Arginine Metabolism in Malolactic Wine Lactic Acid Bacteria and Its Oenological Implications

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ABSTRACT

L-Arginine is a major amino acid found in grapes and wine which is degraded by some wine lactic acid bacteria (LAB). The mechanism of this degradation and its oenological implications were examined in this research.

It was found that wine LAB able to degrade arginine do so by means of the arginine deiminase pathway, demonstrated by measuring the enzyme activities in cell-free extracts: arginine deiminase, ornithine trans-carbamylase and carbamate kinase. These enzymes were present in most heterofermentative lactobacilli and leuconostocs, but were absent in homofermentative lactobacilli and pediococci.

The presence of arginine increased the activities of arginine deiminase pathway enzymes in heterofermenters, but failed to induce these enzymes in homofermenters even under conditions of low glucose concentration (1 g/L). Glucose did not repress arginine utilisation but fructose appeared to do so, as fructose and arginine were metabolised sequentially, with arginine being metabolised mainly after utilisation of the fructose.

Detailed studies on *Leuconostoc oenos* OENO, *Lactobacillus buchneri* CUC-3 and *Lactobacillus brevis* 250 showed that arginine was converted stoichiometrically to ammonia and ornithine as the major end-products and that arginine catabolism could supply energy (ATP) to support growth. It was also demonstrated that citrulline was excreted during arginine catabolism by both the lactobacilli and the leuconostoc. Some of the excreted citrulline was reassimilated and catabolised after arginine depletion by the lactobacilli, but not by the leuconostoc.

The implication of citrulline excretion for the wine industry was explored by studying the formation of the carcinogen ethyl carbamate (urethane) in a synthetic wine and a white wine, since citrulline is a known precursor of ethyl carbamate. During growth of *Lc. oenos* OENO and *Lb. buchneri* CUC-3 in the synthetic wine and wine, significant amounts of ethyl carbamate were

found in the two wine types upon heat treatment of samples. The formation of ethyl carbamate correlated well with arginine degradation and citrulline excretion. Citrulline excretion during arginine degradation is of concern to the winemaker, since the reaction of citrulline and ethanol to form ethyl carbamate has been shown by other workers to occur even at normal wine storage temperatures. Winemakers, therefore, should avoid using arginine-degrading LAB starter cultures for inducing malolactic fermentation (MLF). In addition, spontaneous MLF in wine by undefined LAB strains should be discouraged, as this may lead to formation of ethyl carbamate precursors.

Ammonia detection with Nessler's reagent provides a simple, rapid test to assess arginine degradation by wine LAB in a complex medium, but is useful only for strains showing strong ammonia formation. The more sensitive enzymatic determination of ammonia is required for strains showing weak ammonia formation.

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Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction to the Thesis

Lactic acid bacteria (LAB) are isolated from a variety of sources such as beer, wine, cider, dairy products, meat, vegetables and the intestinal tract. The wine LAB, however, differ from other LAB in that they are aciduric, capable of growth at low pH (pH 4 or below); as well as ethanol-tolerant, capable of growth in the presence of ethanol (at least 10% v/v). As with other LAB, wine LAB are classified as either homofermentative (including pediococci and some lactobacilli) or heterofermentative (including *Leuconostoc oenos* and some lactobacilli). Homofermentative wine LAB normally yield two moles of lactic acid per mole of glucose fermented. Heterofermentative strains usually produce one mole each of lactic acid and carbon dioxide, and varied amounts of ethanol and acetic acid from one mole of glucose.

Wine LAB are responsible for the so-called malolactic fermentation (MLF) in wine. This is a secondary fermentation that normally occurs at the end of alcoholic fermentation of grape juice by yeasts (mainly *Saccharomyces* species). MLF is the conversion of L-malic acid to L-lactic acid and carbon dioxide, but sometimes, associated reactions such as the metabolism of carbohydrates and production of flavourful substances are included in its meaning. There are three beneficial effects of MLF:

1) acidity reduction: Acidity reduction is due to the transformation of dicarboxylic L-malic acid into the weaker monocarboxylic L-lactic acid with the release of carbon dioxide. This is particularly desirable for cool climate areas such as New Zealand, where wines often contain high levels of acids and thus taste too tart. This reduction in acidity improves the palatability of these acidic wines.

2) flavour modification/complexity: Some flavourful compounds, such as diacetyl, acetoin, 2,3-butanediol and ethyl lactate, may be produced during MLF. These compounds are believed to add complexity to the wine flavour or modify it.

3) increased microbial stability: Wines which have undergone MLF before bottling are microbiologically more stable than those without because of

nutrient depletion resulting from growth of LAB. Wines without MLF before bottling are prone to in-bottle MLF, resulting in the formation of undesirable gassiness and haze. However, microbiological stability is not absolute as it has been shown that wine LAB can grow in some wines even after MLF is completed (Davis *et al.*, 1986a,b).

4) promotion of growth of wine LAB: Although the conversion of malic acid to latic acid and carbon dioxide does not involve substrate-level phosphorylation, malic acid has been shown to stimulate growth rate and to increase cell yields of wine LAB (Pilone and Kunkee, 1972; 1976; Kunkee, 1991; Loubiere *et al.*, 1992). This stimulation has been attributed to the energy generated from the efflux of lactic acid through the chemiosmotic mechanism (Cox and Henick-Kling, 1989; 1990; Poolman *et al.*, 1991; Loubiere *et al.*, 1992) as well as the stimulation from small amounts of pyruvate formed from the secondary enzymatic reaction of the malolactic enzyme found in some wine LAB strains (Kunkee, 1991).

MLF can occur naturally by encouraging the development of indigenous wine LAB. Winemakers have traditionally used this method to conduct MLF and some successes have been achieved. Nonetheless, this method has some disadvantages. One of the serious drawbacks of this approach is that the characteristics of the indigenous wine LAB are often unknown. Therefore, this approach renders the process of MLF difficult to control (e.g. timing) and the results are often unpredictable.

It is now a common practice for winemakers to induce MLF by inoculating fermenting musts or wines with pure starter cultures (single or mixed). This approach gives the winemaker better control of MLF and entails the production of starter cultures with known characteristics. Selection of starter cultures requires a better understanding of the physiology and metabolism of wine LAB. However, the fundamental physiology and metabolism of wine LAB are still not well known, despite the research efforts of the past few decades. For instance, the energy source(s) in wine for growth of wine LAB and the undesirable compounds that may result during MLF are largely unknown. The utilisation of carbohydrates in wine (Davis *et al.*, 1986a,b) and in a model wine system (Liu, 1990) has been investigated, but there has been little work done on the nitrogen metabolism of wine LAB. The latter is clearly reflected by a lack of information on the metabolism of nitrogenous compounds in a review on MLF by Henick-Kling (1993) and also in a review

on *Leuconostoc oenos* by van Vuuren and Dicks (1993).

L-Arginine is a major amino acid found in grape juice and wine. It is known that arginine can be utilised by some wine LAB, but little attention has been paid to the mechanism, the enzymes involved, and the practical implication(s) of arginine metabolism. Arginine can be converted by some microorganisms, including some LAB, into ornithine, carbon dioxide, ammonia and adenosine triphosphate (ATP). Hence, arginine is a potential source of energy for wine LAB. This has practical implications for MLF. One problem often encountered when inducing MLF is the slow growth of wine LAB starter cultures. This may be due to the harsh environment of wine (low pH, ethanol and sulphur dioxide). Lack of energy sources may also aggravate this problem, because normal dry wines contain only 0.1 to 0.3 % residual reducing sugars.

In recent years, attention has been drawn to the possible involvement of MLF in the formation of ethyl carbamate (a carcinogen) in wine, but the information published so far is not conclusive. One concern associated with arginine metabolism by wine LAB is the potential excretion of the intermediates, citrulline and carbamyl phosphate, which are known to be direct precursors of ethyl carbamate.

From the above, it is evident that arginine metabolism in wine LAB and its oenological implications have not been thoroughly investigated. An elucidation of arginine metabolism in wine LAB not only would increase our knowledge about these microorganisms, but also generate information important to the wine industry. Consequently, this research project looks into the following aspects of arginine metabolism in wine LAB: 1) development of assays for arginine-degrading enzymes (Chapter 3); 2) occurrence of arginine-degrading enzymes in commercial and laboratory strains of wine LAB (Chapter 4); 3) time-course studies of arginine catabolism and metabolite formation (Chapter 5); 4) energetics of arginine catabolism (Chapter 6); 5) involvement of intermediates of arginine catabolism in the formation of ethyl carbamate (Chapter 7); 6) investigation of a simple test to assess arginine degradation (Chapter 8) and 7) induction of arginine-degrading enzymes (Chapter 9).

Chapter 2

Literature Review

2.1 The pathways of arginine catabolism by microorganisms

L-Arginine can be catabolised by microorganisms via one or more of the following major pathways: 1) the arginase-urease pathway; 2) the arginine transamidinase pathway; 3) the arginine decarboxylase pathway and 4) the arginine deiminase pathway (Abdelal, 1979). In addition, certain microorganisms catabolise arginine by pathways of limited distribution and these pathways have been described elsewhere (Cunin *et al.*, 1986). The main reactions of the first three major pathways are described below, while the complete arginine deiminase pathway is illustrated in Figure 2.1.

1) Arginine $\xrightarrow{\textit{arginase}}$ ornithine + urea $\xrightarrow{\textit{urease}}$ $CO_2 + 2NH_3$

2) Arginine $\xrightarrow{\textit{transamidinase}}$ ornithine + guanidino-derivative

3) Arginine $\xrightarrow{\textit{decarboxylase}}$ CO_2 + agmatine

Among these pathways the arginine deiminase pathway (ADI pathway), which formerly was referred to as the arginine dihydrolase pathway, is widely distributed in procaryotic organisms. This pathway is present in *Pseudomonas*, *Aeromonas*, *Mycoplasma*, *Spiroplasma*, *Spirochaeta*, bacilli, clostridia, halobacteria, cyanobacteria and lactic acid bacteria (reviewed by Abdelal, 1979; and Cunin *et al.*, 1986). By way of the ADI pathway (Figure 2.1), one mole of arginine is converted into one mole each of ornithine, carbon dioxide and ATP, but two moles of NH_3. This pathway involves three enzymes: arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK).

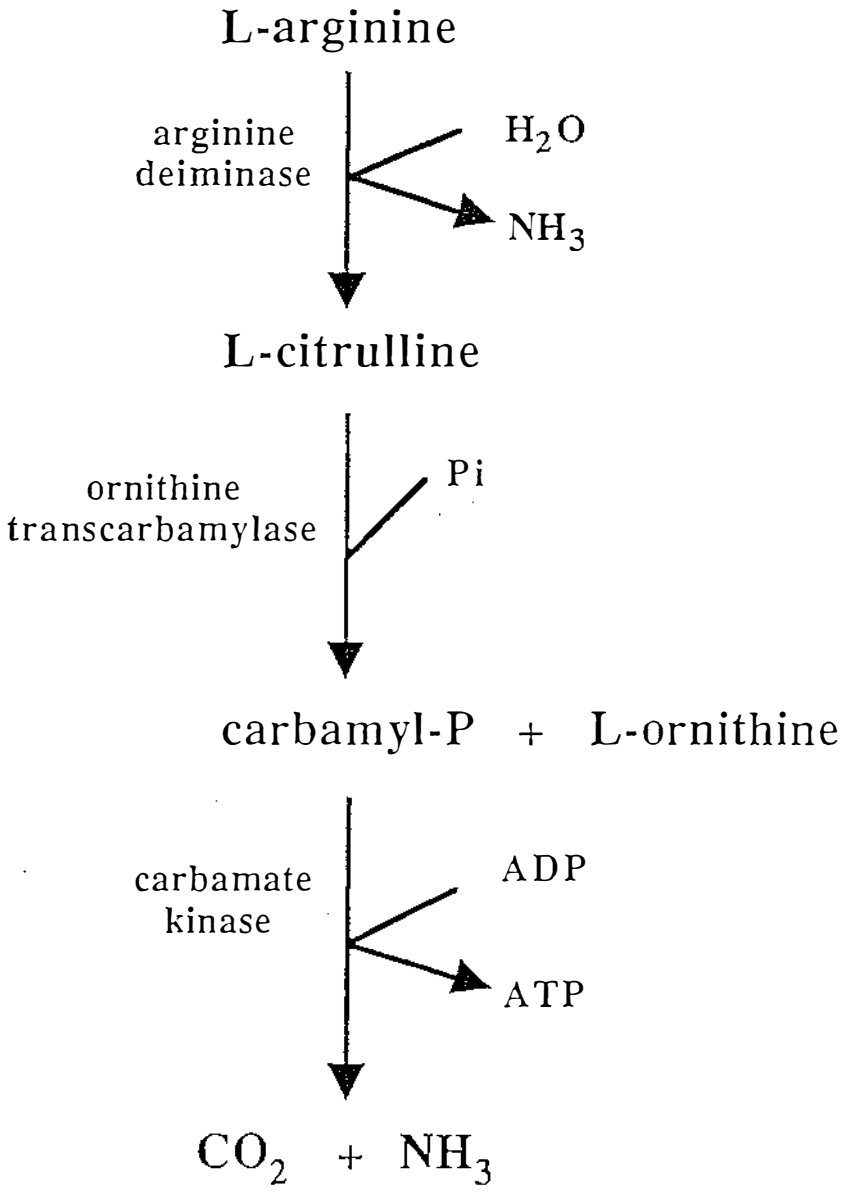

Fig. 2.1 The arginine deiminase pathway

The ADI pathway has been shown to exist in a number of lactic acid bacteria (LAB). For example, it has been found in strains of *Streptococcus lactis* (now *Lactococcus lactis*, Schleifer *et al*., 1987) (Crow and Thomas, 1982), a strain of *Lactobacillus sake* isolated from meat (Montel and Champonier, 1987) and *Lactobacillus buchneri* NCDO 110 (Manca de Nadra *et al*., 1988). To date, there is no evidence for the presence of other pathways of arginine metabolism in LAB.

2.2 Arginine catabolism in wine lactic acid bacteria

Information on arginine catabolism in wine LAB is scant and controversial. Bergey's Manual of Systematic Bacteriology (Vol 2, 1986) states that the genus *Leuconostoc*, including *Leuconostoc oenos*, does not hydrolyse arginine. This contradicts several reports showing that some strains of *Lc. oenos* are, in fact, able to degrade arginine (Kuensch *et al*., 1974; Weiller and Radler, 1976; Garvie and Farrow, 1980; Liu, 1990; and Pilone *et al*., 1991). Probably the ability of *Lc. oenos* strains to degrade arginine varies with strains. That is, the inability to do so is not a general characteristic of the genus *Leuconostoc*.

The limited information available indicates that certain wine LAB do not degrade arginine in the absence of sugars, but do so in their presence. For instance, two strains of *Lc. oenos* and one strain of *Lb. brevis* have been reported not to utilise arginine in a medium lacking sugars, but use the amino acid when present in the medium containing a range of sugars (Liu, 1990; unpublished data). This suggests that the catabolism of arginine in these wine LAB requires the presence of a fermentable substrate.

The concentrations of ornithine and ammonia increased consistently when arginine was degraded by strains of *Lc. oenos* and heterofermentative lactobacilli (Kuensch *et al*., 1974; Weiller and Radler, 1976; Davis *et al*., 1985; Liu, 1990). Pilone *et al*. (1991) also reported that of all the wine LAB tested, most strains of *Lc. oenos* and all heterofermentative wine lactobacilli were indeed able to produce and release ammonia from arginine degradation. However, homofermentative wine LAB do not seem to degrade arginine (Kuensch *et al*., 1974; Liu, 1990; Pilone *et al*. 1991). The reason for this is not known.

Although the degradation of arginine by some wine LAB has been observed

frequently, the metabolic pathway has not been clearly established. The presence of the ADI pathway in wine LAB has been suggested (Weiller and Radler, 1976; Liu, 1990; Pilone *et al.*, 1991) based on the formation of ornithine and ammonia from arginine and the presence of this pathway in other LAB (Figure 2.1). However, Kuensch *et al.* (1974) proposed that *Lc. oenos* metabolises arginine using the urea cycle, based on the formation of ornithine, ammonia and citrulline. This proposition has been extended by Sponholz *et al.* (1991) and Sponholz (1991,1992) to other members of wine LAB. However, these suggestions for the possible pathway of arginine catabolism are not based on critical evidence of the presence of the pathway enzymes. Therefore, it is essential to clarify the pathway of arginine metabolism in wine LAB by providing valid evidence, such as the demonstration of the presence of the enzymes involved.

2.3 Arginine catabolism in non-wine lactic acid bacteria

2.3.1 Occurrence of the arginine deiminase pathway enzymes

The occurrence of the ADI pathway enzymes (ADI, OTC and CK) in non-wine LAB varies with species and strains. Some LAB possess all three enzymes, while others contain only one or two or none of the enzymes. Manca de Nadra *et al.* (1982) surveyed several LAB for the presence of the first two enzymes (ADI and OTC). They found that *Lb. helveticus* strains NCDO 384 and ATCC 15801 and *Lb. jensenii* ATCC 25258 possess only the first enzyme (ADI), while several strains of *Lb. plantarum* were found to lack all three enzymes. Crow and Thomas (1982) detected no activity of ADI, very weak or no activity of OTC, but the definite presence of CK activity in strains of *Lactococcus lactis* subsp. *cremoris*.

2.3.2 Regulation of the arginine deiminase pathway enzymes

The primary function of the ADI pathway is to generate energy (ATP) and, according to Cunin *et al.* (1986), energy depletion is an important signal for inducing the ADI pathway enzymes in pseudomonads. Arginine enhances the inductive response of enzyme synthesis in *Ps. aeruginosa* PAO (Mercenier *et al.*, 1980). Both energy depletion and the presence of arginine are required for the coinduction of the ADI pathway enzymes in *Streptococcus faecalis* ATCC 11700 (Simon *et al.*, 1982). Repression of the pathway occurs under conditions which promote the energy status of the cell. For example, the presence of a fermentable substrate (a carbohydrate) or aerobic and anaerobic respiration, can cause such a repression (Cunin *et*

al., 1986). Repression of the ADI pathway enzymes also seems to be simultaneous, as demonstrated in *Streptococcus sanguis* (Ferro *et al.*, 1983) and *Str. mitis* (Hiraoka *et al.*, 1986).

The synthesis of the ADI pathway enzymes in heterofermentative LAB seems to differ from that in homofermentative LAB. The synthesis of these enzymes in heterofermentative *Lb. buchneri* NCDO 110 apparently is not repressed by glucose (5 g/L) (Manca de Nadra *et al.*, 1986a). This contrasts with the repression of enzyme synthesis by glucose (5 g/L) in homofermentative *Lb. leichmannii* ATCC 4797 (Manca de Nadra *et al.*, 1986b). The repression of arginine hydrolysis in homofermentative lactobacilli by high glucose concentrations also has been noted elsewhere. Sharp *et al.* (1966) reported that when glucose concentrations were high (20 g/L), homofermentative lactobacilli did not hydrolyse arginine, whereas almost all heterofermentative lactobacilli produced ammonia from arginine. As the glucose concentration was lowered, some homofermentative types were able to hydrolyse arginine.

The nature of the fermentable sugar also appears to affect the synthesis of the ADI pathway enzymes. Crow and Thomas (1982) reported that in *Lactococcus lactis* subsp. *lactis*, the specific activities of ADI and OTC were higher when galactose rather than glucose or lactose was the growth sugar, but CK was unaltered. A similar trend was found for all three enzymes in *Lb. buchneri* NCDO 110 (Manca de Nadra *et al.*, 1986a). In *Lb. leichmannii* ATCC 4797, however, the specific activities of ADI and OTC (CK not mentioned) were two or threefold lower when galactose, rather than glucose and lactose, was the carbohydrate source (Manca de Nadra *et al.*, 1986b).

2.3.3 Energetics of arginine catabolism and molar growth yield

One mole of ATP is generated per mole of arginine catabolised by way of the ADI pathway. Therefore, the organism may be able to utilise the energy derived from the degradation of arginine for growth when all three enzymes in the ADI pathway are functional.

Some LAB are known to utilise arginine as an energy source in the presence of fermentable carbohydrates. These include *Str. faecalis* (Bauchop and Elsden, 1960; Deibel, 1964), *L. lactis* (Crow and Thomas, 1982) and a *Lb. plantarum* strain from fish (Jonsson *et al.*, 1983). Some reports also show

that arginine alone does not support the growth of these microorganisms and that a fermentable carbohydrate is required for "sparking" the utilisation of arginine (Bauchop and Elsden, 1960; Jonsson *et al.*, 1983). This agrees with the finding for several wine LAB mentioned in Section 2.2.

The molar growth yield (Y_m) and the Y_{ATP} value are commonly used to predict the amounts of ATP produced when a specific energy source is utilised (ATP moles = Y_m/Y_{ATP}), or to evaluate energy-yielding pathways in a microorganism (Senez, 1962). Molar growth yield is defined as the grams (dry weight) of cells produced per mole of an energy source consumed. Y_{ATP} is the molar growth yield per mole of ATP, and is a biological constant for very different microorganisms (Senez, 1962). The generally accepted value of Y_{ATP} is 10.5 $\pm$ 2 (Bauchop and Elsden, 1960; Senez, 1962; Gottschalk, 1986). For example, a $Y_{ATP(arg)}$ of 8.6 is reported for a strain of *Lb. plantarum* from fish (Jonsson *et al.*, 1983), in addition to the $Y_{ATP(arg)}$ of 10.5 $\pm$ 2 reported for *Str. faecalis* (Bauchop and Elsden, 1960).

Contrary to the reports mentioned above, the generally accepted value of Y_{ATP} (10.5) has been claimed to be erroneously low by Moustafa and Collins (1968). These authors presented an average value of Y_{ATP} of 17 for carbohydrates in certain lactic acid bacteria with a $Y_{ATP(arg)}$ value of 17.8. This compares favourably with the $Y_{ATP(arg)}$ value of 16 in *L. lactis* (Crow and Thomas, 1982). The occurrence of anomalous Y_m and Y_{ATP} values in other microorganisms has been discussed elsewhere (Forrest and Walker, 1971). Despite these irregular data, the value of 10.5 of Y_{ATP} for growth with all the monomers available for biosynthesis is now generally accepted (Gottschalk, 1986).

The discrepancies of Y_{ATP} values can be accounted for by various factors. These include medium composition (complex or synthetic), unknown metabolic pathways, concentrations of energy sources, autolysis, culturing methods (batch or continuous culture; aerobic or anaerobic), and methods of calculating Y_{ATP} values (with or without correcting for unused energy sources). Another factor may be energy uncoupling; that is, ATP formed may not necessarily be coupled to growth. An ineffective coupling between the energy-yielding catabolism and the energy-utilising anabolism may result in energy (ATP) being wasted as heat. Conditions leading to energy uncoupling include nutrient limitations, excess energy sources and high

temperature (Stouthamer, 1969; Forrest and Walker, 1971).

The energy role of arginine catabolism in wine LAB has not been studied, nor has the coupling of energy (ATP) to growth. Strains of wine LAB possessing the complete enzyme system of the arginine deiminase pathway would have the advantage of gaining energy from arginine degradation over other strains containing only part of the enzyme system. However, this may not necessarily be the case if energy uncoupling occurs. One of the aims of this research is to ascertain whether ATP available from the degradation of arginine is indeed used for growth of wine LAB. This can be achieved by determining molar growth yield data for arginine consumption. Conditions that may result in energy uncoupling should also be investigated, because such information would be useful in encouraging or discouraging growth of starter cultures containing the ADI pathway enzymes.

2.3.4 Arginine transport and genetics of the arginine deiminase pathway system

Little is known about the arginine transport system in microorganisms containing the ADI pathway, including LAB, except in *L. lactis* subsp *lactis* ML_3. In membrane vesicles of galactose/arginine-grown cells of this LAB strain, which is known to possess the ADI pathway, arginine is transported into the cell via an antiport system with ornithine (Driessen *et al.*, 1987; Poolman *et al.*, 1987; Konings *et al.*, 1991). This process does not require metabolic energy and is apparently inducible by arginine. It is possible that the arginine-ornithine exchange system also operates in wine LAB, but further research is needed to confirm this.

L. lactis subsp. *lactis* strains are known to metabolise arginine, while *L. lactis* subsp. *cremoris* strains do not normally metabolise this amino acid (Crow and Thomas, 1982). However, *L. lactis* (Arg^+) and *L. cremoris* (Arg^-) can apparently change from being Arg^+ to Arg- and *vice versa*. Heap *et al.* (1978) observed the instability of three *L. lactis* isolates (Arg^+) which on subculture gave rise to arginine-negative (Arg^-) variants. Conversely, Crow and Thomas (1982) obtained a mutant of *L. cremoris* 166 which hydrolysed arginine. In addition, Coventry *et al.* (1984) described a number of phage-resistant derivatives of *L. cremoris* strains that differed from their parent strains (Arg^-) in being able to hydrolyse arginine. Furthermore, Davey and Heap (1993) noted the appearance of the arginine phenotype (Arg^+) in *L. lactis* subsp. *cremoris* 2204 following phage transduction (this phage was

induced from an arginine-positive *L. lactis* subsp. *lactis* 2204 L). This suggests the involvement of phage in the transformation of arginine phenotype, but the exact role of the phage in the phenotypic change remains to be determined. There is, however, no evidence available as to the involvement of plasmid(s) in this phenotypic change.

The genetics of the ADI pathway system is relatively well known in *Pseudomonas aeruginosa*. In this bacterium, the ADI pathway is encoded by a chromosomal four-gene cluster: *arcA* (ADI), *arcB* (catabolic OTC), *arcC* (CK) and *arcD* (arginine uptake), and mutations in any of the genes affect the utilisation of arginine (Mercenier *et al.*, 1982; vander Wauven *et al.*, 1984; Lüthi *et al.*, 1986). It is likely that a similar structural gene cluster may be found in other microorganisms possessing the ADI pathway, since the enzymes of the pathway are coinducible as well as simultaneously repressible, as mentioned in Section 2.3.2. Genetic information would be of use in explaining the lack of some of the pathway enzymes, which was discussed in Section 2.3.1.

2.4 Formation of ethyl carbamate in wine

Ethyl carbamate (urethane) is a carcinogenic compound (Mirvish, 1968) and most fermented foods and beverages, including wine, contain trace amounts of ethyl carbamate (Ough, 1976). The present interest in ethyl carbamate commenced with the discovery of ethyl carbamate at concentrations greatly exceeding that which occurs naturally and the establishment of guidelines to limit this carcinogen in alcoholic beverages (Lee, 1988). Therefore, it is important to keep the ethyl carbamate concentration in wine as low as possible.

The formation of ethyl carbamate is a spontaneous chemical reaction involving ethanol and a compound containing a carbamyl group (N-carbamyl compound) under acidic conditions (pH 4 or below) (Ough *et al.*, 1988). The N-carbamyl compounds that are precursors of ethyl carbamate include urea, citrulline, carbamyl phosphate, allantoin, N-carbamyl-alpha-amino acids and N-carbamyl-beta-amino acids. A number of factors affect the reaction, such as the concentrations of reactants, temperature and pH. The amount of ethyl carbamate produced is proportional to the concentration of the reactants. The ethanolysis of carbamyl phosphate to form ethyl carbamate proceeds rapidly at room temperature (25°C), while heating is required for the rapid

ethanolysis of urea (50°C) and citrulline (60 to 80°C) to form this carcinogen (Ough *et al*., 1988). The ethanolysis of urea and citrulline can also proceed at low to normal storage temperatures, though at a much slower rate (Tegmo-Larsson and Spittler, 1990; Stevens and Ough, 1993).

Current information on the origin of ethyl carbamate in wine is still inconclusive, particularly with regard to the influence that wine LAB have on its formation. Most work done is focused on the involvement of urea. The ethanolysis of urea is a major source of ethyl carbamate in wine (Monteiro *et al*., 1989; Monteiro and Bisson, 1991; Ough *et al*., 1990,1991; Ough, 1991). Urea is the product of arginine metabolism in yeasts, and yeasts of *Saccharomyces cerevisiae* are known to possess arginase, which cleaves arginine into ornithine and urea (Abdelal, 1979). Some urea is excreted out of the yeast cell, then partially reacts with ethanol to form ethyl carbamate, although some of the excreted urea can be reabsorbed. Purines and pyrimidines are also potential sources of urea, but these compounds are not present in sufficient quantity in grape juice to be a significant source of urea in alcoholic fermentation (Monteiro *et al.*, 1989). Besides urea, small amounts of citrulline naturally present in grape juice and wine may also contribute to ethyl carbamate formation (Ough *et al.*, 1988; Tegmo-Larsson and Henick-Kling, 1990b).

Contrary to the above discussion, the role of yeast growth and metabolism in forming ethyl carbamate has been questioned. Tegmo-Larsson and Henick-Kling (1990a) claimed that yeast growth and fermentation seemed to have no influence on ethyl carbamate concentration in wine, except for the increased ethanol content, since non-fermented juices fortified with ethanol produced as much ethyl carbamate as fermented juices. Also, no ethyl carbamate precursors were produced from yeast during extended lees (settled yeast cells) contact after fermentation. The discrepancy between the work of Tegmo-Larsson and Henick-Kling (1990a) and that of other workers (Monteiro *et al*., 1989; Monteiro and Bisson, 1991; Ough *et al*., 1990,1991; Ough, 1991) could be due to the strain of yeast used, the concentration of arginine present and the fermentation conditions.

Yeasts are not the only microorganisms present in wine, as LAB flora may develop at the end of yeast alcoholic fermentation. Few studies are available on the influence of wine LAB on ethyl carbamate formation in wine. Some

researchers report that ethyl carbamate formation is not affected by malolactic fermentation brought about by wine LAB (Tegmo-Larsson *et al.*, 1989; Tegmo-Larsson and Henick-Kling, 1990a). Others claim that the potential of ethyl carbamate formation is higher in wine that has undergone malolactic fermentation and this has been attributed to the excretion of urea formed from arginine metabolism by wine LAB by way of the urea cycle (Sponholz *et al.*, 1991; Sponholz, 1991,1992). However, there is no biochemical evidence for the presence of arginase, the key enzyme for the conversion of arginine to urea, in wine LAB, if the urea cycle were involved (see Figure 5.9 for the urea cycle pathway).

Evidence for the production of ornithine and ammonia from arginine by some LAB led to the suggestion that wine LAB may catabolise arginine by way of the arginine deiminase pathway (Weiler and Radler, 1976; Liu, 1990) and, therefore, may produce ethyl carbamate precursors, citrulline and carbamyl phosphate (Liu, 1990). This suggestion is supported by the evidence that microorganisms defective in or lacking the ADI pathway enzyme OTC do excrete citrulline. This has been demonstrated in strains of *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pseudomonas* (Kakimoto *et al.*, 1971; Yamamoto *et al.*, 1974) and in mutants of *Pseudomonas aeruginosa* (Haas *et al.*, 1979). Excretion of citrulline also has been found in a *Lb. sake* meat isolate containing the enzymes ADI and OTC (Montel and Champomier, 1987), although the presence of CK was not mentioned by the authors. Moreover, other microorganisms with the complete enzyme system of the arginine deiminase pathway also have been shown to excrete citrulline during arginine catabolism; for instance, *Mycoplasma sp.* (Schimke and Barile, 1963). It is possible that wine LAB may excrete citrulline (and carbamyl phosphate) from the degradation of arginine if they degrade this amino acid by means of the ADI pathway. If this is possible, it becomes extremely important to confirm the presence of the ADI pathway in wine LAB used as starter cultures for conducting malolactic fermentation in wine, because of the potential for the formation of ethyl carbamate precursors.

