86 to 110% and had adequate reliability (CV of reproducibility ranged from 2.4 to 8.3% for most of the compounds analysed). This method can be extended to the analysis of carbohydrates and related substances in other alcoholic and non-alcoholic beverages, and food and non-food samples.

10.2 Growth and metabolism of wine lactic acid bacteria

Experiments were carried out to investigate the growth and metabolism of selected wine lactic acid bacteria, including L. oenos 122 and 252, L. plantarum 49 and P. parvulus 93. These bacteria were grown in model wines, model wines with single carbon/energy sources and a red wine. Results achieved from these different media correlated well, suggesting

the suitability of the model wine system as an indicator of metabolism of lactic acid bacteria in true wines.

All the selected LAB grew well at pH 4 with and without malic acid. Strains of L. oenos grew at pH 3.2 to 3.3 with malic acid but their growth was inhibited at the same pH values without malic acid. L. plantarum 49 and P. parvulus 93 did not grow at pH 3.5 either with or without malic acid. These findings imply that pH and malic acid are the key factors in controlling the growth of lactic acid bacteria in wine.

This project substantiated the suggestion that sugars were the main growth substrates for wine LAB and polyols did not serve as growth substrates, except for mannitol. Glucose and trehalose were the preferred growth substrates by all the selected LAB. Malic acid, citric acid and arginine alone did not support the growth of these LAB, but these substances stimulated the growth in the presence of fermentable sugars. Wine LAB varied in their ability to utilize substrates. P. parvulus 93 utilized only mannose, glucose and trehalose. L. plantarum 49 was the only bacterium which utilised fructose, sucrose and mannitol as carbon sources. Strains of L. oenos used fructose as a hydrogen acceptor with the formation of mannitol, while they did not use fructose as a single carbon source. The following sugars were also fermented by L. plantarum 49, L. oenos 122 and 252: ribose, rhamnose, arabinose, mannose and galactose. Xylose was not attacked by any of the selected wine LAB.

Malic acid had a pronounced impact on the growth rate, cell population and substrate utilization of both strains of L. oenos. Yet malic acid

appeared not to affect the substrate utilization in L. plantarum 49 and P. parvulus 93. Malic acid stimulated the growth rate and cell population of L. oenos 122 and 252, but stimulated only the growth rate of L. plantarum 49 and P. parvulus 93. Malolactic fermentation resulted in an increased utilization of sugars in L. oenos 122 and 252. However, malolactic fermentation did not influence trehalose utilization but reduce arabinose utilization in L. oenos 252.

pH greatly affected the metabolism of L. oenos 122 and 252. Very small amounts of glucose were catabolised and, malic and citric acids were partially degraded by strain 122 at pH below 3.31. Utilization of other sugars by this strain at pH below 3.47 was also greatly reduced. L. oenos 252 also used only minor amounts of sugars, but completely degraded malic and citric acids at pH below 3.42. The glucose utilization was not affected by pH, whereas utilization of other sugars at pH below 3.48 was markedly decreased. These results suggest that the nature of the degradable substrate(s) can be controlled by pH.

The role of wine LAB in formation of biogenic amines could be elucidated. L. plantarum 49 appreciably decreased the concentrations of tyrosine and phenylalanine and therefore, may be a potential former of tyramine and phenylalanine. P. parvulus 93 did not markedly reduce the concentrations of any amino acids. Both strains of L. oenos converted arginine to ornithine and ammonia, but did not decrease the concentrations of other amino acids. Arginine degradation by L. oenos 122 and 252 did not occur at pH values below 3.5. These results imply that P. parvulus 93 and strains of L. oenos are not potential amine producers and L. oenos cannot benefit from arginine catabolism at pH below 3.5.

Two potential energy-yielding mechanisms of malolactic fermentation were proposed: ATP generation through pyruvic acid cleavage (substrate level phosphorylation, unreal malolactic fermentation) such as in L. oenos 122, and chemiosmotic ATP synthesis via production of extra lactic acid (non-substrate level phosphorylation, real malolactic fermentation) such as in L. oenos 252, L. plantarum 49 and P. parvulus 93. The biological function of the extra lactic acid can be explained using the chemiosmotic theory, which postulates ATP synthesis via efflux of metabolic end products. Good agreement between the theoretical and actual yields of lactic acid in the absence of malic acid was obtained. The origin of the extra lactic acid is unknown.

Yeast autolysis may play a more important part in inducing MLF in terms of liberation of carbohydrates, in particular trehalose, than simply the release of amino acids.

In conclusion, carbohydrates (mainly sugars) are the growth susbtrates for wine LAB. The growth and substrate utilization of LAB are greatly influenced by and may be regulated by pH and malic acid. The metabolism of organic acids in wine LAB is closely associated with the role, energetics and mechanisms of malolactic fermentation.

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Appendix

1. The recipe of tomato juice broth/agar

Ingredient	Concentration
Tryptone	18 g/L
Peptone	4.5 g/L
Yeast extract	4.5 g/L
Glucose	4.5 g/L
'Tween' 80	0.9 ml/L (of 0.5% solution)
Tomato juice	300 mls
Distilled water	700 mls
Agar	15 g/L

2. The composition of the modified medium

Ingredient	Concentration (g/L)
Basal MRS:	
Peptone	10.0
Yeast extract	4.0
'Tween '80 (straight)	1 ml
di-Potassium hydrogen peroxide	2.0
Sodium acetate.$3H_2O$	5.0
tri-Ammonium citrate	2.0
Magnesium sulphate.$7H_2O$	0.2
Mangnese sulphate.$4H_2O$	0.05
Carbohydrates:	
Glycerol	10.0
Glucose	5.0
Fructose	5.0
Trehalose	5.0
Erythritol	1.0
Threitol	0.5
Ribose	1.0
Rhamnose	0.5
Lyxose	0.3
Arabinose	2.0
Fucose	0.1
Xylose	1.0
Ribitol	1.0
Arabitol	2.0
Xylitol	1.0
Mannose	1.0
Galactose	2.0
Myo-inositol	5.0
Glucitol	1.0
Mannitol	2.0
Galactitol	0.5
Maltose	0.2
Lactose	0.2
Sucrose	1.0
Cellobiose	0.2
Melibiose	0.2