The origin of ethyl carbamate precursors in wine is more complex than previously envisaged. There is still a lack of information on the formation of ethyl carbamate with respect to precursor formation by wine LAB. Therefore, one of the objectives of this project is to investigate the relationship between ethyl carbamate formation and arginine catabolism by wine LAB.

Chapter 3

Verification of Assay Systems for Arginine Deiminase Pathway Enzymes in Selected Wine Lactic Acid Bacteria

3.1 Introduction

Assays for the arginine deiminase (ADI) pathway enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK), are available for some microorganisms. These assays may not be appropriate, however, for the three enzymes from wine lactic acid bacteria (LAB). This is because these enzyme assays were developed under specific conditions using one particular species of microorganism. It is incorrect to assume that such an assay can be used, *per se*, to analyse for that enzymatic activity in a different microorganism, since enzyme properties such as pH optima and kinetic parameters vary appreciably from species to species (Dobrogosz, 1981).

For the purpose of this project, it was necessary to establish enzyme assay conditions appropriate for the wine LAB being investigated. Therefore, the effect of factors such as substrate and enzyme concentration and pH on enzyme activity was investigated in extracts of the wine LAB strains used in the present study. It is essential that the concentrations of substrates used are saturating, that the enzyme activity is linearly dependent on the amount of enzyme added and that the enzyme assays are conducted at their pH optima. The goal here was to establish standard assay conditions appropriate for wine LAB, so that these assays could be used routinely for subsequent experiments.

3.2 Materials and methods

3.2.1 Lactic acid bacterial strains

Strains of wine LAB used in this investigation included strains MCW, OENO and Er1a of *Leuconostoc oenos*, *Lactobacillus brevis* 250 and *Lactobacillus buchneri* CUC-3. All strains are heterofermentative and were ammonia positive when tested using Nessler's reagent (see Section 8.2). The original sources of these strains are listed in Table 4.1, Chapter 4.

3.2.2 Medium preparation, culturing and harvesting of wine LAB

The basal medium (pH 5.5) for culturing wine LAB consisted of the following components in deionised (DI) water per litre: tryptone, 5 g; peptone, 5 g; yeast extract, 5 g; Tween 80 (5% aqueous solution), 1 mL; $MgSO_4.7H_2O$, 0.2 g; $MnCl_2.4H_2O$, 0.05 g and clarified vegetable juice, 200 mL. The clarified juice was prepared from Campbell's V8 Vegetable Juice™ (Campbell's Soups, Australia) with no added sugar. The juice was filtered through Whatman No.1 filter paper and diatomaceous earth filter aid (Witco Corp., Kenite Diatomite 3000). This medium contained three possible sources of L-arginine (yeast extract, tryptone and peptone) at a basal level of approximately 0.5 g/L and was further supplemented with L-arginine and D-glucose to a final concentration of about 5 g/L each. Solutions of arginine and glucose (pH 5.5, 10 g/L each) were first aseptically membrane-filtered (0.45 μm pore-size), then added to equal volumes of the autoclaved basal medium (2-fold concentrated). The supplemented medium was referred to as Tomato Juice Broth-Arginine-Glucose (TJBAG). Before inoculating TJBAG, wine LAB were precultured in vegetable juice or apple juice MRS medium (VJMRS or AJMRS, pH 5.5) using Difco Lactobacilli MRS Broth or Oxoid M.R.S. Broth supplemented with clarified vegetable juice (prepared as above) or apple juice (200 mL/L). Apple juice was prepared from a concentrate (Fresh-up™, the New Zealand Apple and Pear Marketing Board) by diluting to normal strength with DI water.

Actively growing wine LAB in VJMRS or AJMRS were used to inoculate (1%, v/v) TJBAG media, which were then incubated at 30°C. Cells were harvested between the late exponential phase and early stationary phase, as indicated by observing the initial settling of cells. Cell harvesting was achieved by centrifugation at 7000 x g for 10 min at 5°C in a Sorvall RC-5B refrigerated centrifuge. The harvested cells (from 300 mL culture) were divided into three portions, each containing approximately 300 mg wet cells. The wet cells were washed by resuspending in 0.01 M potassium phosphate buffer (pH 7.0) and centrifuging at 7000 x g for 10 min at 5°C. This was repeated two more times, then the washed cell pellets were stored frozen (-13°C) until used for assay. The phosphate buffer (0.01 M) was prepared by dissolving 0.871 g K_2PO_4 in 500 mL DI water, then adjusting to pH 7 with 30% H_3PO_4.

3.2.3 Preparation of cell-free extracts

Frozen cell pellets (about 300 mg wet weight) were thawed and resuspended in 5 mL of 0.01 M potassium phosphate buffer (pH 7.0). Cell disintegration was achieved by two passages through a French pressure cell (Aminco, Maryland, USA) at a pressure of 7,600 PSI. Cell debris was removed by centrifugation at 14,500 x g for 10 min at 5°C and the supernatant extract was used to assay for the three enzymes of the arginine deiminase pathway (ADI, OTC and CK). Enzyme assays were carried out on the day of extract preparation.

3.2.4 Analytical methods

This section describes common analytical methods employed in these and subsequent experiments. Other specific methods are given in Chapters 4, 6, 7, 8 and 9.

3.2.4.1 Determination of protein concentration

Protein concentration was determined using the dye binding method of Sedmak and Grossberg (1977). This method is sensitive, fast, and at least as accurate as the Lowry method (Scopes, 1987). It is based on the conversion of Coomassie brilliant blue G250 in dilute acid from a brownish-orange colour to an intense blue colour upon the binding of the dye to proteins.

The Coomassie blue G250 dye (BDH) was prepared as a 0.06% solution in 3% (w/v) perchloric acid and filtered through Whatman No.1 filter paper. The assay involved mixing 1.0 mL sample, 1.5 mL deionized water (DI water) and 2.5 mL dye solution and measuring the absorbance at 595 nm at room temperature, using a Bausch and Lomb Spectronic-20 Spectrophotometer. A mixture of 2.5 mL each of dye solution and DI water was used as a blank to zero the instrument. A standard solution (20 μg/mL) of bovine serum albumin (Fraction V, Sigma) was used to prepare the standard curve. This was made with 0.5-2.5 mL of protein standard solution and DI water to make up to 2.5 mL and dye solution (2.5 mL).

3.2.4.2 Determination of arginine concentration

Arginine was determined colorimetrically based on the Sakaguchi reaction using the method of Gilboe and Williams (1956).

Reagents

1) Urea (40%): 200 g urea dissolved in a small amount of DI water first and made up to 500 mL with DI water.

2) 8-Hydroxylquinoline (0.02%): 0.2 g 8-Hydroxylquinoline dissolved in 100 mL ethanol (95%) first and made up to 1000 mL with DI water.

3) Sodium hydroxide (10%): 50 g NaOH in 500 mL DI water.

4) Sodium hydroxide (5%): 25 g NaOH in 500 mL DI water.

5) Sodium hypobromite (1%): 0.68 mL liquid bromine in 200 mL 5% NaOH.

6) Arginine standard stock solution (500 μg/mL): 60.50 mg arginine.HCl (Sigma) in 100 mL DI water.

7) Arginine standard working solution (10 μg/mL): 50-fold dilution of arginine standard stock solution in DI water.

Procedure

To a 5 mL sample, 1 mL 0.02% 8-hydroxylquinoline and 1 mL 10% NaOH were added. The solution was mixed thoroughly and placed in an ice bath for 2 min. Then 0.2 mL 1% sodium hypobromite was added rapidly to develop colour. After mixing and within 15 seconds, 1 mL 40% urea was added to destroy excess hypobromite and prevent colour fading. One min after adding hypobromite, 5 mL ice-cold water was added, the solution mixed and the absorbance read within 5 min at 500 nm using a Bausch and Lomb Spectronic-20 Spectrophotometer. A mixture of 5 mL water and reagents was used as the blank to zero the instrument. The arginine standard curve was prepared with the arginine standard working solution (1-4 mL), with DI water to make up to 5 mL.

3.2.4.3 Determination of citrulline concentration

Citrulline was determined using the method of Archibald (1944). This method is based on the formation of a coloured reaction product with diacetyl monoxime in acid solution.

Reagents

1) Sulphuric-phosphoric acid mixture: 1 volume of concentrated sulphuric acid plus 3 volumes of concentrated phosphoric acid (85%).

2) Diacetyl monoxime (3%): 3 g diacetyl monoxime in 100 mL DI water.

3) Citrulline stock standard (100 mg/L): 10.0 mg citrulline (Sigma) in 100 mL DI water. Stored frozen.

4) Citrulline working standard (10 μg/mL): 1 mL stock standard in 10 mL DI water.

Procedure

To a 1 mL sample, 3 mL DI water, 2 mL acid mixture and 0.25 mL diacetyl monoxime reagent were added. A blank containing 4 mL water plus reagents was used to zero the instrument. The test tubes were capped, mixed thoroughly and heated in a covered boiling water bath for 30 min (Spector and Jones, 1963). After cooling for about 10 min, the absorbance was read at 490 nm. The standard curve was prepared with the citrulline working standard (0.5-4.0 mL), with DI water to make up to 4.0 mL.

A heating period of 30 min was employed instead of the 10 min suggested by Archibald (1944). This is necessary for full colour development, since colour development is still proceeding at a linear rate after 10 min (Spector and Jones, 1963). Both L- and D-citrulline isomers react with diacetyl monoxime while arginine does not give a colour reaction (Oginsky, 1957). However, urea reacts with diacetyl monoxime to produce a colour (Archibald, 1944). The possibility of urea interference in the citrulline analysis is considered in Section 5.4, Chapter 5.

3.2.4.4 Determination of ornithine concentration

The concentration of ornithine was determined using an amino acid analyzer (Pharmacia LKB alpha plus). These determinations were carried out by the Department of Chemistry and Biochemistry, Massey University. In some experiments citrulline was also determined using the amino acid analyzer. Amino acids were separated by ion-exchange and estimated by post-column derivatization with ninhydrin.

3.2.4.5 Analysis of ammonia, urea, L-malic acid, glucose and fructose

These compounds were analysed enzymatically using enzyme kits supplied by Boehringer Mannheim, Germany.

3.2.4.6 Measurements of cell mass

Bacterial cell mass was measured as optical density (OD) at 600 nm using a Bausch and Lomb Spectronic-20 Spectrophotometer. When OD readings were above 0.6, samples were diluted with the same but uninoculated media.

3.2.4.7 Measurement of pH

pH was measured using an Orion Research pH meter (Digital Ionalyzer/501) calibrated against pH 7.0 and 4.0 buffers.

3.2.5 Assay for arginine deiminase

Arginine deiminase (ADI) catalyses the hydrolysis of arginine to citrulline and ammonia. Thus, the activity of ADI can be assayed by measurement of either end product, citrulline or ammonia. For this project, ADI activity was measured by determining the rate of citrulline formation, since the determination of citrulline by the procedure of Archibald (1944) as modified by Spector and Jones (1963) is inexpensive, sensitive, and relatively simple. The measurement of ADI activity adopted here is based on the method of Oginsky (1955) with modifications.

Reagents

1) 0.2 M L-arginine (pH 5.8): 1.742 g L-arginine (free base, Sigma) in 50 mL DI water, adjusted to pH 5.8 with conc. HCl.

2) 0.2 M phosphate buffer (pH 5.8): 3.484 g K_2HPO_4 in 100 mL DI water, adjusted to pH 5.8 with 30% H_3PO_4.

3) Enzyme extract: prepared fresh; undiluted or diluted with 0.01 M pH 7 phosphate buffer.

Arginine and buffer solutions were stored at 4°C and used within two weeks.

Procedure

The standard assay mixture (total 6.1 mL) consisted of 4.0 mL 0.2 M potassium phosphate buffer (pH 5.8), 1.6 mL 0.2 M L-arginine (pH 5.8) and 0.5 mL undiluted or diluted extract. The enzymatic reaction (carried out in duplicate) was started by the addition of enzyme extract to a preincubated (approximately 10 min) reaction mixture containing phosphate buffer and arginine held in a water bath at 37°C. At 10 min or 15 min intervals, a 1.4 mL aliquot of the reaction mixture was removed and added to 0.1 mL ice-cold 70% perchloric acid and mixed to stop the reaction. One mL of the supernatant (top layer) of the mixture was then analysed for citrulline. One unit of enzymic activity was defined as that amount of enzyme catalysing the formation of one μmole of citrulline per min. Specific activities were expressed as units per mg extract protein (U/mg).

3.2.6 Assay for ornithine transcarbamylase

The *in vivo* physiological role of catabolic ornithine transcarbamylase (OTC) is to catalyse the conversion of citrulline (resulting from the hydrolysis of arginine by ADI) to ornithine and carbamyl phosphate. However, the equilibrium is much in favour of the formation of citrulline *in vitro* (Stalon *et al.*, 1967). Hence, the activity of OTC is most easily measured by the rate of citrulline synthesis from ornithine and carbamyl phosphate. Although two separate OTC enzymes (one catabolic and one anabolic) exist in *Pseudomonas* (Stalon *et al.*, 1967), apparently only one OTC enzyme with two functions (anabolic and catabolic) is present in *Lb. buchneri* NCDO 110 (Manca de Nadra *et al.*, 1981). To date, there is no evidence for the presence of two separate OTC enzymes in any LAB. With physiologically reversible enzymes, enzymic activities are often similar in both forward and reverse directions (Scopes, 1987). Therefore, for this project, OTC activity was measured in the direction of citrulline synthesis from ornithine and carbamyl phosphate. The procedure described below is a modification of the method of Jones (1962).

Reagents

1) 1 M Tris.HCl (pH 8.2): 7.88 g Tris.HCl (Boehringer Mannheim) in 50 mL DI water, adjusted to pH 8.2 with KOH pellets and 30% KOH.

2) 0.2 M L-ornithine.HCl (pH 8.2): 1.686 g L-ornithine.HCl (Sigma) in 50 mL DI water, adjusted to pH 8.2 with 30% KOH.

3) 0.2 M carbamyl phosphate: 0.122 g di-lithium carbamyl phosphate (Sigma) in 4 mL DI water; prepared fresh.

4) Enzyme extract: prepared fresh; undiluted or appropriately diluted with 0.01 M pH 7 phosphate buffer.

Buffer and ornithine solutions were stored at 4°C and used within two weeks.

Procedure

The standard assay mixture (5.0 mL) consisted of 2.3 mL 1 M Tris.HCl (pH 8.2), 1.2 mL 0.2 M L-ornithine.HCl (pH 8.2), 1.0 mL 0.2 M carbamyl phosphate and 0.5 mL diluted extract. The reaction was initiated with the addition of carbamyl phosphate, followed by adding enzyme extract to the pre-incubated (37°C) mixture of Tris.HCl and L-ornithine.HCl. At 3-min intervals, a 1.2 mL aliquot of the reaction mixture was removed and added to 0.1 mL ice cold 70% perchloric acid and mixed to stop the reaction. One mL of the supernatant (top layer) of the mixture was analysed for citrulline. One

unit of enzyme activity was defined as that amount of enzyme catalysing the formation of one μmole of citrulline per min. Specific activities were expressed as units per mg extract protein (U/mg).

Control assay for OTC

Small amounts of citrulline can be synthesised non-enzymatically from ornithine and carbamyl phosphate at 37°C (Jones, 1962). An OTC assay mixture without enzyme extract represents this non-enzymatic synthesis of citrulline. It was experimentally demonstrated during this investigation that the non-enzymatic synthesis of citrulline was directly proportional to heating time and substrate concentrations. Citrulline synthesised non-enzymatically can result in interference in the assay of OTC using the standard assay mixture under conditions of prolonged heating (>12 min) or when using higher substrate concentrations (60 mM or higher for each substrate). Variation of pH in the range of 7.6-9.1 did not affect the non-enzymatic synthesis of citrulline (data not shown). Very little citrulline is synthesised non-enzymatically under the standard assay conditions described above. Blanks lacking enzyme extracts were often included, particularly when enzyme activities were weak. The amount of citrulline synthesised non-enzymatically was subtracted from the total amount of citrulline. This correction was, however, relatively small.

3.2.7 Assay for carbamate kinase

Carbamate kinase (CK) catalyses the reversible conversion of carbamyl phosphate and ADP to ATP, NH_3 and CO_2. The equilibrium favours ATP synthesis and, therefore, it is easier to measure CK activity by following CO_2 or ammonia production or the disappearance of carbamyl phosphate in the presence of ADP and Mg^{++} (Jones, 1962). However, carbamyl phosphate solutions are not stable (approximate half-life is 50 min at 37°C and 18 hours at 0°C); and are readily decomposed chemically, generating cyanate and phosphate (Jones, 1962). Cyanate is further chemically broken down to CO_2 and NH_3 (Merck Index, 9th edition). Consequently, the chemical decomposition of carbamyl phosphate would lead to serious errors in the measurement of CK activity by following CO_2 or ammonia formation.

For this project, CK activity was measured in the following coupled assay system which determined the rate of ATP production:

1. $$\text{Carbamyl-P} + \text{ADP} \xrightarrow[\text{kinase}]{\text{carbamate}} CO_2 + NH_3 + \text{ATP}$$

2. $$\text{ATP} + \text{Glucose} \xrightarrow{\text{hexokinase}} \text{Glucose-6-P} + \text{ADP}$$

3. $$\text{Glucose-6-P} + NADP^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-P}} \text{6-P-Gluconolactone} + NADPH + H^+.$$

In the presence of excess coupling enzymes, hexokinase and glucose-6-dehydrogenase, CK activity can be followed spectrophoto metrically by determining the rate of NADPH production. This method is based on that of Crow and Thomas (1982) with modifications.

Reagents

1) 0.2 M Tris.HCL (pH 7.9): 1.58 g Tris.HCI (Boehringer Mannheim) in 50 mL DI water.

2) 0.2 M ADP: 0.471 g ADP.Na_2 (Boehringer Mannheim) in 5 mL DI water.

3) 0.2 M Carbamyl phosphate: 0.122 g di-lithium carbamyl phosphate (Sigma) in 4 mL DI water; prepared fresh.

4) 0.2 M $MgCl_2$: 0.404 g $MgCl_2.6H_2O$ in 10 mL DI water.

5) 0.5 M Glucose: 1.802 g D-glucose in 20 mL DI water.

6) 0.1 M $NADP^+$: 83.4 mg $NADP^+.Na_2$ (Boehringer Mannheim) in 1 mL DI water.

7) HK/G6P-DH: 1 mL solution containing 2 mg (272 units) hexokinase (HK) and 1 mg (136 units) glucose-6-P dehydrogenase (G6P-DH) from Boehringer Mannheim. Stored at 4°C.

8) Enzyme extract: prepared fresh; undiluted or appropriately diluted with 0.01 M phosphate buffer.

Reagents were stored frozen (-13°C), unless specified.

Procedure

The standard assay mixture consisted of 2.0 mL 0.2 M Tris.HCI (pH 7.9), 0.2 mL 0.2 M carbamyl phosphate, 0.1 mL 0.2 M ATP, 0.1 mL 0.2 M $MgCl_2$, 0.2 mL 0.5 M glucose, 20 µL 0.1 M $NADP^+$, 10 µL HK/G6P-DH mixture and 0.1 mL cell-free extract. The assay mixture (without carbamyl phosphate and enzyme extract) was equilibrated at room temperature for at least 10 min and further equilibrated in the cuvette compartment (10 min) of the

spectrophotometer at 37°C after adding carbamyl phosphate. The enzymatic reaction was initiated with the addition of 0.1 mL of extract. The changes in the absorbance (340 nm) were monitored continuously at 37°C using a Gilford 260 Spectrophotometer. One unit of enzymic activity was defined as that amount of enzyme catalysing the formation of one µmole of ATP per min, since the amount of ATP produced is equal to the amount of NADPH formed on a molar basis. Specific activities were expressed as units per mg extract protein (U/mg).

Control assay for CK

Myokinase (adenylate kinase) catalyses the formation of ATP from ADP (2ADP ---> ATP + AMP). Therefore, the activity of this enzyme, if present, may interfere with the CK assay in which ATP formation is measured. A CK assay mixture lacking carbamyl-P represents the apparent activity of myokinase. Experience with the CK assay showed that interference by myokinase activity was only significant at high concentrations of cell-free extracts. When high dilutions of extracts were required (50-fold or higher), myokinase activity was so weak that it contributed only a negligible rate of ATP formation. When undiluted or low dilutions of extracts were used (<50-fold), however, the activity of myokinase could interfere significantly with the CK assay. Therefore, when undiluted or low dilutions of extracts were used, CK activity was calculated by subtracting myokinase activity from total activity.

3.3 Results and discussion

Preliminary trials indicated that wine LAB grown in TJBAG broth at 30°C showed measurable activities of the arginine deiminase pathway enzymes, using the unmodified assay procedures of Oginsky (1955), Jones (1962) and Crow and Thomas (1982). Further experiments were then conducted to establish enzyme assay conditions appropriate for the wine LAB.

3.3.1 Enzyme concentration and reaction rate

For an enzyme assay, the product formation should be linear over the time chosen for the assay, so that the reaction rate can be measured accurately. To achieve this, it is crucial that the amount of enzyme used (enzyme concentration) is rate-limiting (*i.e.* that the rate of reaction is linearly dependent on enzyme concentration). Therefore, experiments were carried out to ensure that the amount of enzyme in the cell-free extracts used was

rate-limiting. This was achieved by varying the amount of enzyme extracts added to the assay mixture, then comparing the corresponding reaction rates (slopes of plots of product formation against time, μmoles/min).

Table 3.1 shows the relationship between the enzyme concentrations and ratios of reaction rates for ADI, OTC and CK. Doubling (2:1) the amount of enzyme extract in the assay mixture increased the reaction rates about 2-fold, the increase varying from 1.85 to 2.21-fold. A 1.67-fold increase in the amount of enzyme for ADI and OTC also increased the reaction rates and gave ratios of reaction rates from 1.56 to 1.81. These results suggest that the reaction rates of these enzymes were indeed proportional to the amounts of enzyme extract used. In other words, the amount of extract used was rate-limiting. This procedure was used frequently in subsequent assays and other experiments to check whether the enzyme concentrations used indeed were rate-limiting.

Table 3.1 Relationship between enzyme concentrations and reaction rates[ab]

Enzyme/Strain	Enzyme Ratio	Ratio of Reaction Rate	
		Determined	Expected
ADI			
Lc. oenos OENO	2:1	2.19, 2.21	2.00
Lb. brevi 250	2:1	2.01, 2.08	2.00
Lb. buchneri CUC-3	5:3	1.75, 1.81	1.67
OTC			
Lc. oenos MCW	2:1	2.13, 1.85	2.00
Lc. oenos OENO	2:1	2.12, 2.16	2.00
Lb. brevis 250	5:3	1.56, 1.64	1.67
CK			
Lc. oenos MCW	2:1	1.86, 1.99	2.00
Lc. oenos OENO	2:1	1.95, 2.00	2.00
Lb. brevis 250	2:1	1.87, 1.96	2.00
Lb. buchneri CUC-3	2:1	2.06, 2.03	2.00

[a]The duplicate measurements were made using one or two extracts.
[b]Assay conditions are as described in Sections 3.2.5-3.2.6.

3.3.2 Substrate concentration and enzyme activities

To conduct accurate enzyme assays, it is essential that saturating substrate concentrations are used. This is because when substrate concentrations are saturating, enzyme activities (reaction rates) will not be influenced by small variation of concentration, nor by consumption of substrates during the assays. The following experiments were carried out to ensure that saturating substrate concentrations were used in assays of ADI, OTC and CK. This was achieved by varying the concentration of one substrate while keeping the concentration of other substrates constant, then measuring enzyme activities. The results are presented in Figures 3.1-3.4. It was not the intention of these experiments to determine the K_m values for these enzymes, although it has been suggested that a substrate concentration of at least 10 times the K_m value should be used (Scopes, 1987).

Cell-free extracts from four different strains of wine LAB which are used extensively in subsequent experiments were used as the source of the arginine deiminase pathway enzymes in this study. The buffer pH levels used for measuring the activities of the three enzymes of the ADI pathway found in Figures 3.1-3.4 were not always the same as those found in the study of determining optimum pH for enzyme activity (Section 3.3.3), but were based on preliminary results and values reported in the literature. The optimum pH values were more precisely determined after this study.

Figure 3.1 shows the relationship between arginine concentrations and ADI activities in cell-free extracts from four different strains of wine LAB. This figure shows that increasing the substrate concentration over at least a two-fold range did not cause any significant change in the activities of ADI in cell-free extracts of the four strains of wine LAB. An arginine concentration of 52 mM was used as a standard concentration for assay of ADI activity in extracts from a large number of wine LAB (Chapter 4). The higher concentration of arginine was chosen since a high substrate concentration can alleviate the inhibitory effect caused by the accumulating products (Scopes, 1987). In this assay, the accumulating products are citrulline and ammonia.

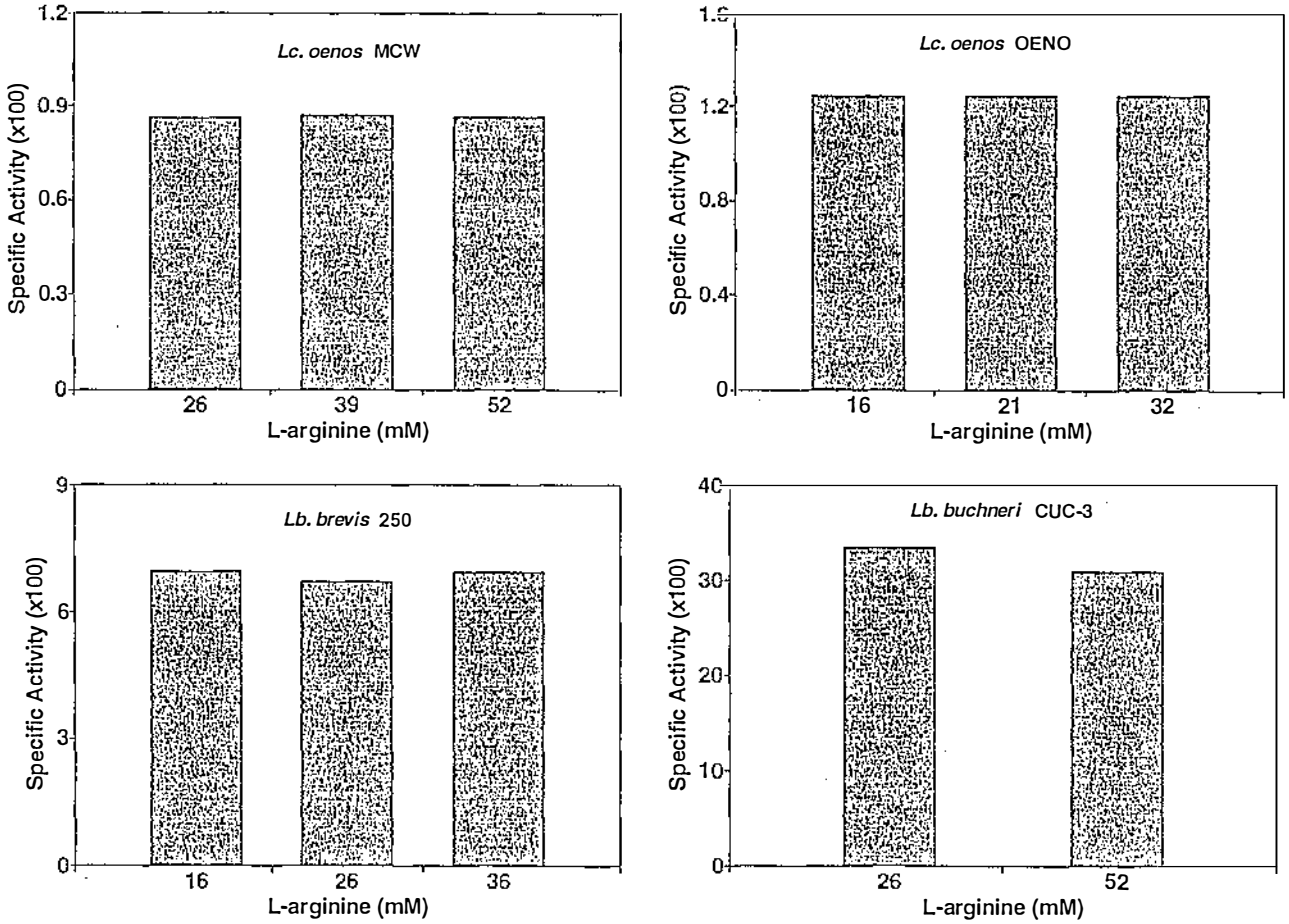

Fig. 3.1 Relationship between arginine concentrations and activities of ADI in extracts of four wine LAB. Assays were carried out using the conditions described in Section 3.2.5, except that arginine concentration was varied as specified. Reported values are based on duplicate measurements from one extract. Specific activities are in U/mg.

Figure 3.2 shows the relationship between ornithine concentration and OTC activity. This figure shows that the activity of OTC from strain MCW increased by 36% as ornithine concentration was increased from 15 mM to 20 mM. Also, the activity of OTC from strain OENO almost doubled (90% increase) when ornithine concentration was increased from 15 mM to 25 mM. This indicates that 15 mM of ornithine was not saturating for OTC from both strains. OTC activities reached a plateau between 20-30 mM for strain MCW and between 25-40 mM for strain OENO. A small increase (14%) in the activity of OTC from strain CUC-3 also occurred from 32 mM to 60 mM of ornithine. This small increase could be due to non-enzymatic synthesis of citrulline from ornithine and carbamyl phosphate, since non-enzymatic synthesis increases with increasing ornithine concentration (Section 3.2.6). With strain 250, the activity of OTC increased by 21% from 32 mM to 48 mM and showed only a very small increase from 48 mM to 60 mm ornithine. Taking into account all the results, an ornithine concentration of 48 mM was used for routine OTC assay.

Figure 3.3 shows the relationship between carbamyl phosphate concentration and OTC activity. The activity of OTC from strain OENO increased by 43% as the concentration of carbamyl phosphate was increased from 15 to 30 mM. 30 mM of carbamyl phosphate was a apparently saturating concentration, since OTC activity did not increase with further increase in substrate concentration to 40 mM. Carbamyl Phosphate at 30-40 mM was also saturating for OTC from strain MCW. For strains 250 and CUC-3, OTC activities were relatively similar from 32 mM to 60 mM carbamyl phosphate. Taking into consideration these results, a carbamyl phosphate concentration of 40 mM was adopted for routine assays.

The relationship between substrate concentration and CK activity is presented in Figure 3.4. This figure indicates that doubling the concentration of ADP caused 17-30% inhibition of the activity of CK from strains MCW, OENO and 250, relative to the activities obtained using the standard ADP concentration (7.3 mM). However, halving the standard concentration of ADP did not change the activity of CK from strain CUC-3. This suggests that the concentration of ADP used in the standard assay mixture (7.3 mM) was saturating. The activities of CK from strains MCW, OENO and 250 varied only slightly upon doubling the concentrations of other substrates, indicating

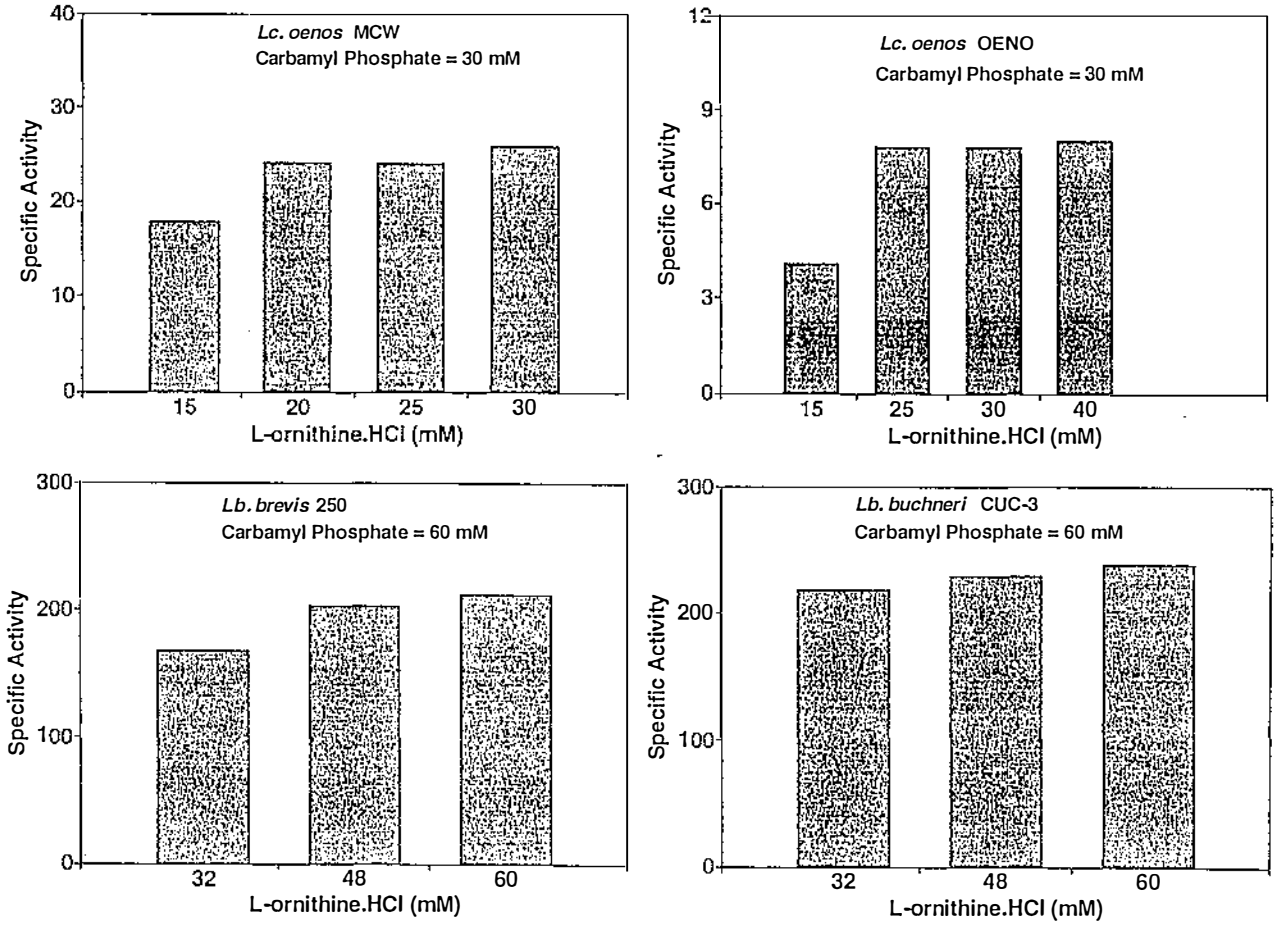

Fig. 3.2 Relationship between ornithine concentrations and activities of OTC in extracts of four wine LAB. Reported values are based on duplicate measurements from one extract. Assays were conducted at pH 7.6 for strains MCW and OENO; pH 8.0 for strain 250; and pH 8.5 for strain CUC-3. Specific activities are in U/mg.

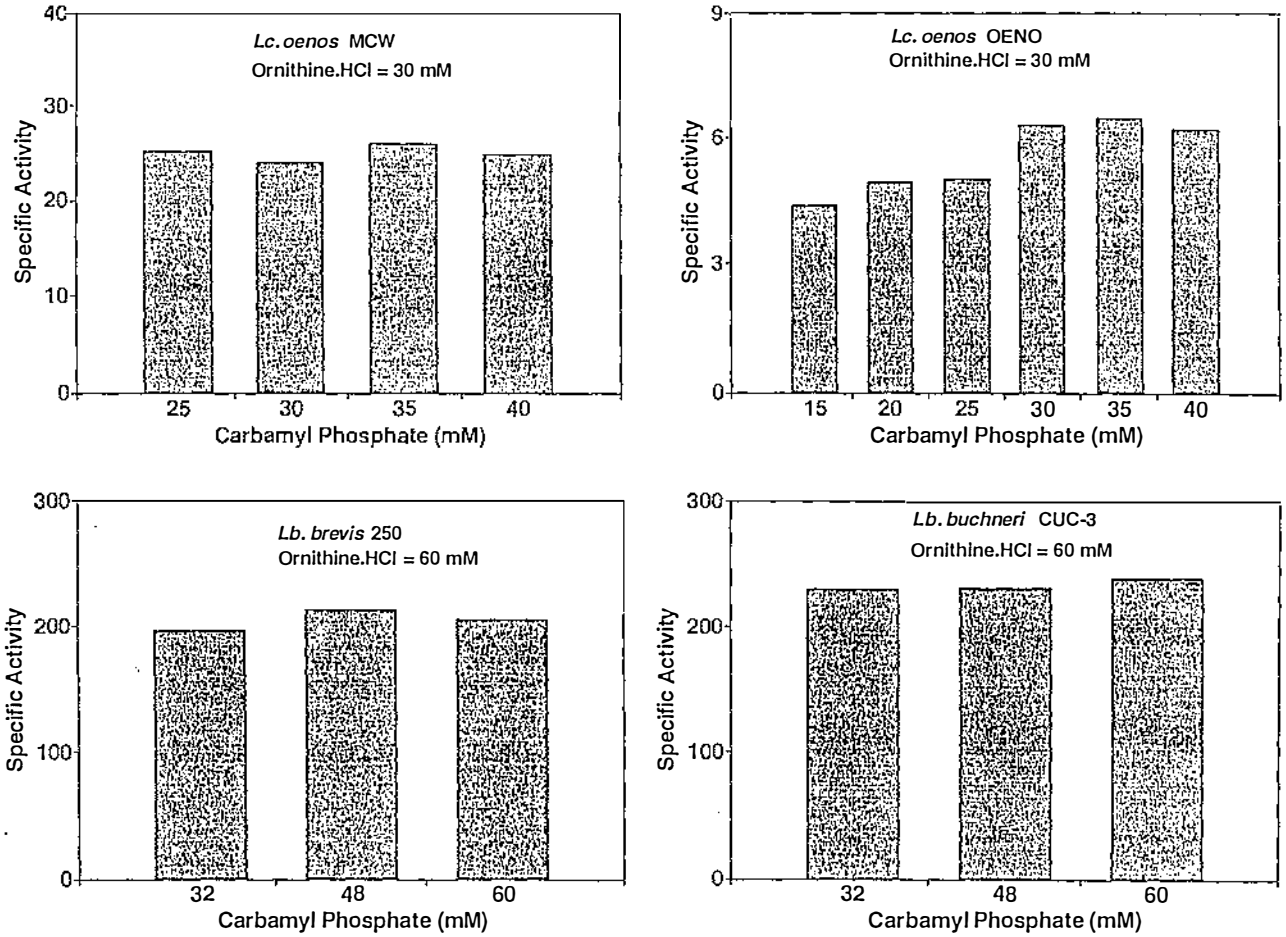

Fig. 3.3 Relationship between carbamyl-P concentrations and activities of OTC in extracts of four wine LAB. Duplicate measurements were made from one extract. Assay pH levels were the same as in Fig. 3.2. Specific activities are in U/mg.

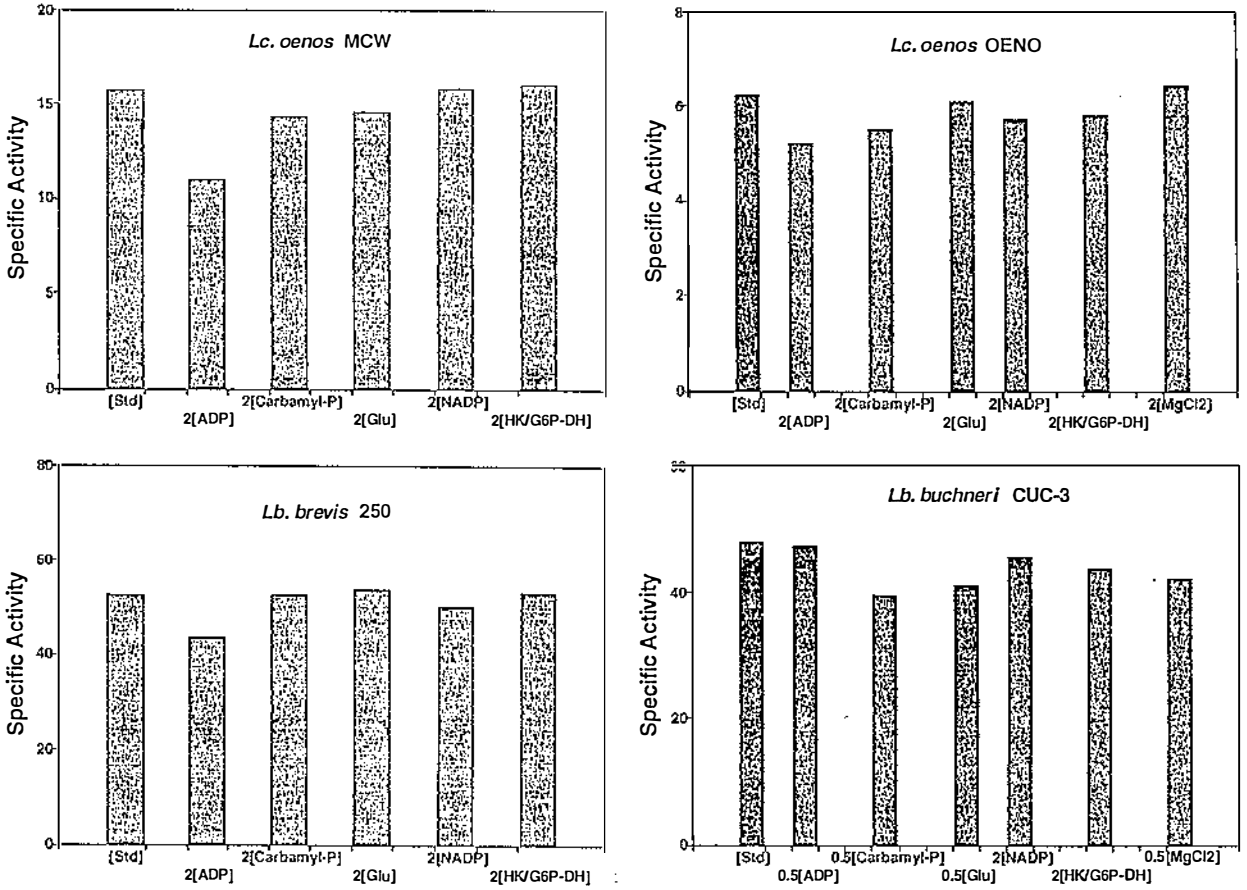

Fig. 3.4 Relationship between substrate concentrations and activities of CK in extracts of four wine LAB. Single measurements were made from one extract. Changes in substrate concentration or coupling enzyme level were made relative to those used in the standard assay (Section 3.2.7). Thus, 2[ ] or 1/2[ ] means a substrate concentration of twice or half that in the standard (std) assay. Assays were conducted at pH 7.6 for strain MCW and pH 7.9 for others. Specific activities are in U/mg.

that these concentrations were saturating as well. The activity of the two coupling enzymes (HK and G6P-DH) was also shown to be not rate-limiting, since doubling the level of these enzymes in the assay mixture did not cause any significant increase in the rate of reaction. Overall, substrate concentrations in the standard assay mixture were considered adequate.

3.3.3 pH and activity of ADI, OTC and CK

The optimum pH of an enzyme activity is regarded as the pH value (or range of pH values) where a maximum reaction rate is obtained. Although data on the pH optima for ADI, OTC and CK from other sources are available, these pH optima may not necessarily be applicable to ADI, OTC and CK from wine LAB. It is important to conduct enzyme assays at their pH optima so that maximal activities can be expressed. Therefore, experiments were carried out to determine the pH optima of ADI, OTC and CK from wine LAB. Results are presented in Figures 3.5-3.7. Measurements at each pH value were made in duplicate from one extract unless otherwise stated. Saturating substrate concentrations were employed for all measurements: arginine, 26 to 52 mM; ornithine.HCl, 30 to 60 mM and carbamyl phosphate, 32 to 60 mM. The standard substrate concentrations (Section 3.2.7) were used for CK assay in this study. Potassium phosphate (131 mM), Tris.HCl (460 mM) and Tris.HCl (147 mM) were used as the buffers in the assay mixtures for ADI, OTC and CK (Sections 3.2.5-3.2.7), respectively. The pH range used was based on values reported in the literature.

Figure 3.5 shows the relationship between pH and activities of ADI in extracts of three different strains of wine LAB. The activity of ADI was maximal at pH 5.8 for all three strains. Therefore, pH 5.8 was chosen as the value for subsequent ADI assays. Various pH values were used for ADI assay in previously published studies. These pH values include 6.0 for *Lb. buchneri* NCDO110 and *Lb. leichmannii* ATCC 4797 (Manca de Nadra *et al.*, 1986a,b); 6.7 for strains of *Streptococcus lactis (Lactococcus lactis*) (Crow and Thomas, 1982) and 5.5 for *Pseudomonas aeruginosa* (Mercenier *et al.*, 1980).

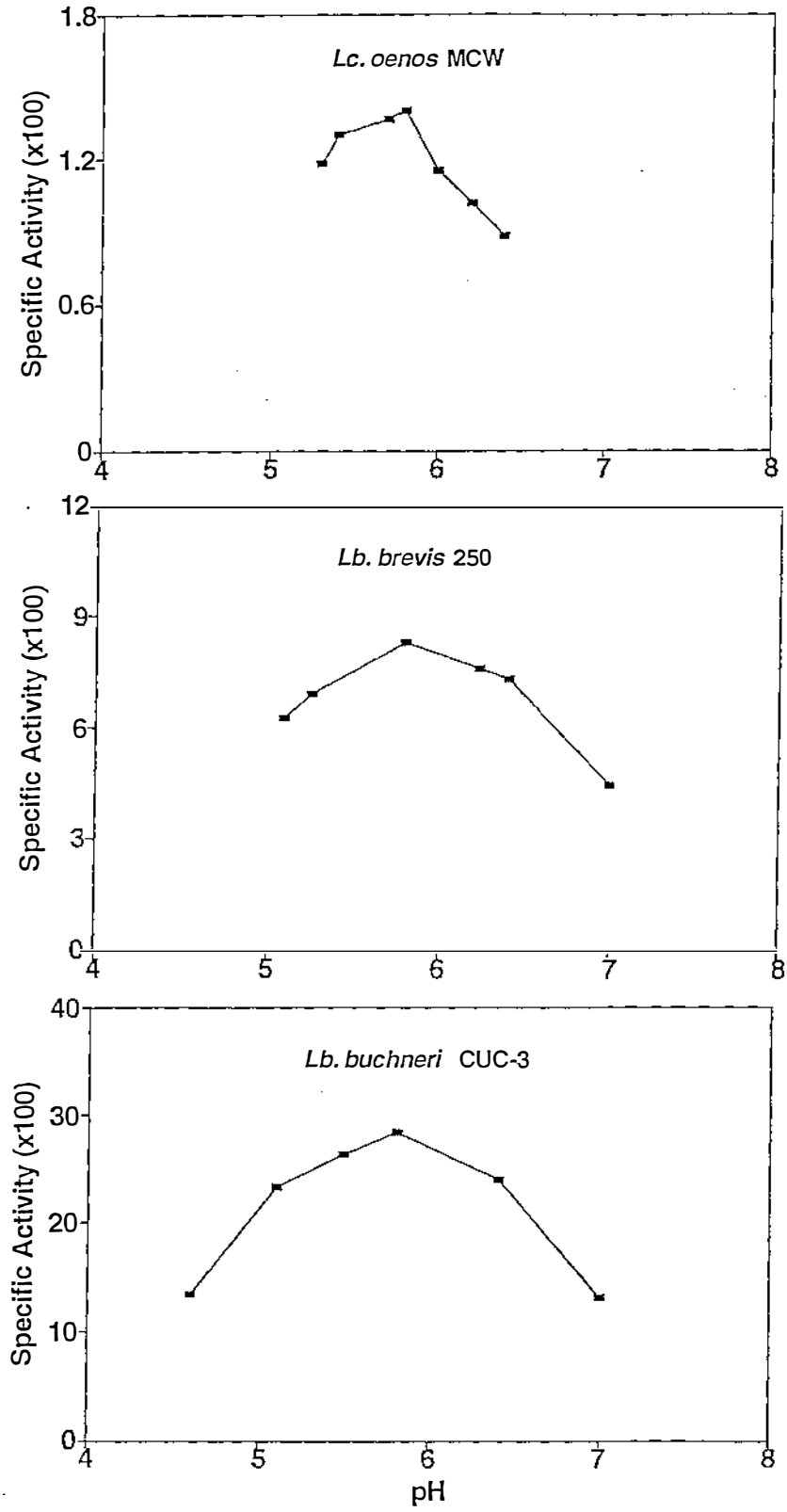

Fig. 3.5 Relationship between pH and activities of ADI in extracts of three wine LAB. Assay conditions are as described in Section 3.2.5. Specific activities are in U/mg.

Figure 3.6 gives the relationship between pH and activities of OTC in extracts of four different strains of wine LAB. This figure shows that the activity of OTC was maximal at pH 8.0 for the two *Leuconostoc oenos* strains, while the activity of OTC was maximal at pH 8.5 for the two lactobacilli. According to the literature, pH 8.5 is commonly used for assaying activities of OTC from various organisms. Examples include *Lb. buchneri* NCDO110 and *Lb. leichmannii* ATCC 4797 (Manca de Nadra *et al.* 1986a,b), strains of *Str. lactis* (Crow and Thomas, 1982) and *Ps. aeruginosa* (Mercenier *et al.* 1980). For convenience, pH 8.2 was chosen for assaying activities of OTC from all wine LAB in subsequent studies. This can be justified, because OTC activity at pH 8.2 is not markedly different from that at pH 8.5 for lactobacilli and at pH 8.0 for leuconostocs.

Figure 3.7 shows the relationship between pH and activities of CK in extracts of four different strains of wine LAB. It should be pointed out that only a single measurement at each pH value was made from one extract for CK from strain OENO. As indicated in this figure, CK activities reached a maximum at pH 7.9 for both *Leuconostoc oenos* strains and *Lb. brevis* 250. The activity of CK from *Lb. buchneri* CUC-3 was maximal at pH 7.6. However, CK activity of this latter strain at pH 7.9 is not significantly different from that at pH 7.6. Therefore, for convenience, pH 7.9 was chosen to assay CK in subsequent experiments. Various pH values have been used for CK assay in previous studies of different organisms, such as pH 8.5 for strains of *Str. lactis* (Crow and Thomas, 1982), pH 5.0 for *Lb. buchneri* NCDO 110 (Manca de Nadra, *et al.* 1986a) and pH 6.15 for *Ps. aeruginosa* (Mercenier *et al.* 1980).

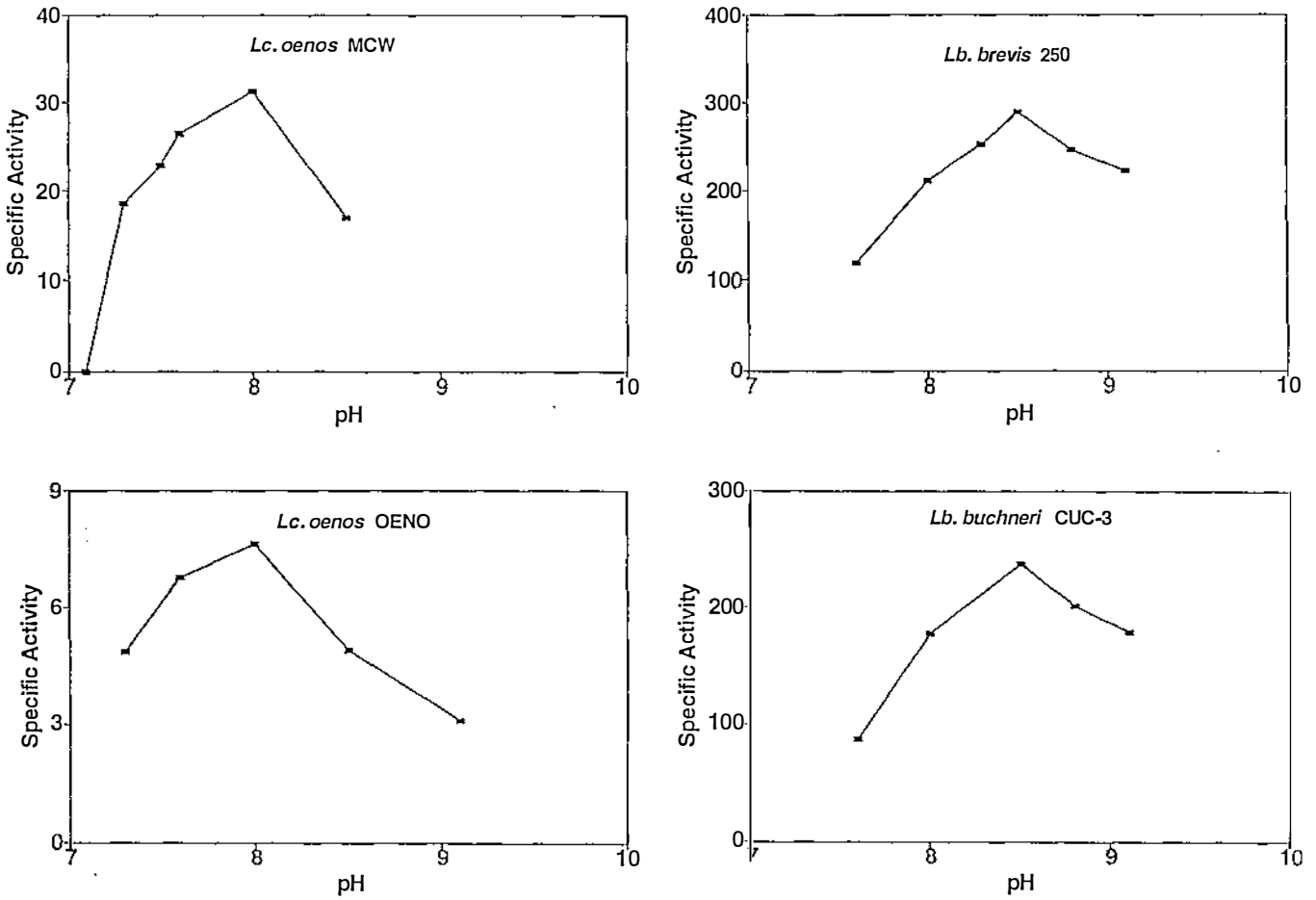

Fig. 3.6 Relationship between pH and activities of OTC in extracts of four wine LAB. Assay conditions are as described in Section 3.2.6. Specific activities are in U/mg.

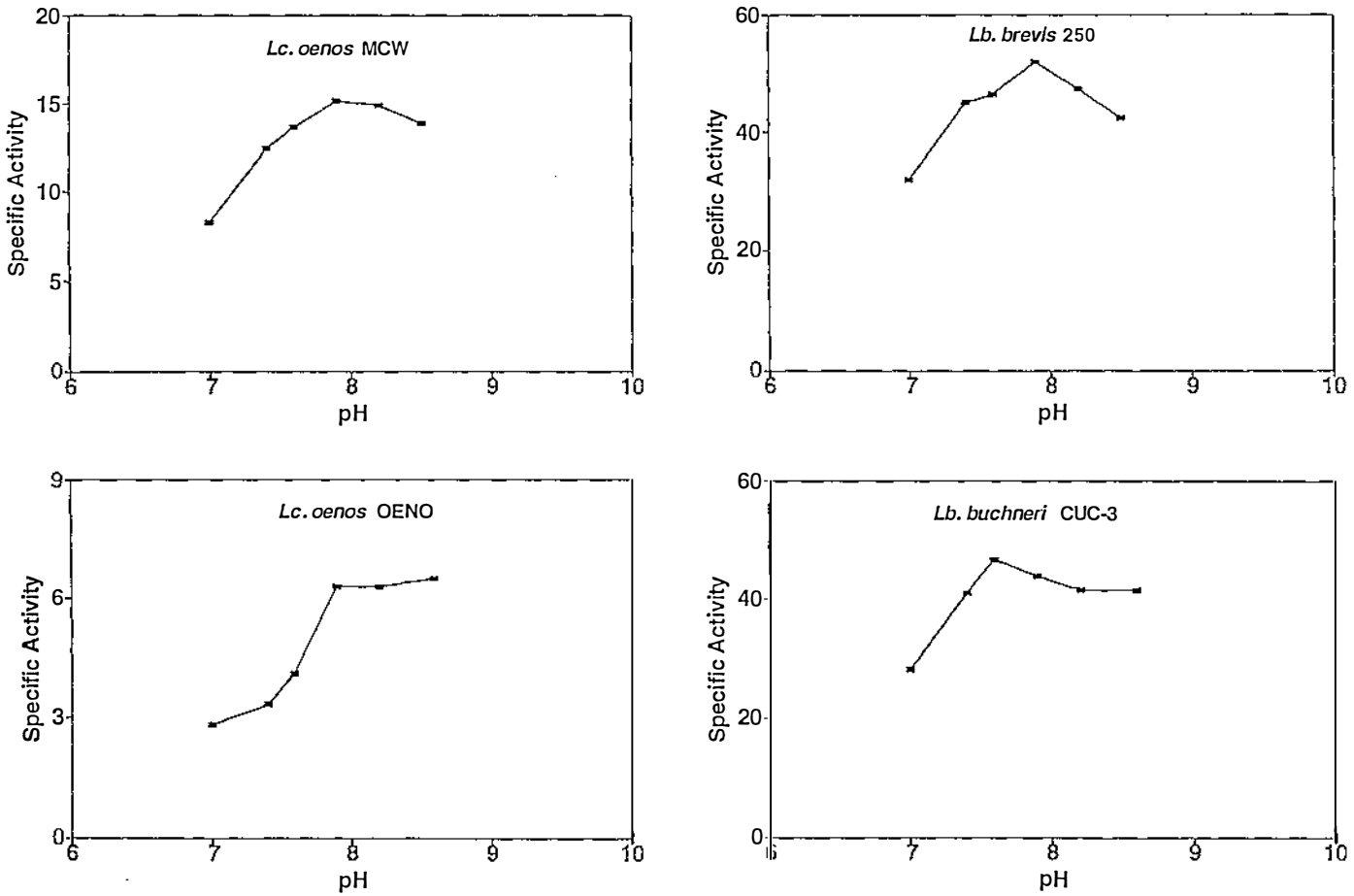

Fig. 3.7 Relationship between pH and activities of CK in extracts of four wine LAB. Assay conditions are as described in Section 3.2.7. Specific activities are in U/mg.

3.3.4 Storage stability of cell-free extracts and whole cells, and effects of freezing on enzyme activities

Table 3.2 presents the stability of cell-free extracts from three different strains of wine LAB during storage under different conditions. Considerable loss of ADI and CK activities occurred upon storage at -13°C for 20 hours, while there was no measurable loss of OTC activity under the same conditions. Loss of CK activity was even greater upon storage at 4°C for 20 hours. However, loss of enzyme activities was insignificant at 0°C (in an ice bath) for 3 hours. OTC is apparently the most stable enzyme. In practice, enzyme assays were completed within 2 hours after cell disintegration and extracts were kept in an ice bath during the assays.

Table 3.2 Storage stability of cell-free extracts[a]

Strain	% Retention of Activity[b]						
	ADI		OTC		CK		
	0°C 3 hrs	-13°C 20 hrs	4°C 20 hrs	-13°C 20 hrs	0°C 3 hrs	4°C 20 hrs	-13°C 20 hrs
Lb. brevis 250		82	100	100	96		77
Lc. oenos MCW		64		100		55	72
Lc. oenos Er1a	98	84			96	74	84

[a]Stability was expressed as percentage retention of specific activities of fresh extracts.
[b]Averages of duplicate determinations from one extract.

Table 3.3 shows the effect of freezing of whole cells on the activities of ADI, OTC and CK from *Lc. oenos* MCW. There were essentially no differences between the specific activities of these enzymes from fresh and frozen cells (-70°C overnight).

The stability of the three enzymes during long-term storage of frozen whole cells is shown in Table 3.4. Specific acitivities of ADI, OTC and CK were in essence unchanged after 7-97 days of storage of whole cells at -70°C. This indicates that these enzymes were stable in whole cells for at least 3 months under such storage conditions. In practice, all enzyme assays were conducted within 3 months after cell harvesting.

Table 3.3 Effect of overnight freezing of whole cells (-70°C) on activities of ADI, OTC and CK from *Lc. oenos* MCW

Enzyme	Specific Activities[a] in Extracts of			
	Fresh Cells		Frozen Cells	
	Sample 1	Sample 2	Sample 1	Sample 2
ADI	11	10	9	10
OTC	33	30	29	33
CK	17	12	17	12

[a]Each value was the average of duplicate determinations from one extract; samples 1 and 2 were from cells harvested at different times.

Table 3.4 Storage stability of whole cells at -70°C

Enzyme	Specific Activities[a]						
	Lc. oenos MCW		*Lb. buchneri* CUC-3		*Lc. oenos* OENO		
	Day 0	Day 97	Day 0	Day 7	Day 0	Day 7	Day 27
ADI	10	9.5	61	65	8	9	9
OTC	31	37	197	195	33	28	34
CK	17	20	42	44	17	19	18

[a]Averages of duplicate determinations from one extract.

Finally, it should be stressed that the investigations described in this chapter were not intended as rigorous enzymological studies of the arginine deiminase pathway enzymes. They were carried out for practical reasons only to define suitable conditions for enzyme assay in cell-free extracts of wine LAB. Therefore, other factors that may affect enzyme activity were not included in this study, such as activators, inhibitors and ionic strength.

Chapter 4

Occurrence of Arginine Deiminase Pathway Enzymes in Wine Lactic Acid Bacteria

4.1 Introduction

Previous work (Chapter 3) verified the presence of arginine deiminase pathway enzymes in selected wine lactic acid bacteria (LAB) and established standard assay conditions for these enzymes. However, little is known about the occurrence of these enzymes and their activities in other wine LAB. For example, it is not known if all wine LAB possess the complete enzyme system, or whether some possess only part of the system. The range of variation in activities of these enzymes from different wine LAB is also unknown. Such information may not only have taxonomic utility, but also practical significance. Therefore, a survey was carried out to define the occurrence and relative activities of the arginine deiminase pathway enzymes in cell-free extracts from a large number of commercial and laboratory wine LAB.

4.2 Materials and Methods

4.2.1 Lactic acid bacterial strains

Wine LAB used in this survey represent members of the genera *Leuconostoc, Lactobacillus* and *Pediococcus*. These bacteria were from the Food and Fermentation Laboratory Culture Collection of the Department of Microbiology and Genetics, Massey University. The original sources of these LAB are listed in Table 4.1.

4.2.2 Culturing and harvesting of wine LAB

In order to make comparisons between different wine LAB, cells should be harvested when activities of ADI, OTC and CK are maximal (optimal harvest time). Thus, preliminary trials were conducted to determine the optimal harvest time. This was achieved by culturing selected wine LAB in TJBAG medium (Section 3.2.2). Wine LAB were first subcultured in pH 5.5 VJMRS broth (Section 3.2.2), then were inoculated (1 % v/v) into pH 5.5 TJBAG medium. Cultures were incubated statically at 30°C. Samples were taken

Table 4.1 Wine lactic acid bacteria and their original sources

Strain	Source[a]	Reference[b]	Comment
HETEROFERMENTERS			
Leuconostoc oenos			
2035	LM	2	Commercially mixed freeze-dried
2001	LM	2	As Above
2043	LM	2	As Above
2008	LM		As Above
MBO	LL		Commercially freeze-dried
MB1	LL		As Above
MB2	LL		As Above
MB3	LL		As Above
DSIR-A	JP		Commercially liquid culture
DSIR-B	JP		As Above
DSIR-C	JP		As Above
Er1a	MD	5,6	Commercially freeze-dried
Ey2d	MD	5,6	As Above
122	CD	4	A laboratory culture
252	CD	4	As Above
MCW	MB		Commercially freeze-dried
OENO	MT		Commercially frozen culture
INO	IO		Commercially freeze-dried
VFO	CP		As Above
ML34	RK	8	Commercially liquid culture
PSU-1	RB	1	As Above
L181	BR	9	A laboratory culture.
Lb. hilgardii			
MHP	LL		Commercially freeze-dried
Lb. buchneri			
CUC-3	RK	7	A laboratory culture
Lb. brevis			
250	CD	4	As Above
ML30	RK	7	As Above
EQ	RK	3	A commercial culture
HOMOFERMENTERS			
Lb. plantarum			
CH2	CP		Commercially freeze-dried
49	CD	4	A laboratory culture
Lb. delbrueckii			
CUC-1	RK	7	As Above

Table 4.1 (Cont'd)

Strain	Source[a]	Reference[b]	Comment
Pediococcus sp.			
44.40	BL	10	Commercially freeze-dried
C5	RK	7	A laboratory culture
CUC-4	RK	7	As Above
Pd. parvulus			
93	CD	4	As Above
272	CD	4	As Above

[a]Sources: BL, Biologicals, Berkeley, Calif., USA; BR, B. Rankine, Austral. Wine Res. Inst., South Australia; CP, C. Prahl, Chr. Hansen's Laboratorium A/S, Hørsholm, Denmark; CD, C. Davis, Uni. of New South Wales, Australia; IO, Institute Oenologique de Champagne, Eperney, France; JP, J. Patel, Hort. and Food Res. Inst., Palmerston North, New Zealand; LL, Lacto-Labo, Dange, France; LM, Lallemand, Montreal, Canada; MB, M. Bannister, Vinquiry, Healdsburg, Calif., USA; MD, M. Daeschel, Dept of Food Sci. and Tech., Oregon State Uni., USA; ML, Microlife Technics, Sarasota, Florida, USA; RB, R. Beelman, Penn. State Uni., USA; RK, R. Kunkee, Dept of Vitic. and Enol., Uni. of Calif., Davis, USA.

[b]References: 1, Beelman *et al.*, 1977; 2, Champagne *et al.*, 1989; 3, Davis *et al.*, 1985; 4, Davis *et al.*, 1986a,b; 5, Henick-Kling *et al.*, 1989; 6, Izuagbe *et al.*, 1985; 7, Pilone *et al.*, 1966; 8, Pilone and Kunkee, 1972; 9, Pilone *et al.*, 1974; 10, Silver and Leighton, 1981.

at various time intervals during incubation for measurement of cell mass (optical density, OD), glucose and activities of ADI, OTC and CK. Cultures for measurement of enzyme activities were centrifuged and stored at -70°C as described in Section 3.3.4. Samples for glucose analysis were stored at -13°C.

For the survey of the arginine deiminase pathway enzymes, wine LAB were cultured as described above. Cultures were grown to the late log/early stationary phase before harvesting, as the preliminary trials had shown that these growth phases were optimal for enzyme activity.

4.2.3 Measurement of enzyme activity

Activities of ADI, OTC and CK were measured using cell-free extracts according to the procedures described in Sections 3.2.3 and 3.2.5-3.2.7.

The activity of acetate kinase (AK) was measured using essentially the same assay conditions as for CK (Section 3.2.7), except for the replacement of carbamyl phosphate with 14.65 mM acetyl phosphate. Thus, 0.2 mL of 0.2 M acetyl phosphate (potassium-lithium salt, Boehringer Mannheim) was added to the standard assay mixture, instead of carbamyl phosphate. The unit of AK activity was the same as CK (μmoles ATP/min/mg protein). One unit of AK was defined as that amount of enzyme catalysing the formation of one μmole of ATP per min (*i.e.* the same as that used for the CK).

4.2.4 Other analyses

Methods for the measurements of cell mass (OD), pH, protein, glucose and citrulline have been described in Section 3.2.4.

4.3 Results

4.3.1 Determination of optimal harvest time

Figures 4.1-4.4 show the time-course of growth, glucose utilisation and activities of ADI, OTC and CK of several wine LAB. These figures show that generally the activities of ADI, OTC and CK were high between the late log and early stationary phases. Enzyme activities were low during early growth, especially for the leuconostocs. The activities tended to decline during the stationary phase, though some enzyme activities remained at or close to the maximal levels. Therefore, it was decided that cells should be harvested between late log and early stationary phase as monitored by OD changes. Although glucose level was low at the end of log phase, this was not always a good indicator of growth and optimal cell harvest time, particularly in strain CUC-3 (Figure 4.4).

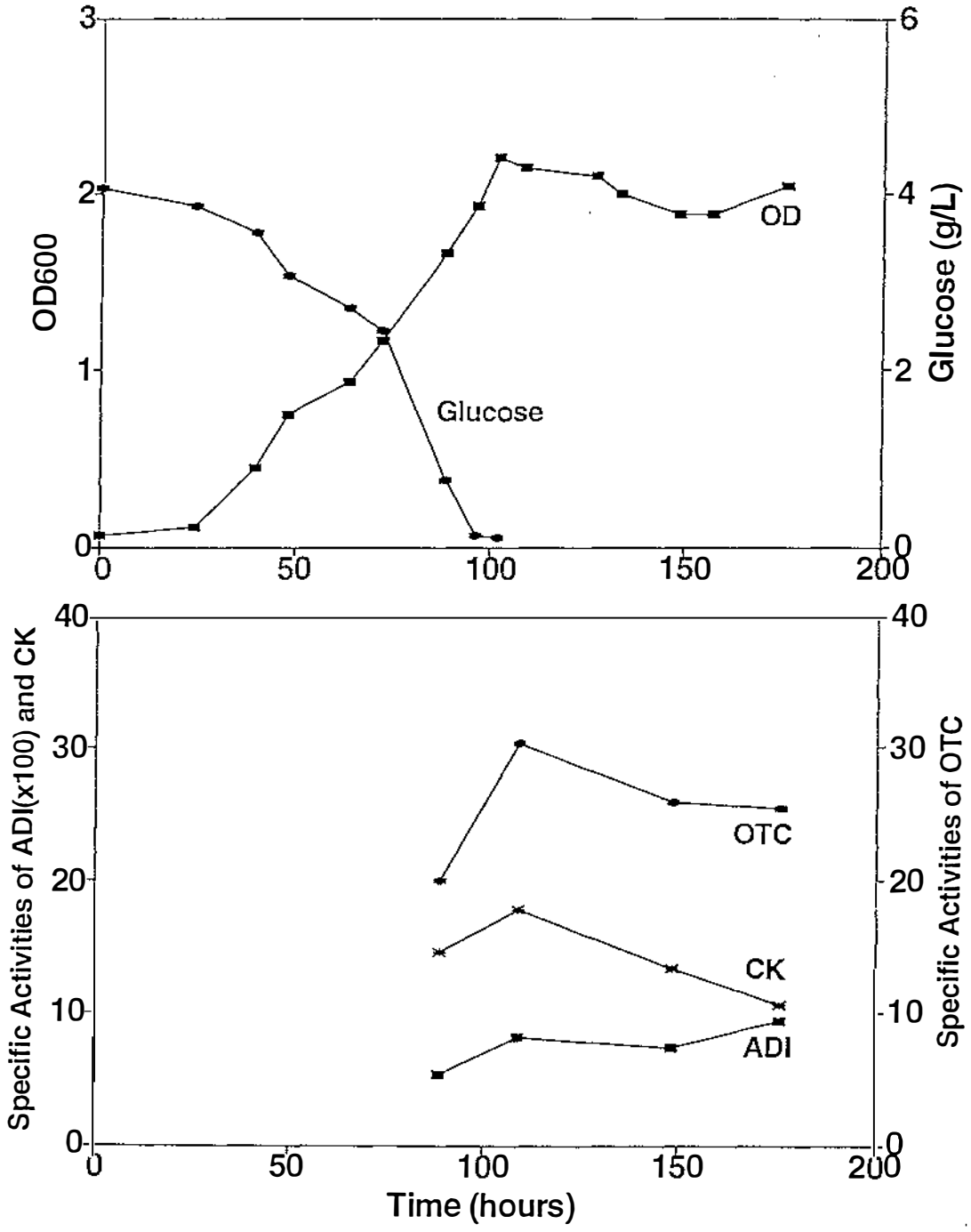

Fig. 4.1 Time-course of activities of arginine deiminase pathway enzymes during incubation (30°C) of *Lc. oenos* Er1a in TJBAG broth. Specific activities are in U/mg.

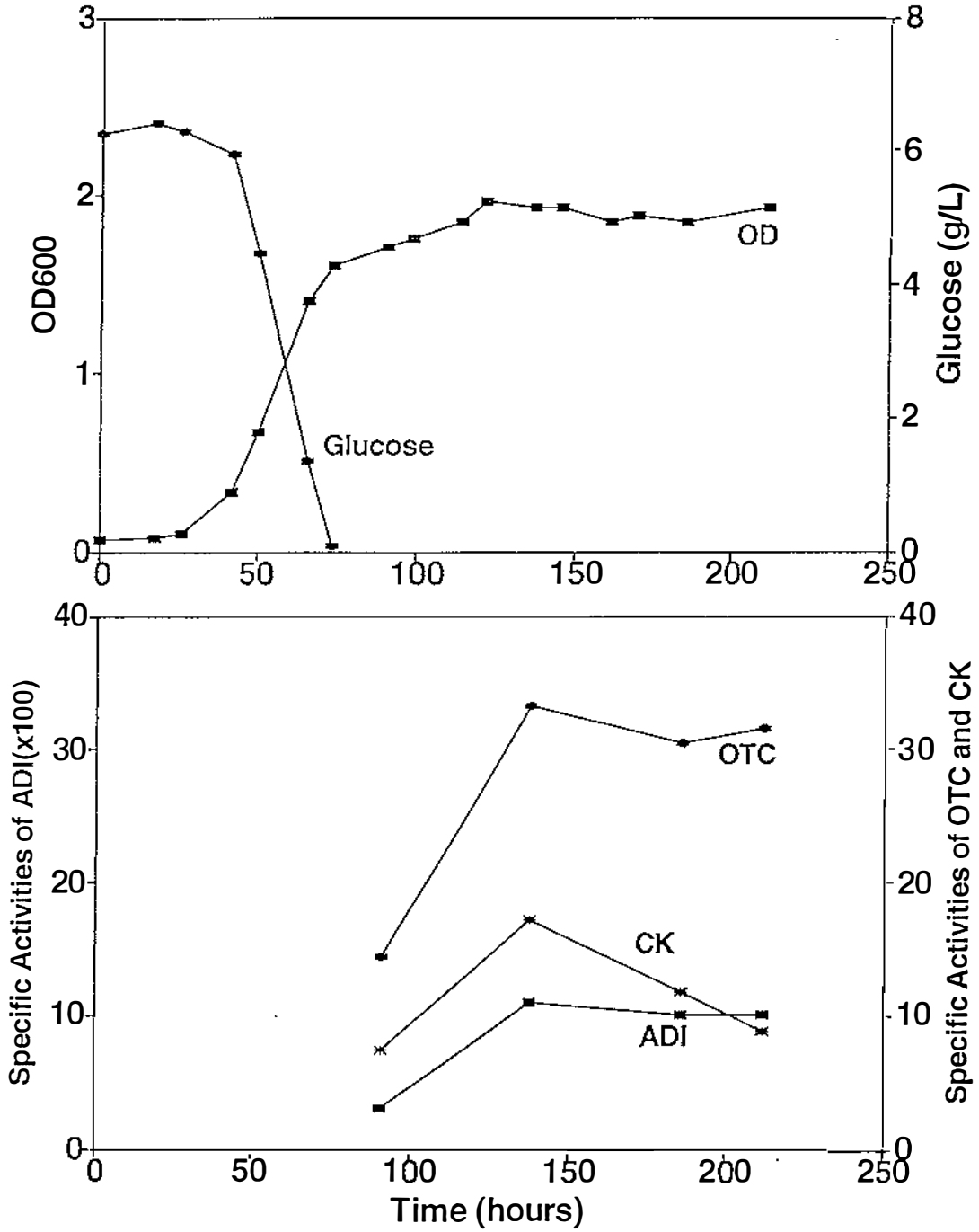

Fig. 4.2 Time-course of activities of arginine deiminase pathway enzymes during incubation (30°C) of *Lc. oenos* MCW in TJBAG broth. Specific activities are in U/mg.

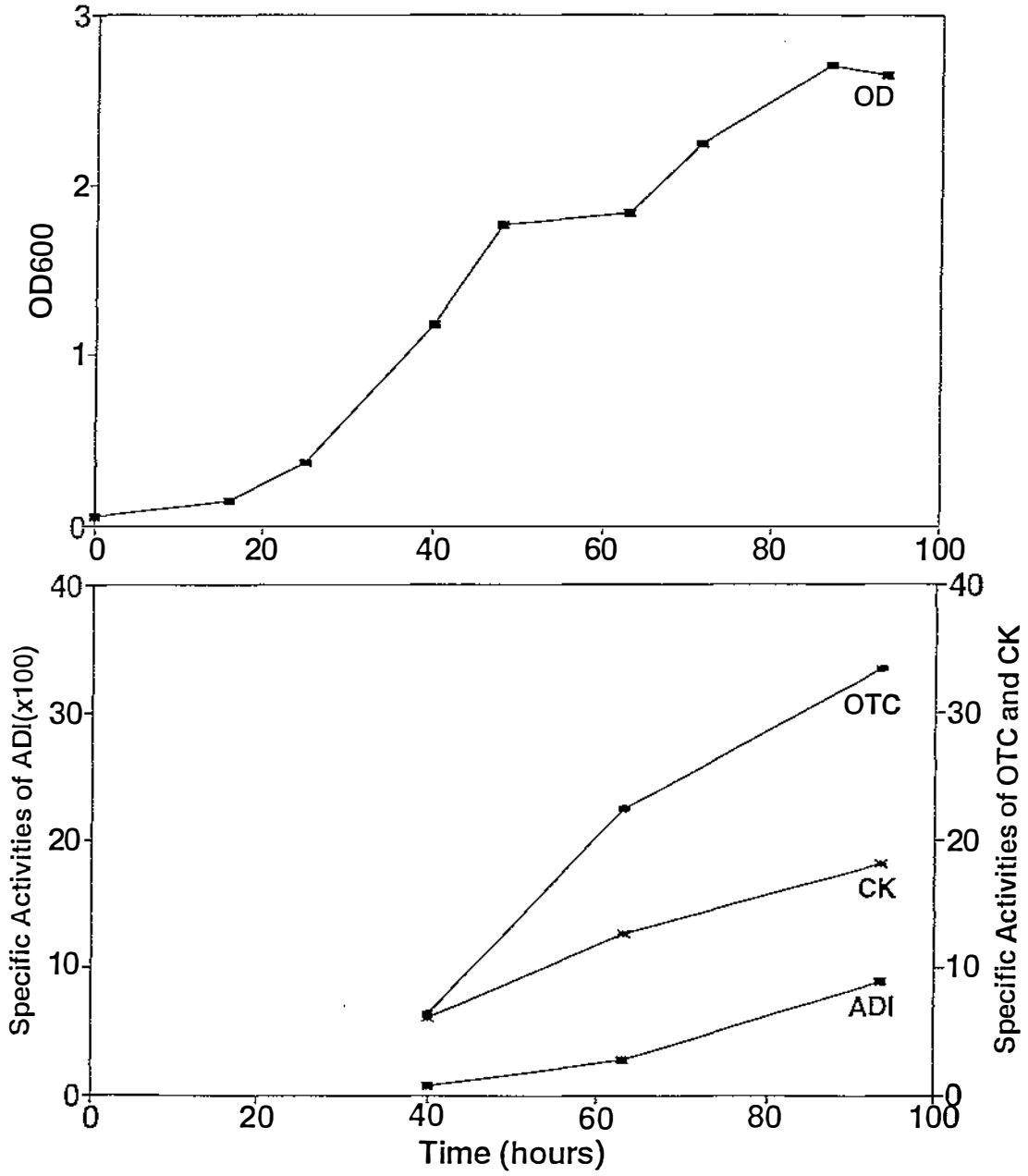

Fig. 4.3 Time-course of activities of arginine deiminase pathway enzymes during incubation (30°C) of *Lc. oenos* OENO in TJBAG broth. Specific activities are in U/mg.

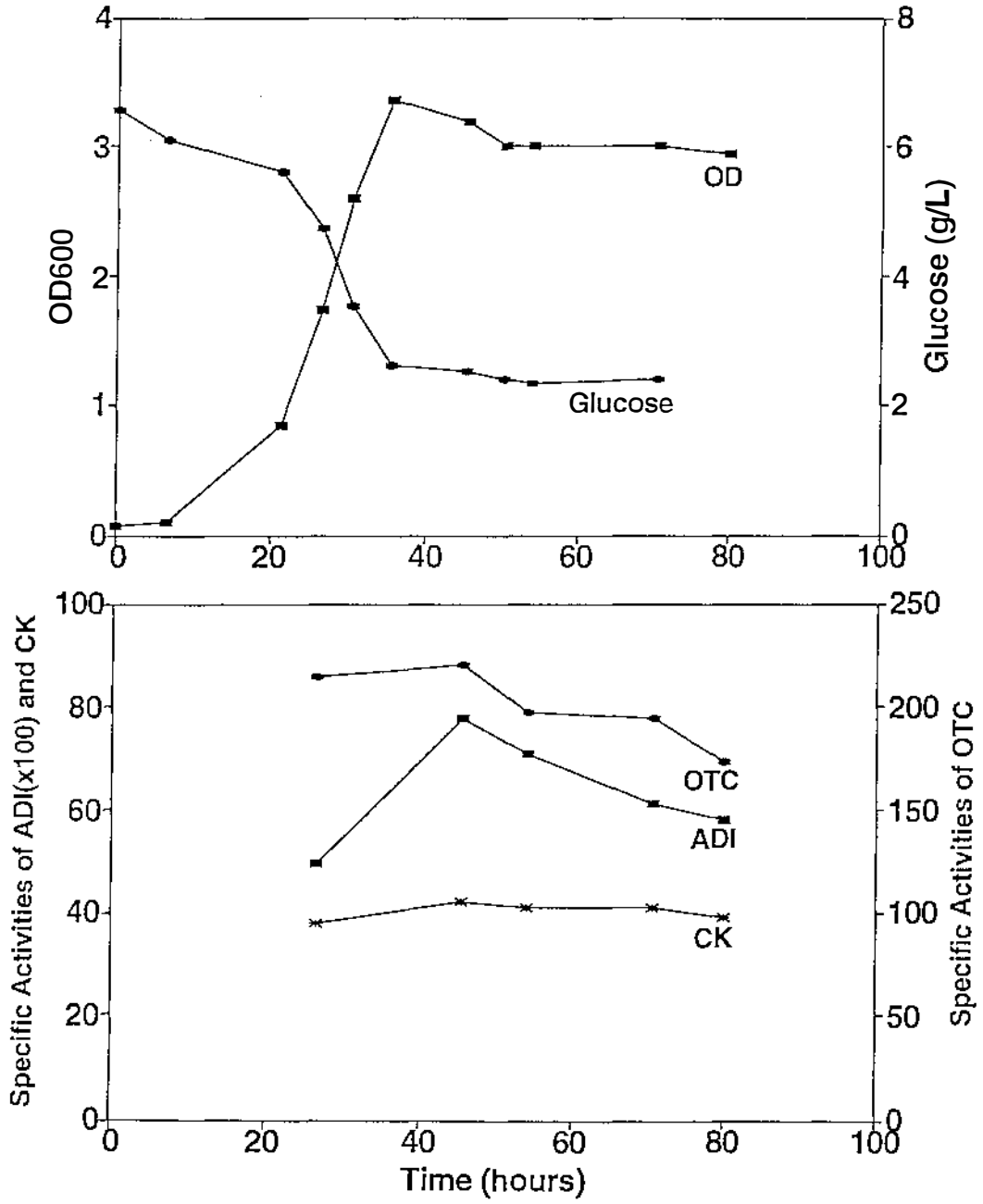

Fig. 4.4 Time-course of activities of arginine deiminase pathway enzymes during incubation (30°C) of *Lb. buchneri* CUC-3 in TJBAG broth. Specific activities are in U/mg.

4.3.2 Occurrence of arginine deiminase pathway enzymes in wine LAB

Table 4.2 shows the specific activities of arginine deiminase pathway enzymes in a wide range of commercial and laboratory wine LAB. The corresponding relative activities are presented in Table 4.3.

As shown in Table 4.2, the activities of the arginine deiminase pathway enzymes varied considerably among species and strains of wine LAB. A high proportion (77%) of leuconostocs and almost all of the heterofermentative lactobacilli tested possessed the complete enzyme system. However, activities of the three enzymes were not found in any of the homofermentative lactobacilli and pediococci under the current extraction and assay conditions. Among the heterofermentative wine LAB, some strains such as MB3, ML34, PSU-1 and ML30 were deficient of one or two of the three enzymes and in all of these cases, the specific activity of the enzymes that were detected was very low relative to the levels found in other strains. Some strains such as L181 and MBO did not have any of the three enzymes.

For the leuconostocs and heterofermentative lactobacilli possessing the complete enzyme system, activities of the three enzymes from the lactobacilli were generally higher than those from the leuconostocs, especially in the case of ADI and OTC. Nevertheless, for those LAB having the three enzymes, the enzyme activities were generally similar within the heterofermentative lactobacilli, as were the enzyme activities within the leuconostocs. This is also shown by the relative activities of the three enzymes in Table 4.3. There were some exceptions, however, such as strain INO in which the activities of the three enzymes were significantly lower than in other strains, and strain VFO in which the ADI activity was considerably lower than in other strains.

Table 4.2 Occurrence of arginine deiminase pathway enzymes in wine lactic acid bacteria

Strains	Specific Activities						n[a]
	ADI(x100)		OTC		CK		
	MEAN	SD	MEAN	SD	MEAN	SD	
HETEROFERMENTERS							
Leuconostoc oenos							
2035	13.2	1.0	51.2	4.9	19.3	1.7	4
2001	13.7	0.4	42.5	6.0	22.6	1.5	6
MB1	12.6	1.3	35.3	2.4	27.3	1.2	6
2008	12.0	0.2	40.5	2.9	18.6	3.2	4
2043	11.7	2.6	47.0	8.1	15.4	3.7	6
MB2	12.5	1.5	32.3	2.7	18.3	2.3	4
DSIR-A	11.2	1.0	35.5	2.8	21.5	2.1	4
122	10.5	0.7	34.9	0.5	19.2	0.4	4
MCW	10.0	1.2	30.1	4.1	17.1	0.7	4
Ey2d	10.6	0.5	33.2	2.4	15.6	2.9	4
OENO	8.8	0.4	32.2	3.1	18.1	1.4	6
DSIR-B	8.4	0.2	31.0	1.0	20.4	3.4	4
DSIR-C	8.9	0.4	30.7	1.3	18.9	2.1	4
Er1a	8.8	0.7	30.2	3.3	18.1	1.6	6
252	9.1	0.5	28.8	1.2	15.0	2.3	6
INO	2.4	0.5	5.1	0.8	4.6	0.6	4
VFO	4.4	1.2	29.0	2.2	16.1	2.2	4
MB3	0		0.2	0.09	0		4
ML34	0		0		0.8	0.11	4
PSU-1	0		0		0.2	0.07	4
L181	0		0		0		4
MBO	0		0		0		4
Lactobacillus spp.							
Lb. hilgardii MHP	92	16	177	7	27.6	1.4	6
Lb. buchneri CUC-3	67	12	217	15	41.5	2.4	6
Lb. brevis 250	76	7.6	229	18	20.8	2.9	6
Lb. brevis EQ	89	5.4	215	14	30.8	1.8	4
Lb. brevis ML30	0		2.2	0.3	3.4	0.2	6
HOMOFERMENTERS							
Lactobacillus spp.							
Lb. plantarum CH2	0		0		0		4
Lb. plantarum 49	0		0		0		4
Lb. delbrueckii CUG1	0		0		0		4
Pediococcus spp.							
44.40	0		0		0		4
C5	0		0		0		4
CUC-4	0		0		0		4
93	0		0		0		4
272	0		0		0		4

[a] n = total number of measurements for the 3 enzymes using at least two separate extracts. Measurements were made in duplicate or triplicate for each extract.

Table 4.3 Relative specific activities of arginine deiminase pathway enzymes in wine lactic acid bacteria

Strains	ADI	OTC	CK	ADI	OTC	CK	ADI	OTC	CK
Lc. oenos									
2035	1.0	388	146	0.003	1.0	0.377	0.007	2.653	1.0
2001	1.0	310	165	0.003	1.0	0.532	0.006	1.881	1.0
MB1	1.0	280	217	0.004	1.0	0.773	0.005	1.293	1.0
2008	1.0	338	155	0.003	1.0	0.459	0.006	2.177	1.0
2043	1.0	402	132	0.002	1.0	0.328	0.008	3.052	1.0
MB2	1.0	258	146	0.004	1.0	0.567	0.007	1.765	1.0
DSIR-A	1.0	317	192	0.003	1.0	0.606	0.005	1.651	1.0
122	1.0	332	183	0.003	1.0	0.550	0.005	1.818	1.0
MCW	1.0	301	171	0.003	1.0	0.568	0.006	1.760	1.0
Ey2d	1.0	313	147	0.003	1.0	0.470	0.007	2.128	1.0
OENO	1.0	366	206	0.003	1.0	0.562	0.005	1.779	1.0
DSIR-B	1.0	369	243	0.003	1.0	0.658	0.004	1.520	1.0
DSIR-C	1.0	331	215	0.003	1.0	0.647	0.005	1.545	1.0
Er1a	1.0	343	206	0.003	1.0	0.599	0.005	1.669	1.0
252	1.0	316	165	0.003	1.0	0.521	0.006	1.920	1.0
INO	1.0	213	192	0.005	1.0	0.902	0.005	1.109	1.0
VFO	1.0	659	366	0.002	1.0	0.555	0.003	1.801	1.0
Lb. hilgardii									
MHP	1.0	192	30	0.005	1.0	0.156	0.033	6.413	1.0
Lb. buchneri									
CUC-3	1.0	324	62	0.003	1.0	0.191	0.016	5.229	1.0
Lb. brevis									
250	1.0	301	27	0.003	1.0	0.091	0.037	11.0	1.0
EQ	1.0	242	35	0.004	1.0	0.143	0.029	6.981	1.0

4.3.3 Distinction of activities of acetate kinase and carbamate kinase

It has been reported that the enzyme acetate kinase (AK) from *Escherichia coli* (Thorne and Jones, 1963; Yashphe and Gorini, 1965) and *Serratia marcescens* (Crane and Abdelal, 1980) can catalyse the reaction between ADP and carbamyl phosphate to produce ATP. Since carbamate kinase (CK) activity was assayed in the present study by following the rate of ATP formation, it is possible that some of the CK activity was due to AK present in the crude extracts. Therefore, the relative activities of CK (assayed as

described in Section 3.2.7) and AK (see Section 4.2.3) were determined in crude extracts of several different wine LAB. The results are shown in Table 4.4. No correlation was found between the activities of AK and CK in the six strains examined. The strain MBO had relatively high AK activity but no detectable CK activity, indicating that AK in this strain is unable to catalyse the reaction between carbamyl phosphate and ADP. It seems very unlikely that the specificity of CK would differ substantially between strains of *Lc. oenos*. Therefore, the result with the MBO strain suggests that CK activities measured in extracts of *Lc. oenos* and probably other wine LAB are not due to AK. In any case, the affinity of AK from other bacterial sources for carbamyl phosphate are low (Thorne and Jones, 1963; Crane and Abdelal, 1980) and, thus, interference due to AK in the CK assays in the current study is unlikely to be significant.

Table 4.4 A comparison of activities of carbamate kinases and acetate kinases from wine LAB

	Specific Activities[a]	
Strains	Carbamate Kinase	Acetate Kinase
Lc. oenos		
ML34	0.8	0.5
Er1a	18.1	6.1
PSU-1	0.2	1.5
MBO	0	13.1
OENO	18.1	30.1
Lb. buchneri		
CUC-3	41.5	4.4

[a]Data for CK were from Table 4.2; AK activities were measured in duplicate using the same extract as for measurements of CK activity.

4.4 Discussion

This survey has investigated the occurrence of the arginine deiminase pathway enzymes (ADI, OTC and CK) in a wide range of homofermentative and heterofermentative wine LAB. The results showed large differences in activities of the three enzymes among different groups of wine LAB.

The ability of *Leuconostoc* to hydrolyse arginine has been a matter of controversy. In Bergey's Manual of Systematic Bacteriology Vol 2, Garvie

(1986) states that the genus *Leuconostoc* does not hydrolyse arginine. However, several reports are available showing the degradation of arginine by strains of *Lc. oenos* (Kuensch *et al.*, 1974; Weiller and Radler, 1976; Garvie and Farrow, 1980; Liu, 1990; Pilone *et al.*, 1991), though there are strains unable to hydrolyse arginine such as ML34 (Pilone and Kunkee, 1972). The hydrolysis of arginine is usually demonstrated by ammonia production using Nessler's reagent (Garvie, 1967). This test, however, has been shown to be insensitive (Pilone *et al.*, 1991), which may partially explain the discrepancies in the reported ability of leuconostocs to hydrolyse arginine. The results of this survey can explain these discrepancies at the enzyme level. Strains capable of hydrolysing arginine and producing ammonia presumably have the functional complete enzyme system, or at least the first enzyme, ADI (Figure 2.1). On the contrary, strains incapable of arginine hydrolysis at least lack ADI and may also be deficient or defective in OTC or CK or both, so that no ammonia can be produced. For instance, strain ML34 is lacking ADI and OTC (Table 4.2), and is reported to be incapable of producing ammonia from arginine (Pilone and Kunkee, 1972).

It is not known whether arginine catabolism in wine LAB is plasmid-borne or chromosomally encoded. Although plasmids have been found in strains of *Lc. oenos* (Janse *et al.*, 1987; Kelly *et al.*, 1993), the metabolic role of these plasmids is not known. Therefore, the reason(s) for the lack of ADI, OTC and CK in some strains is not clear.

Arginine hydrolysis is a common characteristic of heterofermentative lactobacilli and is thought to be an important source of energy for them (Kandler and Weiss, 1986). This common characteristic is supported by the wide occurrence of the arginine deiminase pathway enzymes and their relative high activities in the heterofermentative lactobacilli revealed in this survey (Table 4.2). It is incorrect, however, to assume that arginine can be used as an energy source based solely on the demonstration of arginine hydrolysis and ammonia production. This is because some strains having only the first enzyme (ADI), or ADI and OTC, or ADI and CK, can also hydrolyse arginine (see discussion below). In these cases, energy (ATP) cannot be generated, since OTC and/or CK, or both, is lacking. Further work was conducted to determine if energy (ATP) could be produced from arginine degradation and coupled to bacterial growth. The results of this work are presented in Chapter 6.

It seems that the homofermentative wine lactobacilli and pediococci examined do not contain any of the arginine deiminase pathway enzymes under the present experimental conditions and, thus, are unlikely to degrade arginine. This may, in part, explain the inability of some homofermentative wine LAB to catabolise arginine reported previously (Kuensch *et al.*, 1974; Liu, 1990; Pilone *et al.*, 1991). However, arginine degradation by homofermentative LAB from other sources is well known. Examples include pediococci from fermented sausages (Deibel *et al.*, 1961a,b), a *Lb. plantarum* fish isolate (Jonsson *et al.*, 1983), dairy lactococci (Crow and Thomas, 1982) and strains of *Pd. halophilus* from soy sauce (Matsudo *et al.*, 1993).

According to Sharp *et al.* (1966), glucose concentration is important in the production of ammonia from arginine by lactobacilli. They state that with high glucose concentrations (20 g/L), only heterofermentative strains produce ammonia; but at lower concentrations, some homofermentative strains may also produce ammonia. Although the TJBAG medium used in the current research contained a final concentration of only about 6 g/L glucose, this medium is relatively rich in other nutrients which may affect the ability of the homofermentative wine LAB to degrade arginine. The ability of homofermentative wine LAB to catabolise arginine was subsequently reinvestigated as part of a study on the effects of glucose on arginine metabolism. The results of this investigation are presented in Chapter 5.

Wine LAB examined can be grouped into several phenotypes based on the occurrence of arginine deiminase pathway enzymes. Most leuconostocs and the heterofermentative lactobacilli fall into the phenotype $ADI^+OTC^+CK^+$. All the homofermentative lactobacilli and pediococci tested belong to the phenotype $ADI^-OTC^-CK^-$. This latter phenotype also includes two leuconostocs (strains L181 and MBO). There are also other possible phenotypes such as $ADI^-OTC^+CK^-$ (strain MB3), $ADI^-OTC^-CK^+$ (strains ML34 and PSU-1) and $ADI^-OTC^+CK^+$ (strain ML30). It should be stressed that the enzyme activities in these phenotypes are very low and, therefore, their presence needs further confirmation. Among the phenotypes found in this study, only the phenotype $ADI^+OTC^+CK^+$ is theoretically capable of catabolising arginine. The remaining phenotypes cannot do so, because the key enzyme of the pathway, ADI, is lacking. It should be mentioned that

arginine transport was not assayed in this study and this would also provide further phenotypes to be investigated.

The three other possible phenotypes, $ADI^+OTC^-CK^-$, $ADI^+OTC^+CK^-$ and $ADI^+OTC^-CK^+$, were not found in this survey. These phenotypes are potentially capable of degrading arginine and producing citrulline, or ornithine and carbamyl phosphate. Some of these phenotypes are reported to occur in other LAB. For example, Manca de Nadra *et al.* (1982) found some lactobacilli contain ADI but no OTC. The authors made no mention of the presence of CK in these LAB. Furthermore, excretion of citrulline from arginine catabolism by bacterial strains deficient or defective in OTC has been reported in *Pseudomonas aeruginosa* mutants (Haas *et al.*, 1979) and *Ps. putida* ATCC 4359 (Kakimoto *et al.*, 1971; Yamamoto *et al.*, 1974). Montel and Champomier (1987) also reported excretion of citrulline from arginine catabolism by a *Lactobacillus sake* meat isolate containing ADI and OTC, but they did not report on the presence of CK in this strain.

Citrulline and carbamyl phosphate are known precursors of the animal carcinogen ethyl carbamate (urethane) (Ough *et al.*, 1988). Therefore, the phenotypes $ADI^+OTC^-CK^-$, $ADI^+OTC^+CK^-$ and $ADI^+OTC^-CK^+$ are potential producers of citrulline and carbamyl phosphate, if wine LAB of these phenotypes are selected as starter cultures for malolactic fermentation (MLF) in winemaking. Although these phenotypes were not found amongst the wine LAB used in this survey, their existence in nature cannot be ruled out. Clearly, these strains should not be employed to induce MLF in wine, because of the potential to produce ethyl carbamate precursors. However, evidence is available that the phenotype $ADI^+OTC^+CK^+$ can also excrete some citrulline from arginine catabolism. This has been demonstrated in mutants of *Streptococcus faecalis* (Simon *et al.*, 1982). Further experiments were conducted to ascertain whether strains of the phenotype $ADI^+OTC^+CK^+$ from wine can excrete citrulline from arginine catabolism and the results are presented in Chapter 5.

Finally, from a taxonomic point of view, the inability to hydrolyse arginine can no longer be a criterion to distinguish *Leuconostoc* from other LAB, since many strains of *Leuconostoc oenos* are indeed able to degrade arginine and possess the arginine deiminase pathway enzymes as discussed above.

Chapter 5

Arginine Catabolism and Metabolite Formation in Selected Wine Lactic Acid Bacteria

5.1 Introduction

Enzyme studies in Chapter 3 have confirmed the presence of arginine deiminase pathway enzymes in selected heterofermentative wine lactic acid bacteria (LAB). Results of a survey in Chapter 4 have revealed the wide occurrence of these enzymes in a large number of commercial and laboratory strains of wine LAB. However, there is still a lack of metabolic information on arginine degradation and metabolite formation by these organisms. Although ornithine and ammonia are expected to be excreted as two of the end-products of arginine catabolism according to the pathway (Section 2.1), it is not known whether citrulline, one of the intermediates in the pathway, can be excreted. Also, the relationship between arginine degradation, sugar utilisation and degradation of L-malic acid is not known. Such information will be not only of academic interest, but also of oenological importance. Therefore, a time-course study was carried out to investigate arginine catabolism and metabolite formation by wine LAB in relation to sugar utilisation and L-malic acid degradation. To avoid possible interference in the analysis of metabolites from unknown components in the complex medium TJBAG (Section 3.2.2), a chemically defined medium was employed in this study.

5.2 Materials and Methods

5.2.1 Lactic acid bacterial strains

Members of the genera *Leuconostoc*, *Lactobacillus* and *Pediococcus* were included in this study. Homofermenters used were *Lb. delbruekii* CUC-1 and *Pd. sp.* CUC-4. Heterofermenters used were *Lc. oenos* OENO, *Lb. buchneri* CUC-3 and *Lb. brevis* 250. The original sources of these strains are listed in Table 4.1.

5.2.2 Medium composition and preparation

The growth medium employed in this study was chemically defined and was based on that of Liu (1990) with some modifications. Glucose was the sole

carbohydrate source in most experiments, whereas both glucose and fructose were the carbohydrate sources in others. Concentrations of glucose, fructose and arginine differed from the original medium (Liu, 1990) and are indicated in Table 5.1 and Figures 5.1-5.8 of Section 5.3 (Results). Ornithine and citrulline, two intermediates in the arginine deiminase pathway, were omitted from the original medium to avoid interference in the analysis of these two compounds. Likewise, ammonia salts were not incorporated into the medium. The composition of the modified synthetic medium is given in Table 5.1.

Stock solutions of 10-fold concentrated organic acids, bases, vitamins, minerals (K_2HPO_4 and KH_2PO_4 omitted) and amino acids (arginine and cysteine omitted) were prepared and stored frozen (-13°C) until required. The remaining components were added when preparing the synthetic medium. To prepare the synthetic medium, aliquots of stock solutions and other components predissolved in deionised (DI) water were mixed and the mixture was adjusted to near the required volume. Ethanol was sometimes added before adjusting the volume to prepare synthetic wine for some experiments. The mixture was then adjusted to pH 3.5 for the hetero-fermenters or pH 4.6 for the homofermenters when ethanol was absent, or pH 3.8 when ethanol was present (synthetic wine). After making up to the appropriate volume, the medium was sterilised by filtration through a 0.45 µm pore-size membrane filter (Micro Filtration System, Dublin, California).

5.2.3 Culturing procedures and sampling regime

Wine LAB were first cultured at 30°C in the complex broths (pH 5.5), VJMRS or AJMRS (Section 3.2.2). They were then subcultured (*ca* 1% v/v) in synthetic medium and incubated (30°C) until full growth. This culture was used to inoculate (*ca* 2% v/v) experimental synthetic medium (250 mL) in Duran bottles (full). These cultures were then incubated statically at 30°C. Single-bottle fermentations were performed for each strain. Throughout the fermentation, samples (10 mL) were taken at various times for measurements of cell mass (OD) and pH, followed by storage at -13°C for subsequent chemical analysis.

The synthetic wine fermentations inoculated with *Lc. oenos* OENO and *Lb. buchneri* CUC-3 were part of a study on the formation of ethyl carbamate in relation to arginine catabolism. The synthetic wine was essentially the same

Table 5.1 Composition of the synthetic medium

Compound[a]	mg/L	Compound[a]	mg/L
Carbohydrates		Vitamins	
D-Glucose[b]	1000 or 5000	Thiamine.HCl	1.0
D-Fructose[b]	2500 or 5000	Riboflavin	1.0
		Pyridoxine.HCl	1.5
Organic acids		Pantothenic acid (calcium salt)	5.0
L-Tartaric acid	3000		
L-malic acid	3000	Nicotinic acid (free)	2.0
		d-Biotin	0.05
Minerals		*p*-aminobenzoic acid	0.05
$MgSO_4.7H_2O$	200	Folic acid	0.05
$MnSO_4.4H_2O$	50	Cyanocobalamine (Vitamin B_{12})	0.07
$FeSO_4.7H_2O$	50		
K_2HPO_4	2000	*myo*-Inositol	20
KH_2PO_4	2000		
		Bases	
Amino acids		Adenine.sulphate	10
L-Arginine	5000	Guanine.HCl	10
L-Alanine	75	Thymine	10
L-Aspartic acid	50	Uracil	10
L-Cystine	50	Cytosine	10
L-Glutamic acid	200	Xanthine	5
L-Glycine	20		
L-Histidine	20	Others	
L-Isoleucine	30	Tween 80	100
L-Leucine	30	Ethanol[b] (95%)	84 mL
L-Lysine.HCl	40		
L-Methionine	25		
L-Phenylalanine	20		
L-Proline	100		
L-Serine	40		
L-Threonine	100		
L-Tryptophane	10		
L-Valine	20		
L-Tyrosine	20		
L-Cysteine	500		

[a]All chemicals were of analytical grade.
[b]Concentrations of glucose and fructose, and the presence or absence of fructose and ethanol were dependent on the experimental design.

as the synthetic medium, except that the former contained ethanol, while the latter did not (see Section 5.2.2). This study is described in detail in Chapter 7, but some of the results from these fermentations are presented here.

5.2.4 Analysis

Methods for the determinations of cell mass (optical density, OD), pH, glucose, fructose, arginine, citrulline, ammonia, urea and L-malic acid have been described in Chapter 3 (Section 3.2.4). Single determinations were normally made, but duplicate determinations were sometimes made to check analytical reproducibility. As a rule, adequate agreement was achieved between duplicate determinations.

5.3 Results

5.3.1 Growth and metabolism of heterofermentative wine LAB in the absence of fructose

Three strains of wine LAB, *Lc. oenos* OENO, *Lb. brevis* 250 and *Lb. buchneri* CUC-3, were cultured in the synthetic medium or synthetic wine to assess growth, metabolism of arginine, glucose and malic acid, and formation of citrulline, ornithine and ammonia. A concentration of 5 g/L glucose was initially used in the synthetic medium inoculated with strain OENO (Figure 5.1). The concentration of glucose was reduced to 1 g/L for all strains in subsequent experiments with synthetic medium (Figures 5.2-5.4), in case glucose inhibits (represses) arginine metabolism. In the synthetic wine fermentation with strain CUC-3 (Figure 5.5), 5g/L glucose was employed because earlier results (Figure 5.1) indicated that this concentration did not inhibit arginine metabolism. It should be made clear that the synthetic wine fermentation was a preliminary trial for the experiments described in Chapter 7.

5.3.1.1 *Lc. oenos* OENO

Figures 5.1 and 5.2 show the growth and metabolism of strain OENO in the synthetic medium containing 5 g/L and 1 g/L glucose, respectively. This strain grew rapidly after inoculation at 5g/L glucose, but OD (growth) decreased immediately upon reaching a maximum when arginine was depleted in the medium (Figure 5.1). This indicates the occurrence of cell lysis, which was presumably the result of energy depletion. Surprisingly, a secondary growth was observed and this may be attributed to nutrients released upon cell lysis. This strain responded quite differently at 1 g/L glucose (Figure 5.2). Growth was initiated rapidly after inoculation, but

followed by a plateau, resumption of growth and another plateau. As at 5 g/L glucose, cell death occurred when arginine was exhausted. The growth curve is not diauxic, though there is a resemblance. This is because the secondary growth occurred after a long plateau when approximately 50% arginine had been used.

The medium pH rose significantly during growth with increments of about 1.3 units at 5 g/L glucose (Figure 5.1) and 2.5 units at 1 g/L glucose (Figure 5.2). The increases in pH (*ca* 0.2 units) during very early stages of growth were contributed by the degradation of L-malic acid, rather than arginine, since arginine had hardly been attacked at that time. The major increases in pH, however, correlated well with the release of ammonia from the catabolism of arginine.

Malic acid was totally degraded during very early stages of growth at both 5 g/L (Figure 5.1) and 1 g/L (Figure 5.2) glucose. Glucose was metabolised concurrently with arginine utilisation at 5 g/L glucose (Figure 5.1), suggesting that glucose does not inhibit (repress) arginine catabolism. However, glucose was used up before arginine at both glucose concentrations, particularly at 1 g/L glucose. Arginine metabolism and growth continued after the exhaustion of glucose, indicating that arginine was being used as a source of energy for cell growth.

The complete degradation of arginine corresponded well with the production of ammonia, citrulline and ornithine at both glucose concentrations. The formation of ammonia, citrulline and ornithine from arginine paralleled with each other at 5 g/L glucose (Figure 5.1). In contrast, at 1 g/L glucose, the formation of the three metabolites from arginine was in parallel only before 280 hours and arginine was converted to ammonia and citrulline only, not ornithine, after this time (Figure 5.2). Only a small proportion of arginine was converted to and excreted as citrulline, approximately 10% at 5 g/L glucose and 16% at 1 g/L glucose. Urea was not found in the medium at both glucose levels, analysed using enzyme test kits supplied by Boehringer (Section 3.2.4).

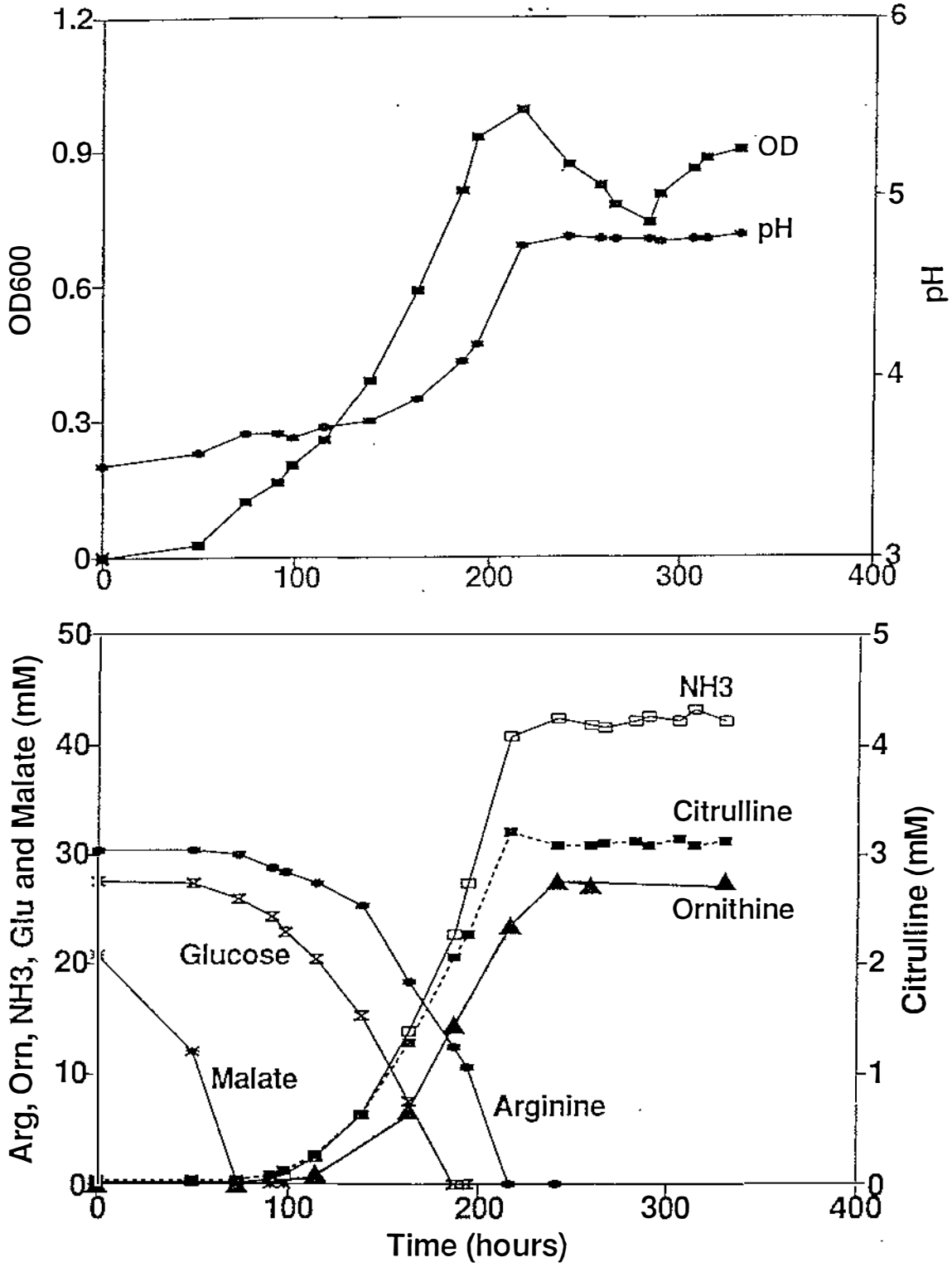

Fig. 5.1 Growth (30°C) and metabolism of *Lc. oenos* OENO in a synthetic medium containing 5 g/L each of glucose and arginine.

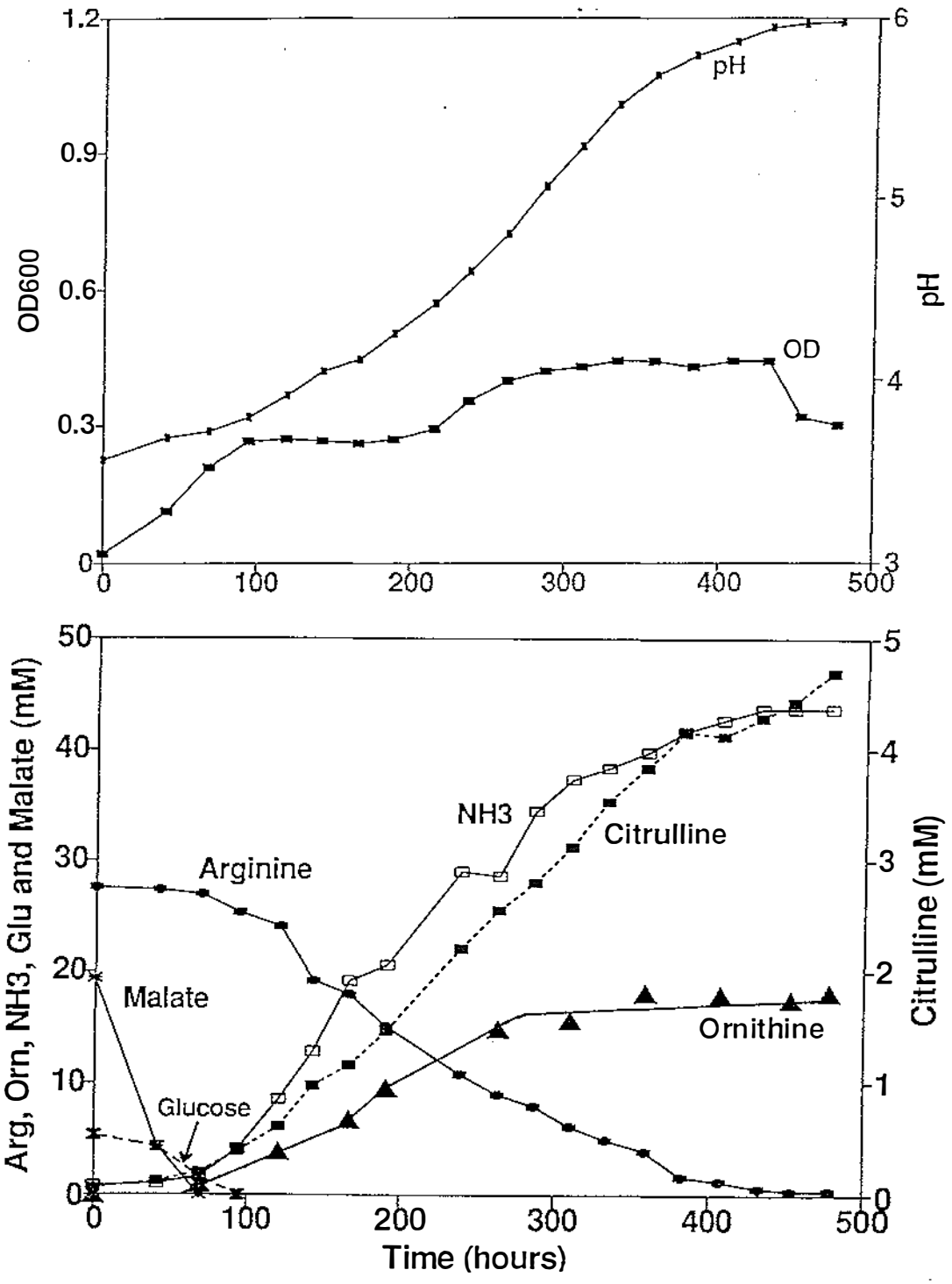

Fig. 5.2 Growth (30°C) and metabolism of *Lc. oenos* OENO in a synthetic medium containing 1 g/L glucose and 5 g/L arginine.

5.3.1.2 *Lb. brevis* 250 and *Lb. buchneri* CUC-3

Figures 5.3 and 5.4 show the growth and metabolism of the two lactobacilli in the medium containing 1 g/L glucose. The two lactobacilli grew rapidly after inoculation, but OD (growth) declined sharply immediately after arriving at a maximum, especially with strain 250 (Figures 5.3 and 5.4). The OD decrease indicates that cell lysis occurred. The medium pH also rose significantly during growth with increments of 1.7 units (Figure 5.3) and 2.0 units (Figure 5.4). The increases in pH were mainly attributable to the formation and release of ammonia from the catabolisn of arginine but with little contribution from the decarboxylation of malic acid, since the degradation of this acid by both strains was very small (Figures 5.3 and 5.4).

Glucose and arginine were used up simultaneously by the two lactobacilli (Figures 5.3 and 5.4), again suggesting that glucose does not repress arginine metabolism. Arginine was completely catabolised corresponding with the formation of ammonia, citrulline and ornithine. However, the excreted citrulline was reassimilated and recatabolised after the disappearance of arginine: strain 250 recatabolised citrulline by about 50% and strain CUC-3 virtually recatabolised all the citrulline. This did not occur with *Lc. oenos* OENO (Figures 5.1 and 5.2). As with strain OENO, urea was not found in the media inoculated with the lactobacilli.

In both lactobacilli, the complete degradation of arginine corresponded with the maximal formation of citrulline, not with the growth maximum. Further growth occurred in correlation with the catabolism of citrulline, suggesting that citrulline was being used as a source of energy.

Figure 5.5 shows the growth and metabolism of *Lb. buchneri* CUC-3 in the synthetic wine containing 5 g/L glucose. Arginine was used up while there was little metabolism of glucose, indicating the preferential utilisation of arginine. Moreover, the degradation of arginine was dramatic, corresponding with the sharp increases in OD, pH and the concentration of citrulline. Citrulline was reutilised after the exhaustion of arginine, confirming the previous finding (Figure 5.4). After the depletion of arginine, this strain entered the stationary phase during which citrulline and glucose were slowly co-metabolised. However, neither compounds were completely utilised even by the end of the experiment. These results are in contrast with those found in the synthetic medium containing 1 g/L glucose (Figure 5.4).

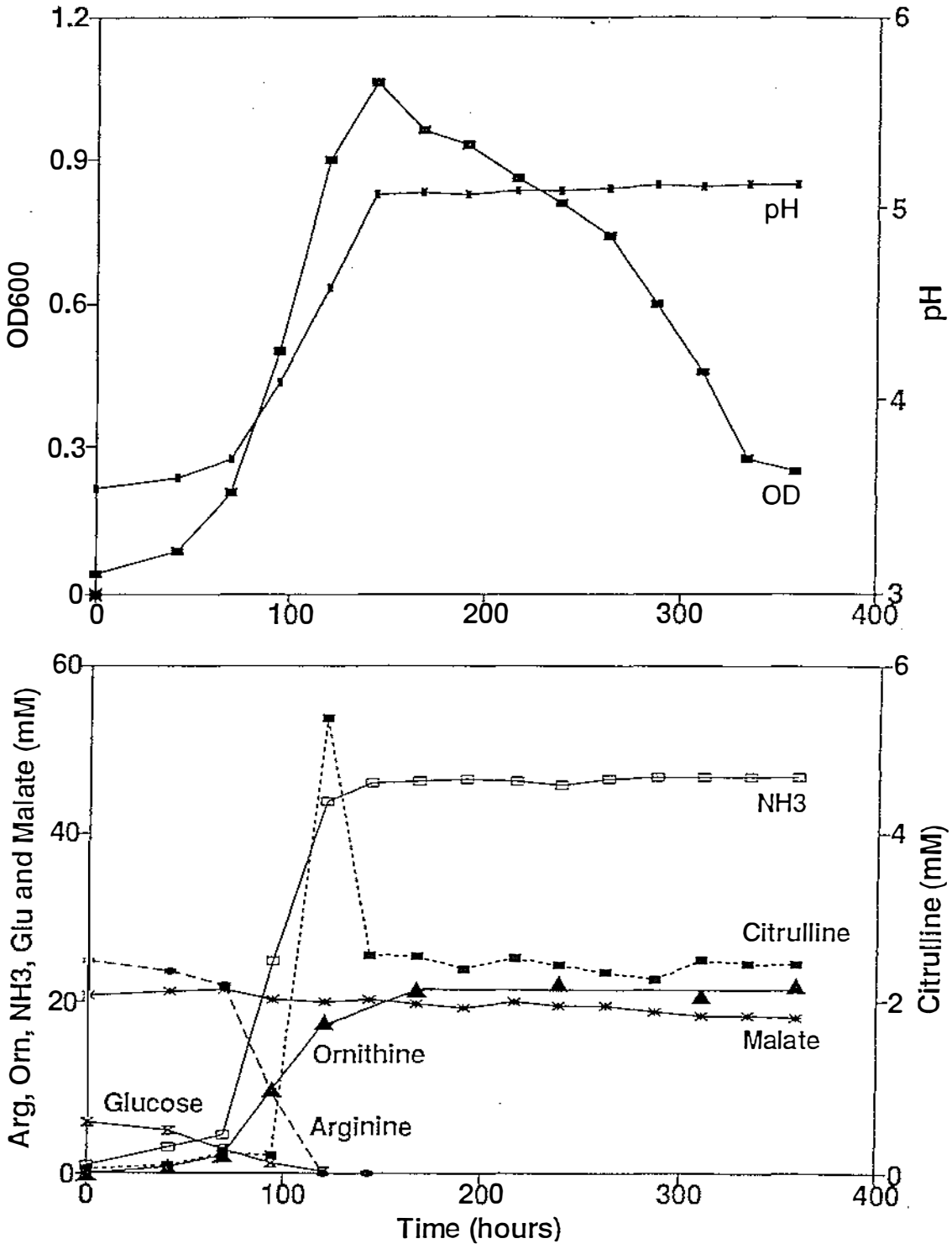

Fig. 5.3 Growth (30°C) and metabolism of *Lb. brevis* 250 in a synthetic medium containing 1 g/L glucose and 5 g/L arginine.

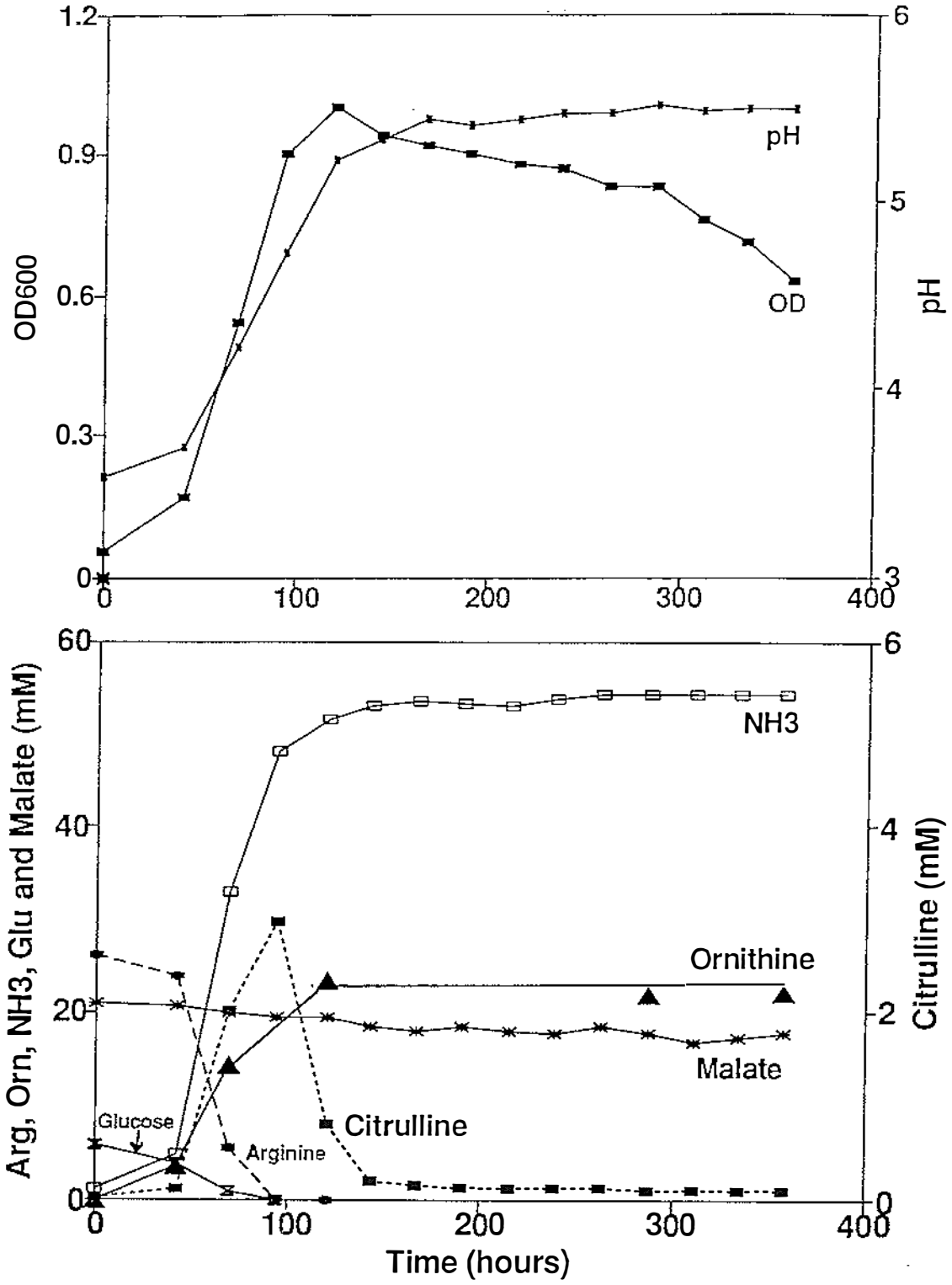

Fig. 5.4 Growth (30°C) and metabolism of *Lb. buchneri* CUC-3 in a synthetic medium containing 1 g/L glucose and 5 g/L arginine.

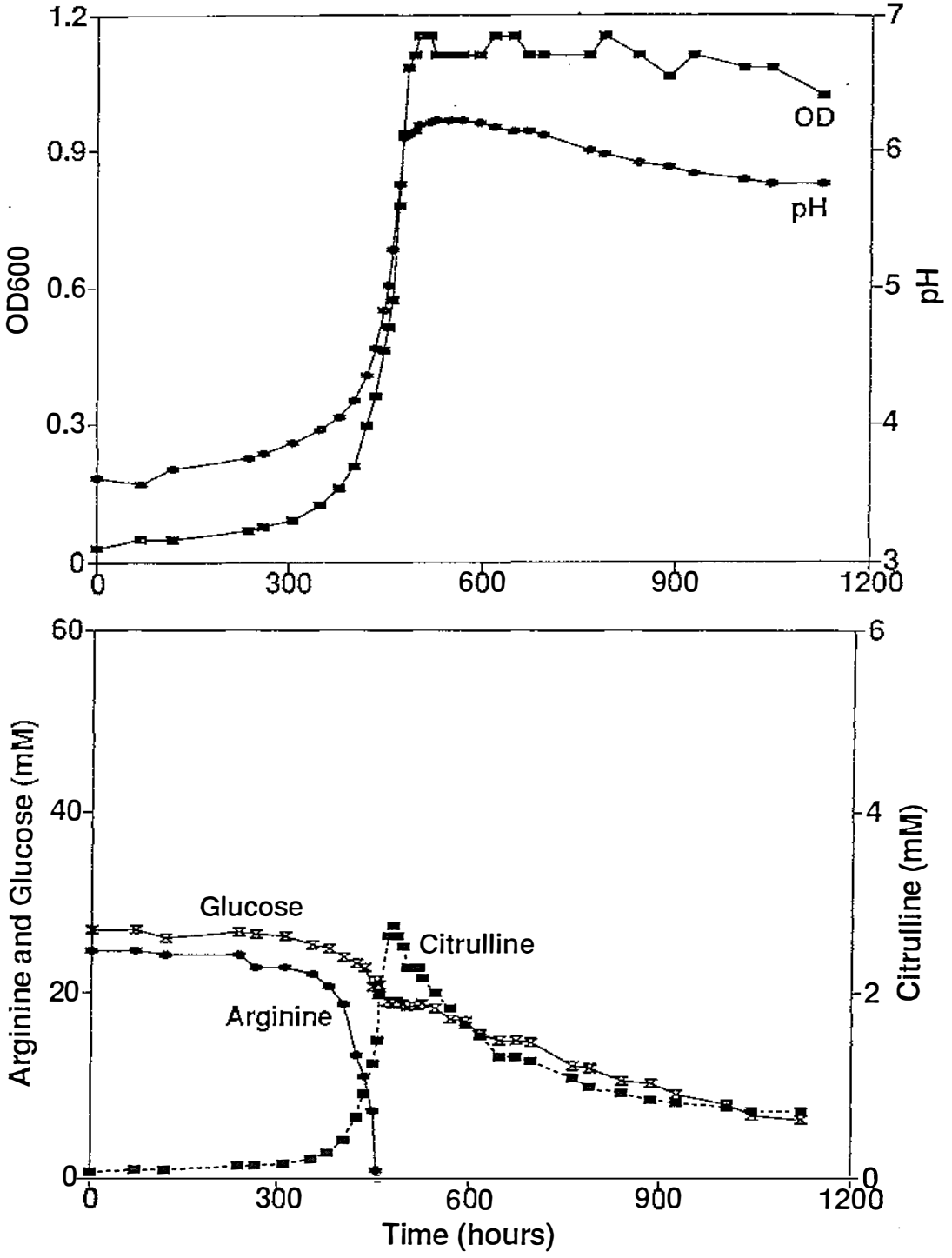

Fig. 5.5 Growth (30°C) and metabolism of *Lb. buchneri* CUC-3 in a synthetic wine containing 5 g/L each of glucose and arginine.

5.3.1.3 Stoichiometry of arginine catabolism

According to the arginine deiminase pathway (Section 2.1), one mole of arginine is converted into one mole each of ornithine, carbon dioxide and ATP, and two moles of ammonia. Carbon dioxide and ATP were not determined in these experiments because of technical complexity. Table 5.2 gives the stoichiometry of arginine catabolism and metabolite formation of the three heterofermentative LAB strains. The experimentally found molar ratios of ornithine to arginine ranged from 0.94 to 1.01, very close to the expected ratio of 1.0. The experimentally found molar ratios of ammonia to arginine ranged from 1.82 to 2.01, also approaching closely to the expected ratio of 2.0. Although the differences between some experimentally found and expected molar ratios were statistically significant, these differences were small and probably arose from analytical errors. The stoichiometry of arginine catabolism and the presence of the arginine deiminase pathway enzymes (Chapters 3 and 4) strongly suggest that the arginine deiminase pathway is active in wine LAB.

Table 5.2 Stoichiometry of arginine catabolism

Strain	Molar Ratio							
	Ornithine/Arginine				Ammonia/Arginine			
	Expected[a]	Experimental[b]			Expected[a]	Experimental[b]		
		Mean	SD	n		Mean	SD	n
Lc. oenos OENO	1.0	1.01	0.07	4	2.0	1.82***	0.05	10
Lb. brevis 250	1.0	0.99	0.03	6	2.0	1.91***	0.01	8
Lb. buchneri CUC-3	1.0	0.94*[c]	0.02	3	2.0	2.01	0.02	10

[a]Expected ratios are based on the pathway (Section 2.1).
[b]Experimental ratios were obtained after correcting for unused arginine, and excreted citrulline.
[c]Significance was determined using t-test; *, p = 0.05; ***, p = 0.005.

5.3.2 Growth and metabolism of heterofermentative wine LAB in the presence of glucose and fructose

Fructose, along with glucose, is one of the major sugars present in wine. Therefore, in some experiments a mixture of glucose and fructose was supplied in the medium. Figures 5.6 and 5.7 show the growth and metabolism of three heterofermentative wine LAB in synthetic media containing glucose and fructose. Good growth was obtained, especially by the lactobacilli. Arginine, glucose and fructose were metabolised by all strains. Arginine was totally degraded after the disappearance of glucose in the case of strains OENO and CUC-3 (Figure 5.6), but before the depletion of glucose in the case of strain 250 (Figure 5.7). Close examination of Figures 5.6 and 5.7 revealed that arginine degradation did not commence until fructose concentration was low (<1g/L), particularly with *Lc. oenos* OENO (Figure 5.6). This interesting sequential metabolism of fructose and arginine and the concurrent metabolism of glucose and arginine are discussed further in Section 5.4. Figure 5.7 also shows the excretion and reutilisation of citrulline by *Lb. brevis* 250, confirming the finding in Figure 5.3 for this strain.

5.3.3 Growth and metabolism of homofermentative wine LAB

Figure 5.8 shows the growth and metabolism of the homofermentative *Lactobacillus delbrueckii* CUC-1 and *Pediococcus sp.* CUC-4 in synthetic media which contained a low concentration of glucose (1 g/L), but a high arginine concentration (5 g/L) at an initial pH 4.6. This pH value was chosen to allow for some pH decrease, so that pH did not become low enough to inhibit bacterial growth if arginine was not catabolised. This is because lactobacilli and pediococci are susceptible to inhibition of growth at low pH (Wibowo *et al.*, 1985; Liu, 1990). Both strains grew in this medium, but neither degraded arginine. Glucose and L-malic acid were completely catabolised by both strains between the end of log phase and early stationary phase. The pH increased as a result of L-malic acid degradation, but declined after the disappearance of L-malic acid. This pH fall was probably the result of further formation of acid by fermentation of glucose.

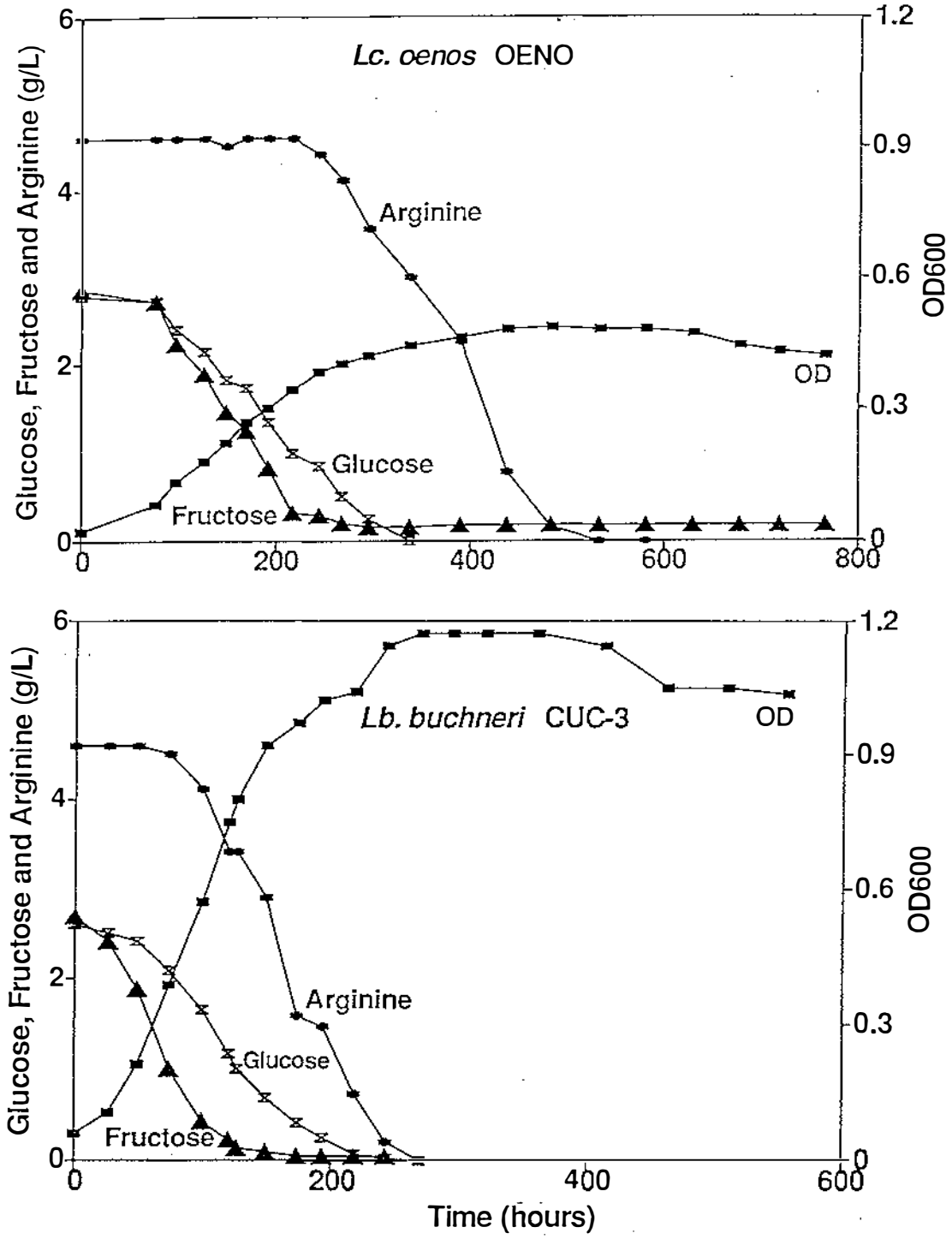

Fig. 5.6 Growth (22°C) and metabolism of *Lc. oenos* OENO and *Lb. buchneri* CUC-3 in a synthetic wine containing glucose and fructose (2.5 g/L each).

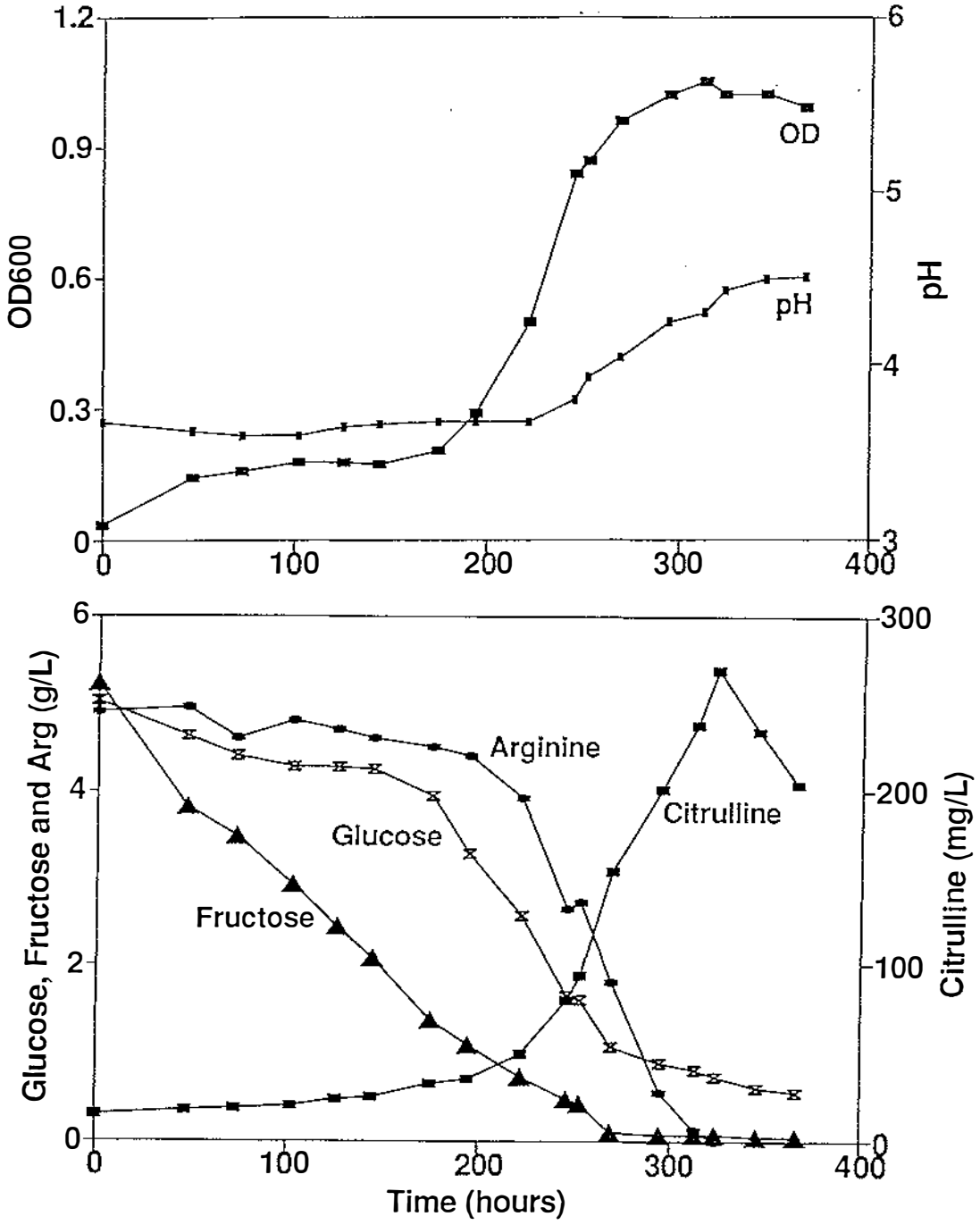

Fig. 5.7 Growth (30°C) and metabolism of *Lb. brevis* 250 in a synthetic medium containing glucose and fructose (5 g/L each).

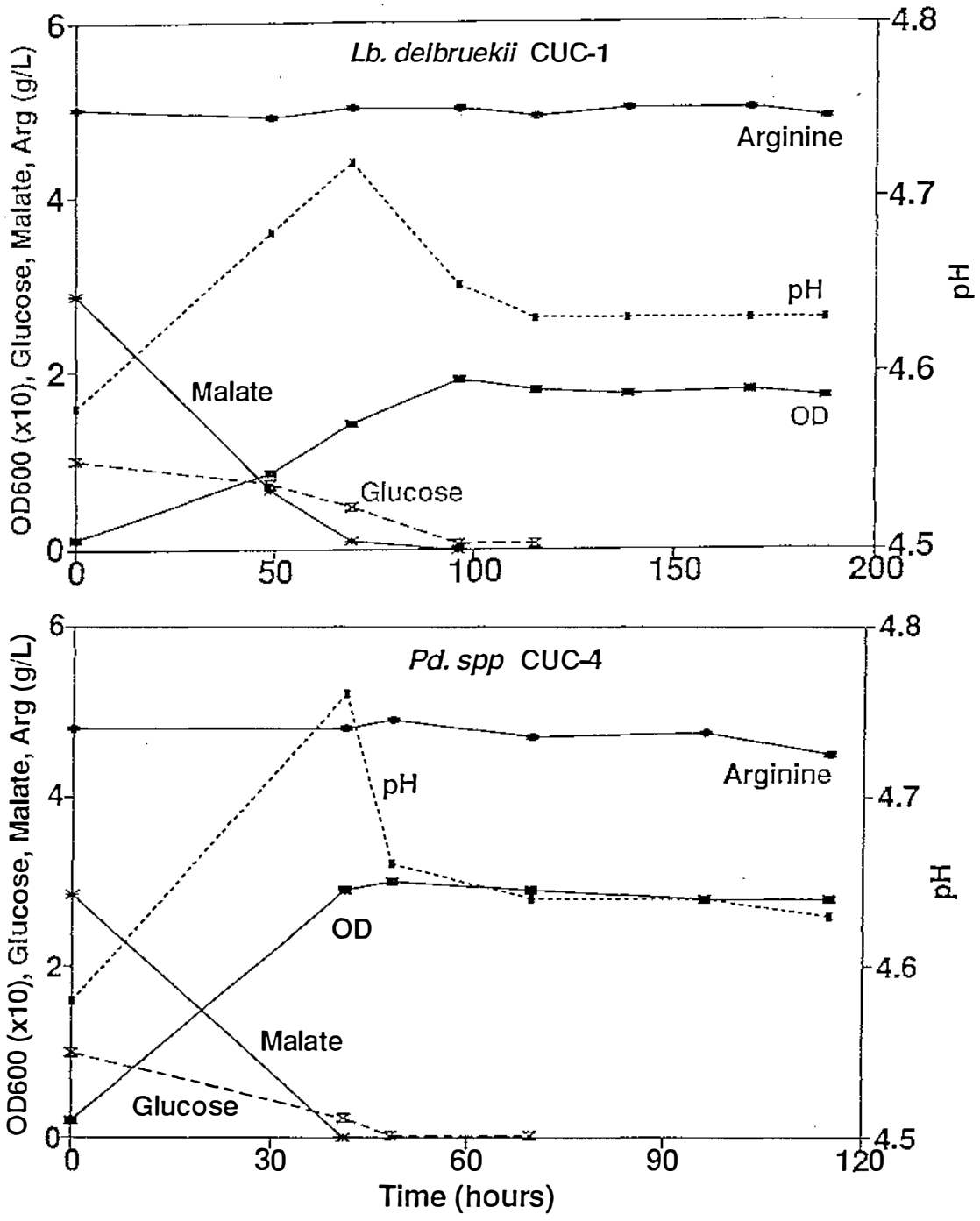

Fig. 5.8 Growth (30°C) and metabolism of *Lb. delbruekii* CUC-1 and *Pd. sp.* CUC-4 in a synthetic medium containing 1 g/L glucose and 5 g/L arginine.

5.4 Discussion

The time-course study has investigated the catabolism of arginine and metabolite formation in several heterofermentative wine LAB strains, including *Lc. oenos* OENO, *Lb. brevis* 250 and *Lb. buchneri* CUC-3. As expected from the arginine deiminase pathway (Section 2.1), ammonia and ornithine were found in the media as arginine was catabolised (Figures 5.1-5.4). The production of ornithine and ammonia from catabolism of arginine has also been reported in other wine LAB (Kuensch *et al.*, 1974; Weiller and Radler, 1976; Liu, 1990; Sponholz, 1992). However, this production has been attributed to the urea cycle being active in wine LAB (Kuensch *et al.*, 1974; Sponholz *et al.*, 1991; Sponholz, 1991;1992). According to this concept, arginine is first converted to ornithine and urea by arginase. Urea is then split by urease to ammonia and carbon dioxide. The urea cycle is shown in Figure 5.9.

The presence of the urea cycle in wine LAB has not been supported by valid evidence such as demonstration of the presence of arginase and urease. The findings from the current research project strongly suggest that in heterofermentative wine LAB, arginine is catabolised through the arginine deiminase pathway (the arginine dihydrolase pathway). This suggestion is supported by demonstration of the presence of the arginine deiminase pathway enzymes (Sections 3.3 and 4.3) and the stoichiometric conversion of arginine to ornithine and ammonia (Section 5.3.1.3). Further evidence to be presented in Section 6.3 suggests that arginine catabolism yields energy (ATP) for cell growth. This would be consistent with the arginine deiminase pathway, but not with the urea cycle.

Arginase has not been reported to be present in any LAB, according to a review by Cun in *et al.* (1986). The presence or absence of arginase in wine LAB was not investigated in this research. However, urea was analysed using enzyme test kits and urea was not found in the samples obtained in the experiments described in this Chapter (Section 5.3.1.2), as well as in other samples (to be presented in Section 8.3), suggesting the absence of arginase in wine LAB. This agrees with findings in other microorganisms possessing the arginine deiminase pathway. For example, arginase is not found in *Mycoplasma sp.* (Schimke and Barile, 1963) and urea is not formed from arginine degradation by a *Lb. plantarum* fish isolate (Jonsson *et al.*,

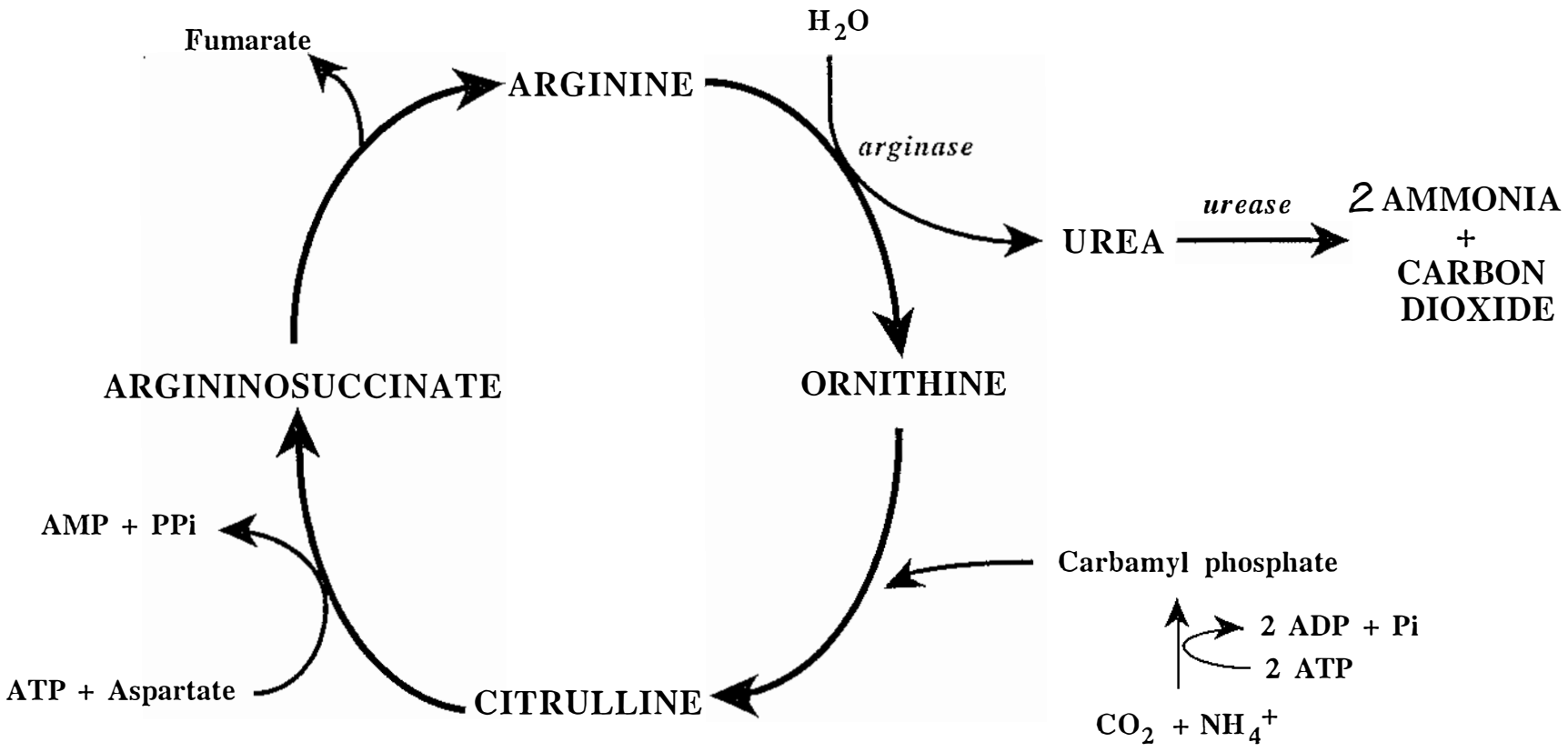

Fig. 5.9 The urea cycle (modified after Stryer, 1981).

1983). The evidence above strongly suggests that the urea cycle does not exist in wine LAB. Sponholz (1992) reported the production of urea from arginine by some wine LAB (strains of *Lb. brevis* and *Lb. buchneri*), contrary to the findings of the current studies. However, the author did not describe the materials and methods used in the determination of urea or, indeed, of any of the metabolites investigated in this study. Thus, it is not certain whether the reported "urea" was indeed urea. From a stoichiometric point of view, the production of urea from arginine is not likely, since arginine is completely degraded through the arginine deiminase pathway and thus, there is no arginine available to go through a urea cycle. On the other hand, if arginase alone of the urea cycle enzymes was present, there would be a stoichiometric conversion of arginine to ornithine, but urea would also be present in a 1:1 stoichiometry unless urease was also present. However, there is no evidence on the presence of urease in wine LAB. In the Sponholz study, the reported "urea" is present at very low levels in relation to the amount of arginine catabolised, indicating that it may not be urea.

It should be stressed that urea, if present, would interfere with the citrulline analysis used in this study, because diacetyl monoxime, one of the reagents, reacts with urea to give a coloured product (Archibald, 1944). However, since urea was shown to be absent in the fermentations, this interference can be ruled out.

The excretion of small amounts of citrulline (10-15% on a molar basis) from arginine catabolism (Figures 5.1-5.6) was found, in addition to the production of ammonia and ornithine. Kuensch *et al.* (1974) also noted a slight production of citrulline associated with arginine degradation and attributed the production of citrulline to the transformation of ornithine in the urea cycle. However, the time-course of citrulline production in the present study is not consistent with this proposal. In fact, the time-course of citrulline formation correlated well with that of arginine degradation and ornithine production (Figures 5.1-5.6). This provides additional evidence against the existence of the urea cycle in wine LAB. The production of citrulline from arginine catabolism has been reported in other microorganisms possessing the arginine deiminase pathway, such as *Streptococcus faecalis* mutants (Simon *et al*, 1982), *Lactobacillus sake* INRA 300 from meat (Montel and Champomier, 1987) and *Clostridium botulinum* Okra B (Patterson-Curtis and Johnson, 1992).

The physiology of citrulline excretion is not clear and only some speculations can be made at this stage. In *Lactococcus lactis* subsp. *lactis* ML_3 known to possess the arginine deiminase pathway, arginine is transported into the cell via an antiport system in a 1:1 stoichiometry with ornithine export (Driessen *et al.*, 1987; Poolman *et al.*, 1987). This process does not require metabolic energy. It is likely that this arginine-ornithine antiport process functions in the transport of arginine into the cell of wine LAB. It is possible that arginine uptake may also be coupled to export of citrulline via the same antiport system, but only to a small extent. The excretion of citrulline would result in loss of ATP, since the excreted citrulline could not be catabolised further along the ADI pathway to produce ATP. Alternatively, citrulline excretion may involve an independent transport system.

Some of the excreted citrulline can apparently be recatabolised by the lactobacilli strains (Figures 5.3-5.6), but not by the *Lc. oenos* strain (Figures 5.1 and 5.2). This suggests that citrulline can be transported back into cells by lactobacilli, but not by the leuconostoc, which raises the question of the physiological difference between these organisms. Another question to be posed is why do the lactobacilli excrete citrulline, then reassimilate and recatabolise it? These questions remain to be answered.

The time-course study found that glucose and arginine were metabolised concurrently in heterofermentative wine LAB (Figures 5.1-5.7). Concomitant degradation of glucose and arginine has also been reported in *Str. faecalis* ATCC 11700 (Simon *et al.*, 1982). Concurrent utilisation of glucose and arginine indicates that glucose does not repress the synthesis of arginine deiminase pathway enzymes. Lack of glucose repression has been shown with *Lb. buchneri* NCDO 110 (Manca de Nadra *et al.*, 1986a) and *Mycoplasma fermentans* PG18 (Olson *et al.*, 1993). Conversely, a sequential metabolism of glucose and arginine is found in some homofermentative LAB such as *Lactococcus lactis* H1 (Crow and Thomas, 1982) and *Lb. plantarum* from fish (Jonsson, *et al.*, 1983). Sequential utilisation of glucose and arginine suggests that glucose does repress the synthesis of the arginine deiminase pathway enzymes. This repression has been demonstrated in *Lb. leichmannii* ATCC 4797 (Manca de Nadra *et al.*, 1986b) and *Lb. sake* INRA 300 (Montel and Champomier, 1987).

Although glucose and arginine were found to be metabolised concurrently in this investigation, when a mixture of glucose, fructose and arginine was supplied, the arginine was not catabolised until the fructose level was low (<1g/L) in the heterofermentative wine LAB (Figures 5.6 and 5.7). This does not appear to have been reported in other microorganisms. This finding suggests that fructose represses the synthesis of the arginine deiminase pathway enzymes. The mechanism of the repression of arginine catabolism by fructose is not known.

It is known that the ATP pool size is an important signal in the regulation of the arginine deiminase pathway in *Pseudomonas aeruginosa* PAO1, *Str. faecalis* ATCC 11700 and other bacteria (Mercenier *et al.*, 1980; Simon *et al.*, 1982; Cunin *et al.*,1986). Repression of the ADI pathway occurs under conditions which promote the energy status of the cells; for instance, the presence of a fermentable substrate or other energy sources or both, as well as aerobic and anaerobic respiration (Cunin *et al.*, 1986). Conversely, energy depletion results in induction of the pathway enzymes. Hence, the sequential utilisation of fructose and arginine in heterofermentative wine LAB could conceivably be due to the generation of additional ATP from fructose metabolism relative to that available from glucose fermentation.

According to Kandler (1983), fructose can act as a hydrogen acceptor for reoxidation of NADH and NADPH by the reduction of fructose to mannitol during heterofermentation, and mannitol formation from fructose has been demonstrated in heterofermentative wine LAB (Pilone *et al.*, 1991). The acetyl phosphate formed from the phosphoketolase reaction could therefore be converted to ATP and acetate, rather than being reduced to ethanol. Consequently, an additional ATP is generated when fructose is reduced. It is perhaps this additional ATP per mole of substrate oxidised that may have repressed arginine catabolism observed in this study (Figures 5.6 and 5.7). Further work would be required to substantiate this hypothesis. Alternatively, the difference between the fructose and glucose effects on arginine metabolism may be a consequence of differences in the mechanism or rate of transport of the two sugars.

This study indicates that arginine catabolism does not seem to affect malic acid degradation or vice versa. Complete utilisation of malic acid was achieved with *Lc. oenos* OENO, but not with the lactobacilli. The incomplete

utilisation of malic acid by the lactobacilli may not necessarily be due to arginine catabolism, since incomplete degradation of malic acid by the lactobacilli also occurred in AJMRS containing 0.5 g/L arginine (Section 3.2.2) and in synthetic media containing only 0.17 g/L arginine (unpublished data). In a wine containing 4 g/L malic acid and 1.3 g/L arginine, Sponholz (1992) found that malic acid was completely degraded by *Lb. brevis* B22, but incompletely by a strain of *Lb. buchneri*, while arginine was almost used up by both strains. This reflects strain variation in the ability to degrade malic acid, rather than the influence of arginine catabolism.

Activities for arginine deiminase pathway enzymes were not found in homofermentative wine LAB (Chapter 4). The results presented in the present chapter confirm that arginine catabolism does not occur in the homofermenters cultured in the synthetic medium containing low levels of glucose (1 g/L) and high levels of arginine (5 g/L) (Figure 5.8). This suggests that homofermentative wine LAB are indeed incapable of catabolising arginine in contrast to other homofermenters (Crow and Thomas, 1982; Jonsson *et al.*, 1983; Manca de Nadra *et al.*, 1986b; Montel and Champomier, 1987). The reason for this is unclear.

Comparisons of cell mass (OD) reveal the poorer growth of the homofermenters (Figure 5.8) compared to that of the heterofermenters, particularly in the case of the lactobacilli (Figures 5.2-5.4), when cultured in the synthetic medium containing 1 g/L glucose and 5 g/L arginine. Furthermore, growth and arginine catabolism continued after the exhaustion of glucose with *Lc. oenos* OENO (Figures 5.1 and 5.2). With the lactobacilli, growth also continued while citrulline was reutilised after the exhaustion of glucose and arginine (Figures 5.3 and 5.4). These observations suggest that arginine and citrulline catabolism may contribute energy for growth of the heterofermenters. A more thorough study, therefore, was carried out on the growth responses and energetics of arginine and citrulline catabolism in wine LAB and the results are presented in Chapter 6.

The findings of this research have important oenological implications. Ammonia formed from arginine catabolism leads to an increase in the pH value and the extent of such a pH rise depends on the amount of arginine present in the medium. The effect of ammonia formation from arginine metabolism on pH increase may be more significant than the contribution of

malic acid degradation at high arginine levels. It is possible to find wines with arginine over 2 g/L (Sponholz, 1991) and grape juice containing 10 g/L arginine (Eggenberger, 1988). The pH increases can be considerable and cause wine spoilage if the arginine level is high.

The finding of citrulline excretion in this study is also oenologically significant, since citrulline is a precursor of the carcinogenic ethyl carbamate (Ough *et al.*, 1988). An experiment on the formation of ethyl carbamate and citrulline excretion from arginine catabolism was subsequently conducted in synthetic wine and wine inoculated with heterofermentative wine LAB. The results of this investigation are presented in Chapter 7.

Carbamyl phosphate, another intermediate of arginine catabolism and a precursor of ethyl carbamate (Ough *et al.*, 1988), was not determined in the time-course study. This is because carbamyl phosphate is chemically unstable and no reliable ready-to-use method is available for the determination of this compound. Carbamyl phosphate is decomposed to phosphate, ammonia and carbon dioxide at pH levels from 2-4; and to phosphate and cyanate at pH levels from 6-8, the cyanate being further decomposed to ammonia and carbon dioxide (Jones, 1962; Allen and Jones, 1964). Carbamyl phosphate is commonly determined by differential phosphate analysis using the Fiske-SubbaRow method (Leloir and Cardini, 1957). However, this method is not applicable for the determination of carbamyl phosphate excreted (if any) in the synthetic medium, since a high concentration of potassium phosphate (4 g/L) is present in the medium (Table 5.1). It remains to be investigated whether carbamyl phosphate can be excreted.

Chapter 6

Energetics of Arginine and Citrulline Catabolism in Selected Wine Lactic Acid Bacteria

6.1 Introduction

Arginine degradation by means of the arginine deiminase pathway (see Section 2.1) results in the production of ATP. Thus, arginine is a potential source of energy if the three enzymes of the pathway are functional. Previous experiments (Chapters 3-5) have confirmed that certain wine lactic acid bacteria (LAB) catabolise arginine through the deiminase pathway. However, it is still not known whether the ATP derived from arginine catabolism is used by these wine LAB to support growth. The results in Chapter 5 show some further growth of *Lc. oenos* OENO (Figures 5.1 and 5.2) after the disappearance of glucose, which is correlated with continuing arginine degradation. Similarly, some further growth of *Lb. brevis* 250 and *Lb. buchneri* CUC-3 (Figures 5.3 and 5.4) is correlated with citrulline reassimilation and utilisation after the exhaustion of arginine and glucose. In addition, these arginine-degrading heterofermentative wine LAB grew to higher cell yields (Figures 5.2-5.4) than non-arginine-degrading homofermentative strains (Figure 5.8). These results suggest that ATP generated from the catabolism of arginine and citrulline may be coupled to growth of the heterofermentative strains. Therefore, further work was conducted in an attempt to confirm the energy role of arginine and citrulline catabolism by studying the growth response and molar growth yield for selected wine LAB cultures metabolising arginine and citrulline.

6.2 Materials and methods

6.2.1 Lactic acid bacterial strains

The following heterofermentative wine LAB strains were used: *Leuconostoc oenos* OENO, *Lactobacillus brevis* 250 and *Lactobacillus buchneri* CUC-3. The original source of these strains is given in Table 4.1.

6.2.2 Media composition and preparation

A chemically defined synthetic medium was employed in this study. The composition and preparation of the medium are as described in Section

5.2.2, except that fructose and ethanol were omitted. The initial concentrations of glucose, arginine and citrulline and the initial pH of the media were dependent on the experimental design and are indicated in Section 6.2.3.

6.2.3 Culturing procedures

To test the growth response to arginine at different initial pH values, wine LAB strains, subcultured in pH 5.5 VJMRS broth (Section 3.2.2), were directly inoculated into the synthetic medium containing glucose (5 g/L) with or without arginine (5 g/L) at pH 3.2, 3.5 and 5.5.

To determine the growth-limiting glucose concentration and molar growth yield when using glucose as the sole energy source, strains were cultured in the synthetic medium (pH 5.5) containing no arginine, but with additions of 0-40 mM of glucose. The initial pH value of the synthetic medium was adjusted to pH 5.5. This high initial pH value allowed for some pH reduction from glucose catabolism in the weakly buffered medium before becoming too low and inhibiting growth. Strains were first subcultured in pH 5.5 VJMRS broth (Section 3.2.2), followed by subculturing in pH 3.5 synthetic medium containing glucose (5 g/L) and arginine (5 g/L), before being inoculated into the test media.

The growth response to various concentrations of arginine and citrulline was tested using the synthetic medium (pH 3.5) containing 13.3 mM glucose (growth-limiting, see Section 6.3.2) and various concentrations of arginine (0-21.8 mM) or citrulline (0-20.5 mM). The initial pH value of the synthetic medium was adjusted to pH 3.5. This low initial pH value was chosen to allow for some pH increase from arginine catabolism in the weakly buffered medium before becoming too high and inhibiting growth. Strains were first subcultured in pH 5.5 VJMRS broth (Section 3.2.2), followed by subculturing in a pH 3.5 synthetic medium containing glucose (5 g/L) and arginine (5 g/L), before being inoculated into the test media. The results of the growth response to arginine and citrulline were used to calculate molar growth yields (Section 6.2.5).

All cultures were incubated at 30°C in screw-capped test tubes containing 10 mL medium. Single fermentations were performed for the experiments on growth response to arginine at different initial pH values and on the

determination of growth-limiting concentrations of glucose. However, triplicate fermentations were performed for the more critical experiments on the growth response to various concentrations of arginine and citrulline. Inoculation of media was at about 1% (v/v) for subcultures from VJMRS broth and 2% for subcultures from the synthetic medium. Only cultures between late log and early stationary phases were used as an inoculum as judged visually from turbidity changes. The carry-over of glucose, arginine and citrulline was negligible, because these compounds either would have been metabolised during growth in the subculture medium (in the case of glucose and arginine) or the concentrations were low at the time of transfer (in the case of citrulline). Growth was monitored by optical density (OD) readings at 600 nm from culture tubes fitting directly into the cuvette holder of a Bausch and Lomb Spectronic-20 Spectrophotometer. Cultures were mixed by vortexing before taking OD readings.

6.2.4 Determination of cellular dry weight

Cellular dry weight was determined by relating optical density (OD_{600}) readings to standard calibration curves of *Lc. oenos* OENO or *Lb. brevis* 250. A standard curve was not prepared for *Lb. buchneri* CUC-3, since this strain was morphologically similar to strain 250 and it was assumed that the standard curve of strain CUC-3 would be similar to that of strain 250. Thus, the dry weight of strain CUC-3 was obtained from the standard curve of strain 250.

To prepare the standard curves, six separate cultures of each strain were grown (30°C) in 200 mL of synthetic medium (5 g/L each of glucose and arginine) at pH 3.5 in medical flat bottles (full). A 175 mL sample of each culture was vacuum-filtered through a pre-dried and pre-weighed 0.45 µm pore-size membrane filter (Micro Filtration System, Dublin, California). The cells collected on the membranes were washed by passing two aliquots (175 mL each) of DI water. The membrane was then dried at 75°C in a vacuum oven to a constant weight (133.5 $\pm$ 7.3 µg/mL, n = 6 for strain OENO; and 182.3 $\pm$ 23 µg/mL, n = 5 for strain 250). The remaining 25 mL culture was diluted (from no dilution to 10-fold) with the same but uninoculated synthetic medium and OD at 600 nm determined. Regression analyses were made for the plots of diluted dry weights against the corresponding OD readings for each culture. The following averaged regression equations were found: dry weight (µg/mL) = 338 ($\pm$ 12.1) x OD - 6.0 ($\pm$ 0.4) for *Lc. oenos* OENO, and

dry weight (μg/mL) = 305 (± 20.3) x OD - 11.1 (± 1.5) for *Lb. brevis* 250. The R-squared values were above 99% for all cultures. The regression equations were used to calculate the dry weights from OD readings in subsequent experiments.

6.2.5 Determination of molar growth yields and YATP

Molar growth yield ($Y_{m)}$ is defined as the cellular dry weight (grams) formed per mole of an energy source consumed (Stouthamer, 1969; Gottschalk, 1986). In the present experiments, the energy source was glucose, arginine or citrulline. In practice, Y_m is the slope of the plot of cellular dry weights (μg/mL) against the amounts of the energy source consumed (μmol/mL). Regression analyses were made to obtain the slope (regression coefficient) of the plots of dry weights versus the amounts of the energy sources consumed in the experiments described in Section 6.2.3. Y_{ATP} is the molar growth yield achieved per mole of ATP. Y_{ATP} is calculated by dividing Y_m by the moles of ATP known to be generated per mole of the energy source on the basis of the established pathways of metabolism for the particular strain.

To determine Y_m, samples were taken at the point of maximum growth. For each sample, cellular dry weight was calculated from OD readings using the equations described in Section 6.2.4. The concentration of arginine, citrulline and glucose was determined from which the amount of arginine and glucose metabolised was calculated. Assuming the same $Y_{m(glucose)}$ in the presence of arginine as that determined in its absence, the contribution of total cell yield due to glucose metabolism can be calculated (this also assumes that $Y_{m(glucose)}$ is the same at pH 5.5 and pH 3.5).

6.2.6 Analysis

Methods for the determination of glucose, arginine, citrulline and pH have been described in Section 3.2.4.

6.3 Results

6.3.1 Growth in presence of arginine at different initial pH values

Figure 6.1 shows the growth of *Lb. brevis* 250, *Lb. buchneri* CUC-3 and *Lc. oenos* OENO in the synthetic medium containing glucose (5 g/L) with or without arginine (5 g/L) at various initial pH values. None of the strains grew

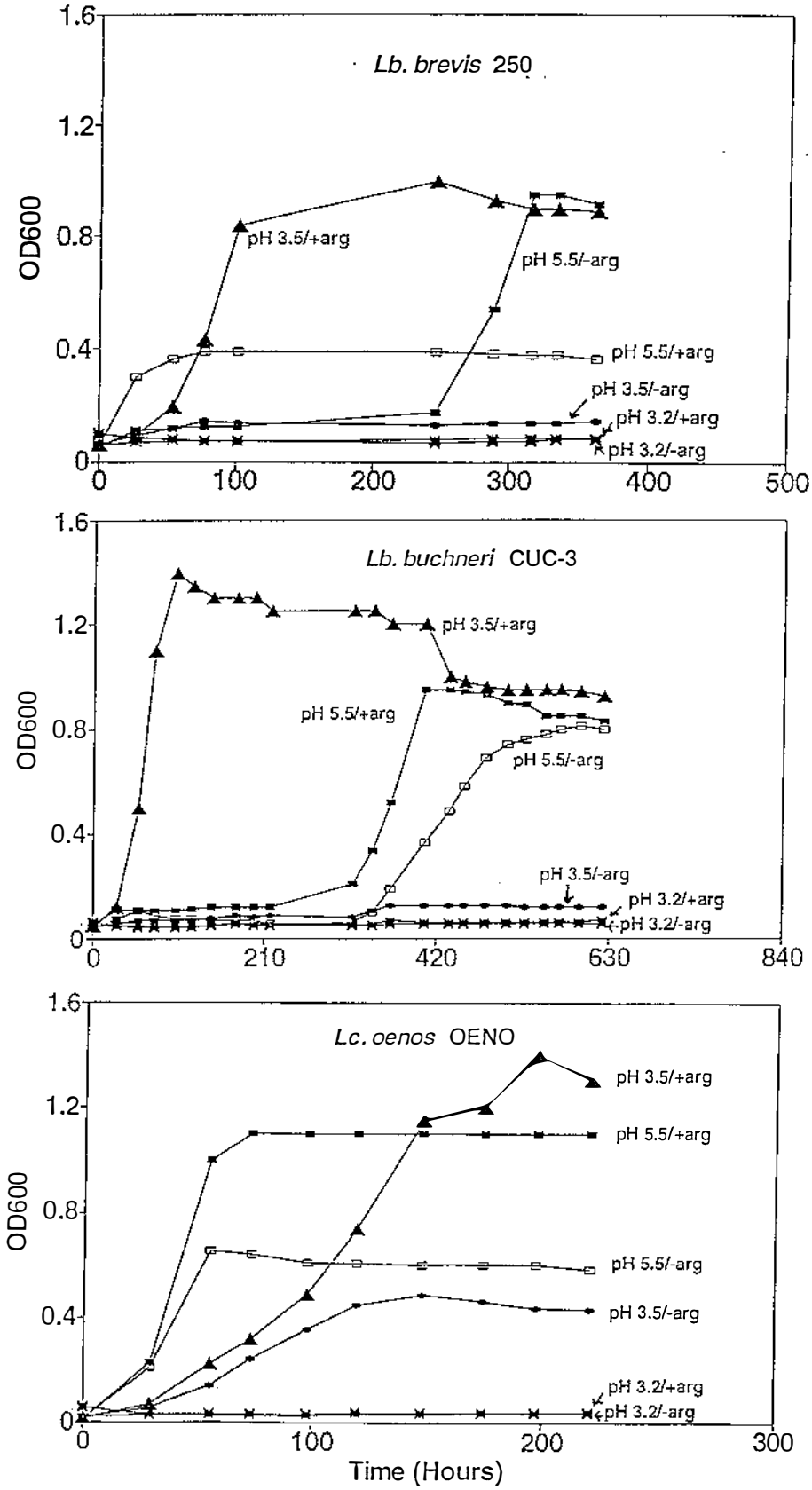

Fig. 6.1 Growth of *Lb. brevis* 250, *Lb. buchneri* CUC-3 and *Lc. oenos* OENO at different initial pH values in synthetic media containing glucose with or without arginine.

at pH 3.2 with or without arginine. The inhibition of growth of the lactobacilli at this pH was not unexpected, since lactobacilli are known to be sensitive to low pH (Wibowo *et al.*, 1985). However, the inhibition of strain OENO at this pH was unexpected, because leuconostocs are generally tolerant of low pH (Wibowo *et al.*, 1985; Liu, 1990). A combination of nutrient limitation in the synthetic medium and low pH may be the reason for the inhibition.

The three strains responded differently at pH 3.5. Good growth was achieved by strain OENO in the medium with or without arginine, although growth was better in its presence. The lactobacilli grew only slightly in the absence of arginine, but the same strains achieved excellent growth in its presence. The inhibition of growth of the lactobacilli in the the medium without arginine is probably due to the reduction in pH due to acid formation from glucose. Furthermore, the lactobacilli degraded malic acid poorly as shown in Chapter 5 (Figures 5.3 and 5.4) and, hence, malic acid degradation would not have effectively counteracted the pH decrease. The excellent growth of the three strains in the presence of arginine clearly demonstrates the beneficial effects of arginine catabolism. The beneficial effects are probably largely attributable to the pH increases from the formation of ammonia as demonstrated in Chapter 5 (Figures 5.1-5.6) and energy (ATP) generation. The beneficial effect of pH rise on growth is especially evident with the lactobacilli. Since little growth was achieved by the lactobacilli in the absence of arginine, a comparison of cell yield (OD) is not possible. This comparison, however, is possible with strain OENO. Although the maximum cell yield with strain OENO is more than twice as high in the presence of arginine as in its absence, this may well be due to an enhanced glucose utilisation as a result of the increase in pH due to ammonia production from arginine. Since the pH and residual glucose concentration were not determined in the experiment, it is not possible to ascertain the extent to which the production of ATP from arginine also contributed to the increased growth yield.

All strains grew at pH 5.5 with or without arginine, but the growth pattern was different for each strain. Strain 250 initiated growth about 20 hours after inoculation in the presence of arginine, but growth did not begin until about 250 hours later in its absence. Thus, the presence of arginine clearly stimulates growth initiation with this strain. On the other hand, strain CUC-3 initiated growth only slightly earlier in the presence of arginine than in its

absence. However, neither strain achieved higher cell yields in the presence of arginine. On the contrary, strain 250 actually achieved higher cell yields in the absence of arginine. This difference could again be due to pH changes during growth. Since the initial pH was already 5.5, the release of ammonia from arginine catabolism may have raised the pH further to a value that might have inhibited the growth of the lactobacilli. In contrast to the effect of arginine on lactobacilli, the time of growth initiation of *Lc. oenos* OENO was similar in the presence and absence of arginine, but the maximum cell yield with arginine was almost twice that without arginine. These findings raise the question as to why strain OENO was not inhibited by the increasing high pH and the lactobacilli strains 250 and CUC-3 clearly were?

6.3.2 Determination of growth-limiting glucose concentration and molar growth yield of glucose-grown cultures

The growth-limiting concentration of glucose was determined for subsequent use in the study of growth in the presence of arginine and citrulline (see Section 6.3.3), since a fermentable sugar is required for the catabolism of arginine in wine LAB strains (Liu, 1990). Information is not available on the requirement of sugar for the catabolism of citrulline, but it was assumed that the presence of a fermentable carbohydrate is also needed.

Figure 6.2 shows the cell yield of *Lb. brevis* 250 and *Lc. oenos* OENO at various glucose concentrations. For strain 250, cell yield (dry weight) was proportional to glucose concentration up to at least 20 mM. For strain OENO, cell yield increased only up to 16 mM glucose. The regression analysis for the linear portion of the plot of dry weights versus glucose concentrations gave a slope of 11.9 (R-squared value = 98.8%) for strain 250 and 12.5 (R-squared value = 95%) for strain OENO. By definition, $Y_{glucose}$ is the equivalent of the slope. Therefore, it can be estimated that for both strains, approximately one mole of ATP was generated per mole of glucose fermented using the accepted Y_{ATP} value of 10.5 $\pm$ 2 (Gottschalk, 1986). The linear dependence of cell yield on glucose concentration up to 16-20 mM indicates that concentrations below this will be growth-limiting. For convenience, a growth-limiting concentration of 13.3 mM glucose was used for all strains in the study of growth in the presence of arginine and citrulline (Section 6.2.3). This relatively high, but growth-limiting concentration of glucose was chosen to ensure that a sufficient cell yield at each level of arginine and citrulline was achieved.

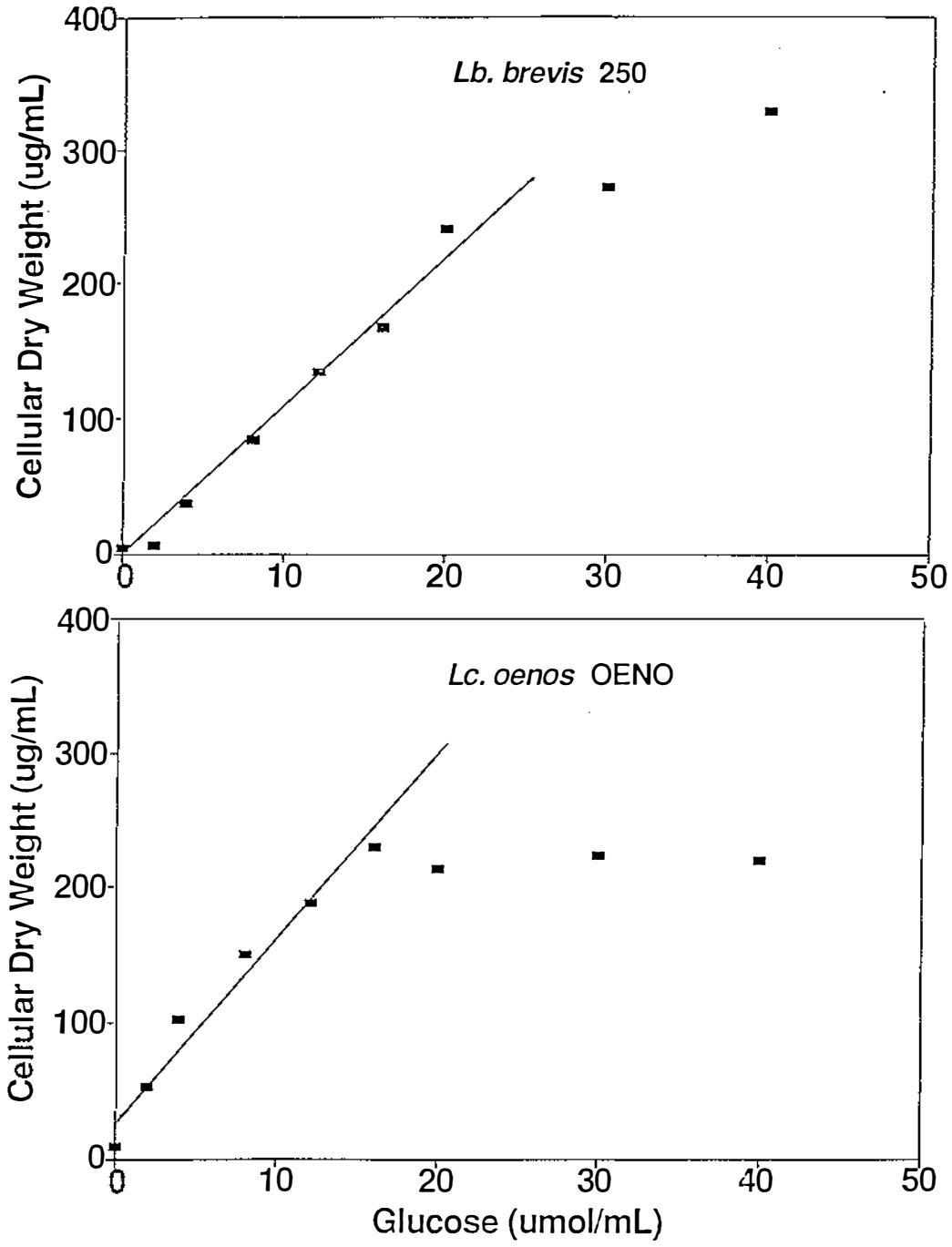

Fig. 6.2 Cell yield of *Lb. brevis* 250 and *Lc. oenos* OENO in synthetic media containing glucose at increasing concentrations (initial pH 5.5, 30°C).

6.3.3 Growth at different concentrations of arginine and citrulline

Figure 6.3 shows the growth of *Lb. brevis* 250 and *Lb. buchneri* CUC-3 at various initial concentrations of arginine at initial pH 3.5. As mentioned in Section 6.2.3, this low initial pH value was chosen to allow for pH increases due to ammonia formation from arginine, instead of the high initial pH 5.5 in the absence of arginine (to allow for pH decreases due to acid formation from glucose). It should be pointed out that these concentrations were the amounts of arginine added into the media, not necessarily the actual amounts consumed. The maximum cell yield of strains 250 and CUC-3 increased with increasing arginine concentrations. Neither strain grew in the absence of arginine, confirming the previous observations of growth at pH 3.5 (Figure 6.1). Strain 250 failed to grow at 4.6 mM arginine, presumably due to pH reduction that resulted from acid formation from glucose fermentation. It is interesting to note that the time lag to reach the maximum growth by both strains at 21.8 mM arginine was longer than at lower concentrations, except at 12.3 mM for strain 250. Data for strain OENO are not shown here, since growth of this strain did not follow a consistent pattern with varying arginine concentration.

Figure 6.4 shows the growth of *Lb. buchneri* CUC-3 and *Lc. oenos* OENO at various initial citrulline concentrations at pH 3.5. The maximum cell yield of strain CUC-3 increased with increasing citrulline concentrations. In contrast, the growth yield of strain OENO was not affected by increasing citrulline concentrations. The different response of these two strains to the addition of citrulline is consistent with the finding reported in Chapter 5, that strain CUC-3 reassimilates the excreted citrulline at the later stages of fermentation, while strain OENO does not metabolise the excreted citrulline. As with arginine, the highest level of added citrulline (20.5 mM) caused a significant delay in the onset of the exponential growth phase in both strains CUC-3 and OENO. *Lb. brevis* 250 failed to grow at any of the citrulline concentrations, even though this strain has been shown to be capable of metabolising the excreted citrulline (Figures 5.3 and 5.6). The reason for the failure of strain 250 to grow is not clear.

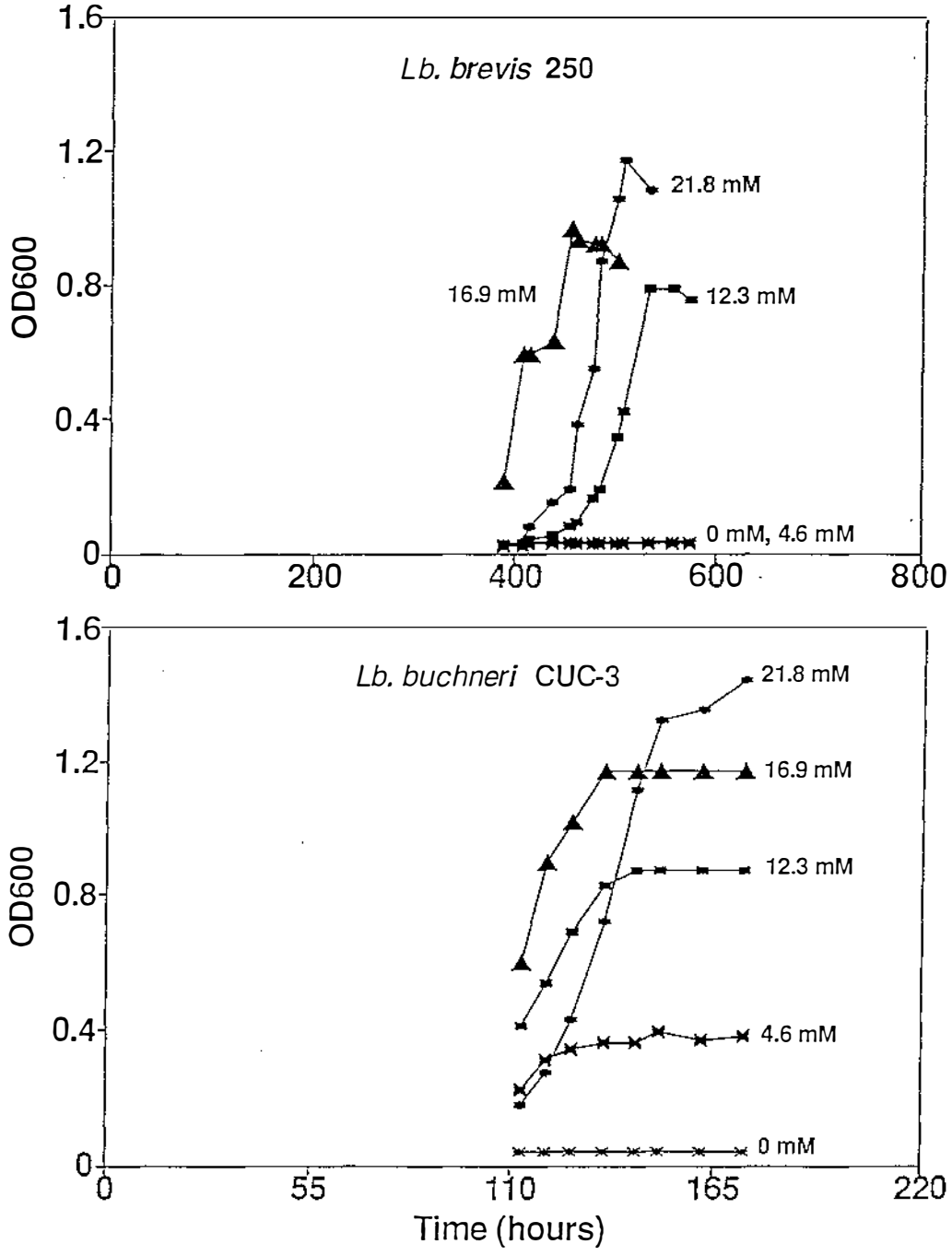

Fig. 6.3 Growth (30°C) of *Lb. brevis* 250 and *Lb. buchneri* CUC-3 in synthetic media containing glucose at 13.3 mM and at various concentrations of arginine (initial pH 3.5)

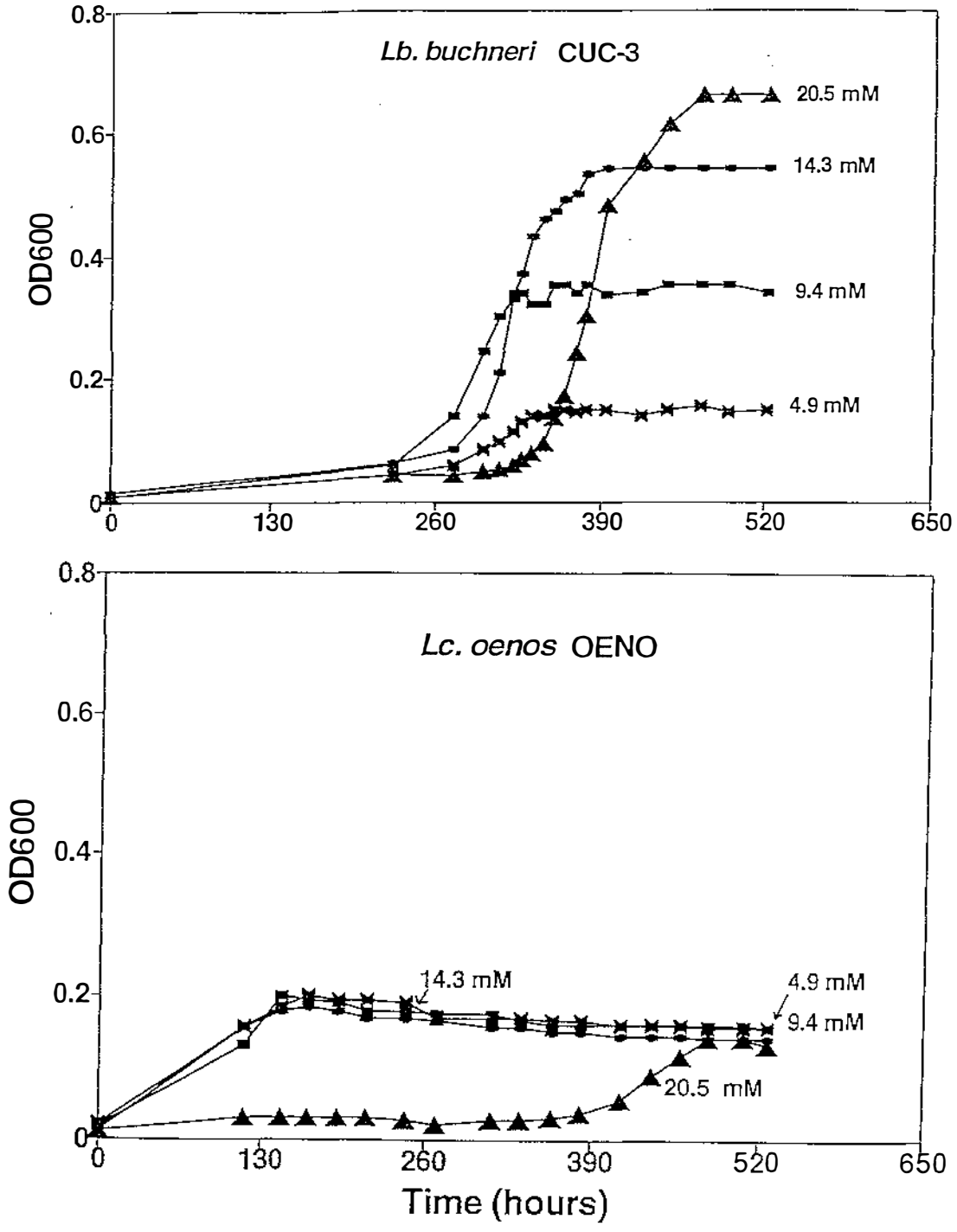

Fig. 6.4 Growth (30°C) of *Lb. buchneri* CUC-3 and *Lc. oenos* OENO in synthetic media containing glucose at 13.3 mM and at various concentrations of citrulline (initial pH 3.5)

6.3.4 Molar growth yields of arginine and citrulline

Cellular dry weight at maximum growth for each level of arginine and citrulline was calculated from OD readings using the equations described in Section 6.2.4. The relationship between dry weights of strains 250, CUC-3 and OENO and arginine consumption was found after correcting for unused arginine, excreted citrulline and the contribution to cell yield from limited glucose consumption (see below). Figure 6.5 shows that this relationship is linear, confirming that energy (ATP) was indeed produced from the catabolism of arginine, and that this energy was coupled to cell biosynthesis.

The relationship between dry weights and citrulline consumption in strain CUC-3 was found after correcting for unused citrulline and the contribution to cell yield from limited glucose consumption (see below). Figure 6.6 shows that this relationship was also linear, demonstrating that energy was generated from the catabolism of citrulline, and that this energy was also coupled to bacterial growth.

In these experiments it was found that glucose utilisation (data not shown) was related to the amount of arginine and citrulline consumed. That is, the more arginine or citrulline was consumed, the more glucose was utilised. This is presumably due to the increase in pH from the release of ammonia as a result of consumption of the two amino acids. Therefore, the contribution to cell yield from glucose needed to be subtracted from the total dry weights, in order to obtain the true dry weights resulting from the metabolism of arginine or citrulline only. [It should be emphasised that the glucose concentration (13.3 mM) used was growth-limiting (Figure 6.2)]. The dry weight from glucose contribution was calculated by multiplying the amount of glucose consumed (μmol/mL) by the molar growth yield (g/mole = μg/μmole) of glucose ($Y_{glucose}$). The experimental $Y_{glucose}$ values of 11.9 and 12.5 were used for strains 250 and OENO, respectively. Since $Y_{glucose}$ value for strain CUC-3 was not obtained experimentally, a value of 10.5 was used, assuming that one mole of ATP was produced per mole of glucose fermented by this strain.

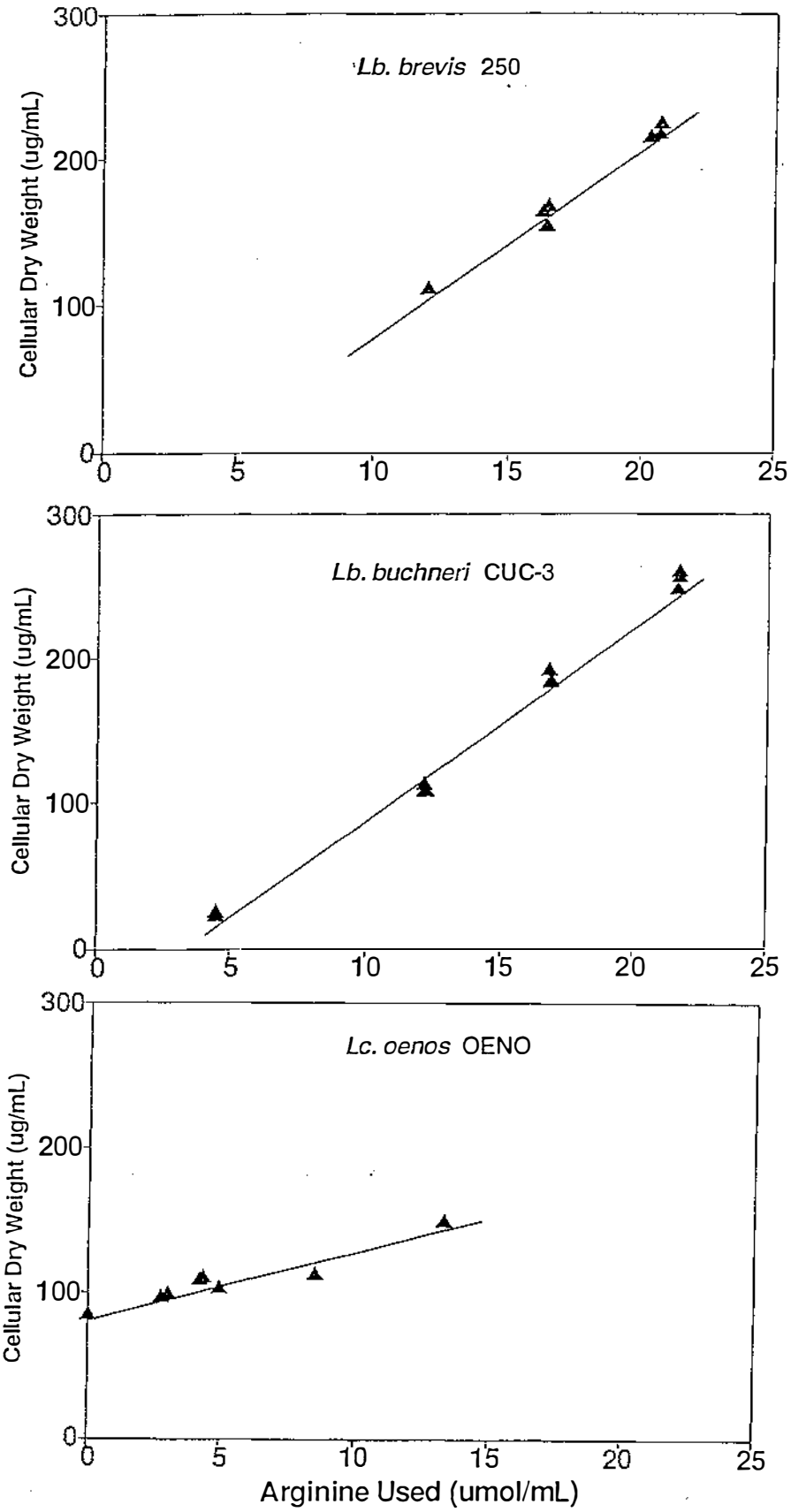

Fig. 6.5 Cell yield from arginine consumption in *Lb. brevis* 250, *Lb. buchneri* CUC-3 and *Lc. oenos* OENO.

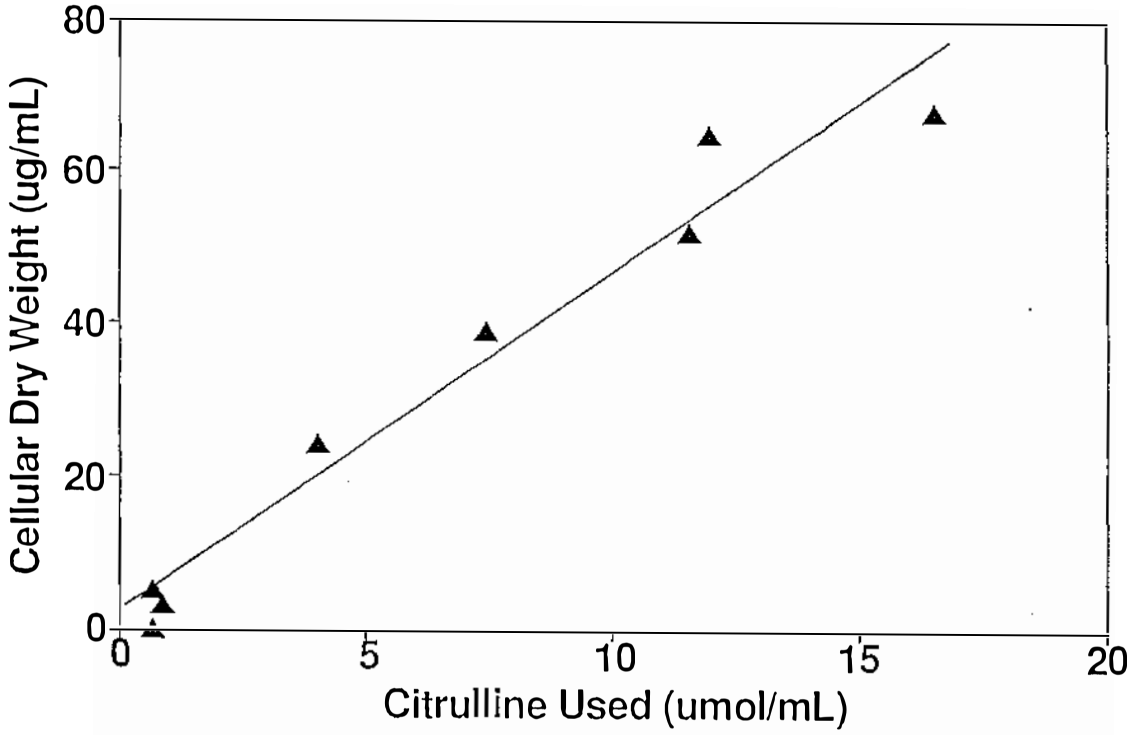

Fig. 6.6 Cell yield from citrulline consumption in *Lb. buchneri* CUC-3.

Table 6.1 shows the molar growth yields of arginine (Y_{arg}) and citrulline (Y_{cit}) with or without corrections for glucose consumption for comparison. Y_{arg} and Y_{cit} must equal Y_{ATP}, since one mole of ATP is generated per mole of arginine catabolised by means of the arginine deiminase pathway (Section 2.1). The Y_{arg} value of strain 250 with (11.7) or without (13.6) correcting for glucose contribution is reasonably consistent with the accepted value of 10.5 $\pm$ 2, since glucose was essentially used up at each arginine concentration. Indeed, the discrepancy between the corrected and uncorrected Y_{arg} values was experimentally insignificant. The corrected Y_{arg} value (14.4) of strain CUC-3 is in reasonable agreement with the accepted value of 10.5 $\pm$ 2, and analytical errors may account for the difference. However, the uncorrected Y_{arg} value (18.6) of this strain was much higher than the corrected value. This discrepancy can be accounted for by the variation in glucose utilisation at each level of arginine tested. More glucose was utilised when more arginine was consumed by this strain. In contrast, the corrected Y_{arg} value (4.4) of strain OENO was significantly lower than that of strains 250 and CUC-3 and the accepted value of 10.5 $\pm$ 2. The uncorrected Y_{arg} value (11.7), however, was more than twice the corrected value, and this could be due to the variation in glucose consumption at each level of arginine tested, as with strain CUC-3.

Table 6.1 Molar growth yields of arginine and citrulline with or without correcting cell yield from glucose consumption

	Y_{arg}				Y_{cit}			
Strain	Corrected[a]	R^2	Uncorrected	R^2	Corrected	R^2	Uncorrected	R^2
Lb. brevis 250	11.7	99%	13.6	98%	NG[b]			
Lb. buchneri CUC-3	14.4	99%	18.6	99%	4.5	96%	12.2	93%
Lc. oenos OENO	4.4	94%	11.7	82%	NA[c]			

[a]Method of correction is described in text.
[b]NG, no growth.
[c]NA, not applicable, as citrulline cannot be catabolised.

In the case of the cultures with added citrulline, only with *Lb. buchneri* CUC-3 was the growth yield related to citrulline concentrations (Figures 6.4 and 6.6). Therefore, the molar growth yield of citrulline was calculated for this strain only (Table 6.1). Both the corrected and uncorrected Y_{cit} values of strain CUC-3 were markedly lower than the corresponding Y_{arg} values. This is not expected, and the reason(s) for this is not known. The uncorrected Y_{cit} value was much higher than the corrected value, presumably due to variations in glucose utilisation at each level of citrulline tested as mentioned above.

6.4 Discussion

This study has confirmed that the heterofermentative wine LAB can indeed utilise the energy derived from arginine metabolism for cell biosynthesis. This trait may be important in the selection of starter cultures for conducting malolactic fermentation in wine. The investigation also has found that *Lb. buchneri* CUC-3 can use the energy derived from the degradation of citrulline for growth. This finding implies that the excretion of citrulline from the catabolism of arginine by leuconostocs (Chapters 5 and 7) may provide an extra source of energy for lactobacilli able to utilise this amino acid, in addition to the small amounts of citrulline present in grape juice. This is of oenological interest as it may contribute to a secondary growth of potential spoilage microorganisms such as lactobacilli.

Arginine appeared to stimulate the initiation of growth of the lactobacilli, but not *Lc. oenos* OENO at initial values of pH 3.5 and 5.5, especially at pH 3.5 (Figure 6.1). This stimulation may be attributable to the ability of the lactobacilli to derive energy for growth from the catabolism of arginine, as well as to the increases in pH due to the release of ammonia. It is not known, however, why the initiation of growth of strain OENO was not stimulated by arginine.

The significant influence of the formation of ammonia from arginine on the increase in pH (Figures 5.1-5.6) has been demonstrated previously. This increase in pH was conducive to glucose consumption and, thereby, partially contributing to higher cell yields. The increase in pH arising from ammonia released may also protect the lactobacilli against the lethal effects of acid production from glucose fermentation. This is suggested by the survival and

subsequent excellent growth of the lactobacilli in the presence of arginine, and the inhibition of these strains in the absence of arginine at the same initial pH. This, of course, increases the potential for spoilage of wine. The role of the arginine deiminase system in reducing the damaging effect of acid environments has also been reported in *Str. lactis* ML3 (Thomas and Batt, 1969) and oral streptococci (Marquis *et al.*, 1987; Casiano-Colón and Marquis, 1988).

The time required to reach maximum growth by *Lb. buchneri* CUC-3 and *Lc. oenos* OENO was longer when high concentrations of arginine and citrulline were used (Figures 6.3 and 6.4). This delay indicates a potential inhibition of growth by high arginine and citrulline concentrations. The inhibition of growth by high concentrations of arginine (1% or above) has been reported in *Lb. buchneri* NCDO 110 (Manca de Nadra *et al.*, 1981), but this is the first report of inhibition by citrulline. The delay was still observed with strain OENO, even though this strain does not catabolise citrulline (Figure 6.4). Possibly the uptake of glucose is inhibited by high concentrations of citrulline or citrulline at high concentrations may be toxic in other ways to this strain?

The molar growth yield of arginine (Y_{arg}) has been determined in other bacteria that catabolise arginine by the deiminase pathway, but there are large variations in the reported values. Bauchop and Elsden (1960) found values of 10 and 10.5 in *Streptococcus faecalis* NCTC 6783, and a value of 8.6 was found by Jonsson *et al.* (1983) in *Lactobacillus plantarum* from fish. Others report high values of 17.0 in *Str. faecalis* NCTC 6783 (Moustafa and Collins, 1968), and 17.8 in *Str. lactis* (Crow and Thomas, 1982). Methodological differences may explain the discrepances. However, comparisons show that the higher values of 17.0 and 17.8 are very close to the uncorrected value of 18.6 in strain CUC-3 (Table 6.1). This may indicate that utilisation of other energy source(s) including glucose might have contributed to the high values, since undefined media were used and corrections for these contributions were not mentioned by these researchers.

This study found low Y_{arg} (4.4) for strain OENO and low Y_{cit} (4.5) for strain CUC-3 compared with the expected 10.5 ± 2. This suggests that there was low effective coupling between the energy-yielding catabolism of arginine and citrulline and the energy-consuming anabolism in these strains. This may be due to energy uncoupling which occurs under conditions such as

nutrient limitations (Stouthamer, 1969; Forrest and Walker, 1971). Some of the ATP generated could also be hydrolysed by ATPase (Senez, 1962) or used for maintenance (Forrest and Walker, 1971). The low Y_{arg} for strain OENO agrees with and explains the poorer growth of this strain (Figure 5.2), compared with strains 250 and CUC-3 (Figures 5.3 and 5.4) under the same conditions. This again indicates the spoilage potential of lactobacilli able to degrade arginine.

This was the first attempt to determine Y_{arg} and Y_{cit} for wine LAB strains using batch cultures. The determinations were, however, complicated by pH changes (increases) which influenced glucose utilisation. This could be avoided by carrying out the experiments at controlled pH values. Continuous culture also should be employed to determine Y_{arg} and Y_{cit} to confirm the results obtained from batch cultures in the current research. Further investigations of Y_{arg} and Y_{cit} should also take into consideration the contribution of malic acid degradation (malolactic fermentation, MLF) to cell yields, as MLF has been shown to form energy through the chemiosmotic mechanism (Cox and Henick-Kling, 1989; 1990; Poolman *et al.*, 1991; Loubiere *et al.*, 1992). Perhaps malic acid should be absent.

changes. For *Lc. oenos* ML34, only samples before and after growth were taken. Samples for ethyl carbamate analysis were stored at -70°C. These samples were filtered through 0.45 μm pore-size membrane filters before despatch for analysis by an outside analytical service.

7.2.4 Growth measurement and analyses

Methods for the determination of arginine, citrulline, L-malic acid, and measurement of pH and growth (as optical density) have been described in Section 3.2.4.

The analysis of ethyl carbamate was carried out by ETS Laboratories, St. Helena, California. The method used was that of Canas *et al.* (1993) using gas chromatography with mass selective detection. The relative standard deviations for repeatability were reported to be 3.7 to 7% for table wines. Samples were analysed before and after heating (71°C/48 hours). Heat treatment is a standard procedure in the analysis of alcoholic beverages to rapidly convert slow ethanol-reacting precursors to ethyl carbamate (Ough *et al.*, 1988; Tegmo-Larsson and Henick-Kling, 1990a). This ethanolysis in wine has recently been shown to occur also during prolonged storage at low to normal wine storage temperatures (Tegmo-Larsson and Spittler, 1990; Stevens and Ough, 1993). Single determinations of the samples were made unless noted otherwise.

7.3 Results

7.3.1 Growth, L-malic acid degradation and pH changes

Two strains of wine LAB, *Lb. buchneri* CUC-3 and *Lc. oenos* OENO were cultured in the synthetic wine and wine. Good growth was achieved, particularly by strain CUC-3, in both synthetic wine and wine (Figures 7.1A-7.4A). L-malic acid was completely degraded by strain OENO during early stages of growth (Figures 7.1B and 7.2B), while it was only partially degraded by strain CUC-3 by the end of fermentation in both media (Figures 7.3B and 7.4B). The pH of both the synthetic wine and wine increased significantly after growth of both organisms (Figures 7.1A-7.4A). The synthetic wine pH increased by about 1.5 units, and the wine pH rose by approximately 0.6 unit. This large increase in pH was the result of ammonia formation from arginine catabolism as demonstrated in Chapter 5 (Figures 5.1-5.4 and 5.6) with some contribution from malic acid degradation.

7.3.2 Arginine catabolism and citrulline production

Arginine was completely catabolised by both *Lc. oenos* OENO and *Lb. buchneri* CUC-3 in the synthetic wine and wine (Figures 7.1B-7.4B). Citrulline was excreted into the media by both strains (Figures 7.1B-7.4B). The production of citrulline corresponded well with the catabolism of arginine in both organisms. However, strain CUC-3 reassimilated and catabolised some of the excreted citrulline after the exhaustion of arginine in both the synthetic wine and wine. This catabolism of excreted citrulline did not occur with strain OENO.

7.3.3 Ethyl carbamate formation

Figures 7.1C-7.4C show the formation of ethyl carbamate in synthetic wine and wine with and without post-fermentation heat treatment. Generally, only insignificant amounts of ethyl carbamate (<10 ng/g) were formed in both media without heat treatment. In the wine inoculated with *Lb. buchneri* CUC-3, ethyl carbamate concentrations in unheated samples were below the minimum detectable value (*ca* 1 ng/g or 1 ppb) and therefore not quantifiable (Figure 7.4C). However, in the synthetic wine and wine inoculated with *Lc. oenos* OENO and the synthetic wine inoculated with strain CUC-3, low concentrations of ethyl carbamate were detected in unheated samples (Figures 7.1C-7.3C). These low concentrations of ethyl carbamate may arise from reaction of carbamyl phosphate with ethanol during fermentation at the incubation temperature of 22°C, or they could be due to slow reaction between citrulline and ethanol even at this temperature. Carbamyl phosphate was not analysed because it is chemically unstable (Jones, 1962; Allen and Jones, 1964), and no reliable ready-to-use method is available, as discussed earlier in Section 5.4.

In the wine inoculated with strain OENO, the concentrations of both citrulline excreted and ethyl carbamate formed on subsequent heating increased throughout the fermentation period (Figures 7.2B,C). In the synthetic wine inoculated with this strain, a very high concentration of ethyl carbamate formation in the heated samples was attained, which corresponded with a high level of citrulline excretion (Figures 7.1B,C). In both the synthetic wine and wine inoculated with strain CUC-3, where the citrulline concentration

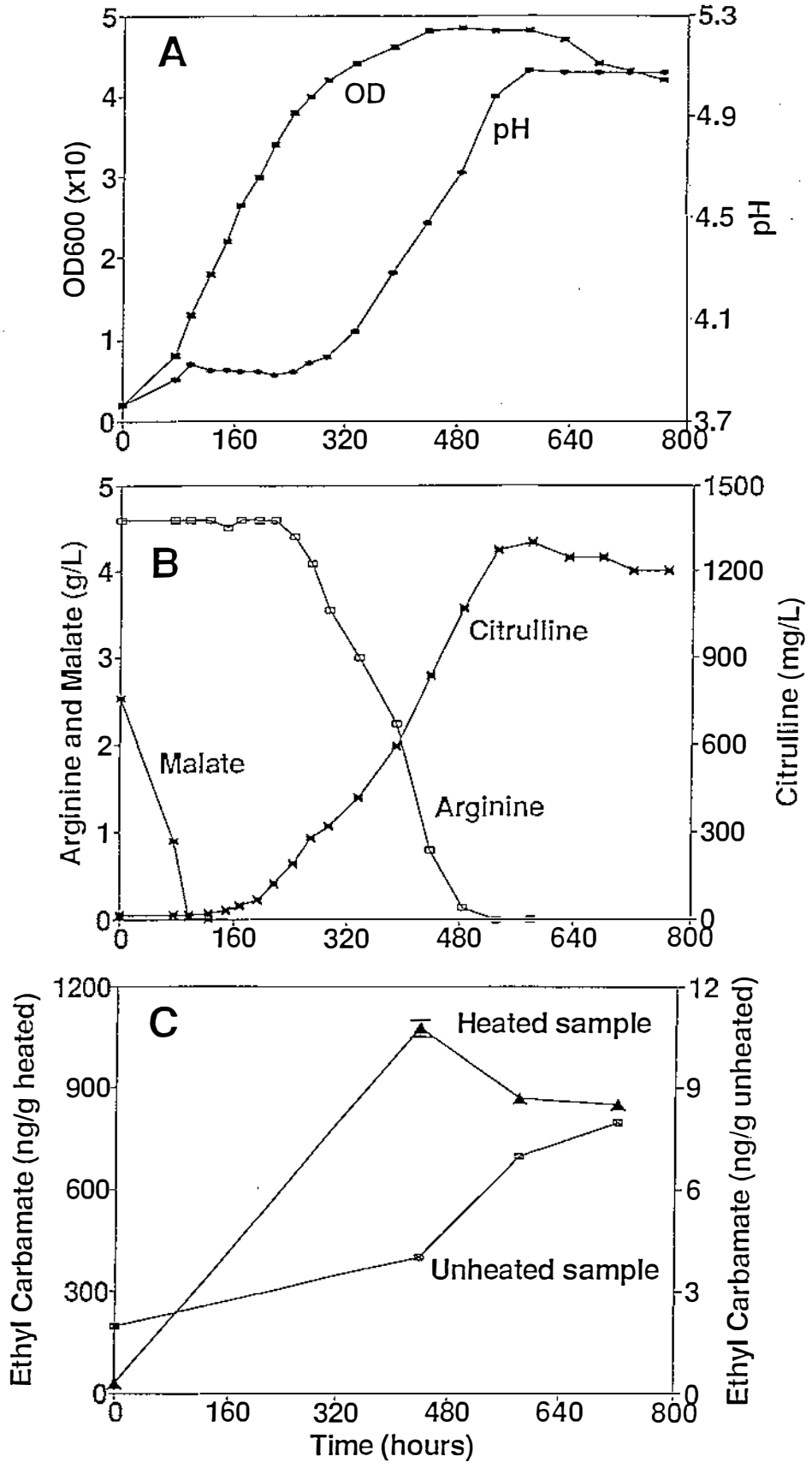

Fig. 7.1 Arginine and L-malic acid catabolism and metabolite formation during incubation (22°C) of *Lc. oenos* OENO in synthetic wine. Samples for measuring ethyl carbamate taken at the times indicated were determined with and without subsequent heating (71°C/48 hrs). In graph C, the bars at 437 hours indicate the range of duplicate determinations.

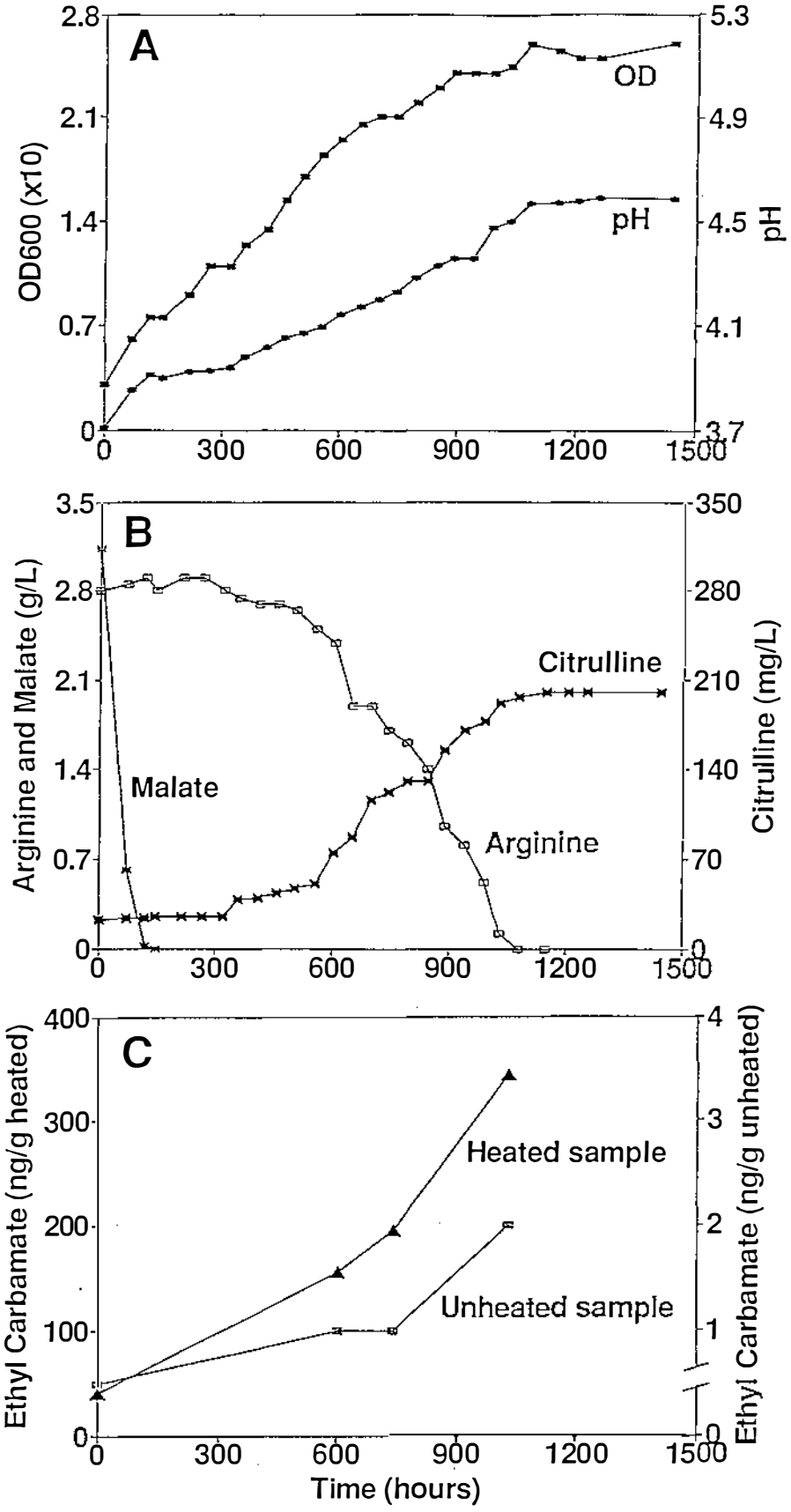

Fig. 7.2 Arginine and L-malic acid catabolism and metabolite formation during incubation (22°C) of *Lc. oenos* OENO in a white wine. Samples for measuring ethyl carbamate taken at the times indicated were treated as in Fig. 7.1.

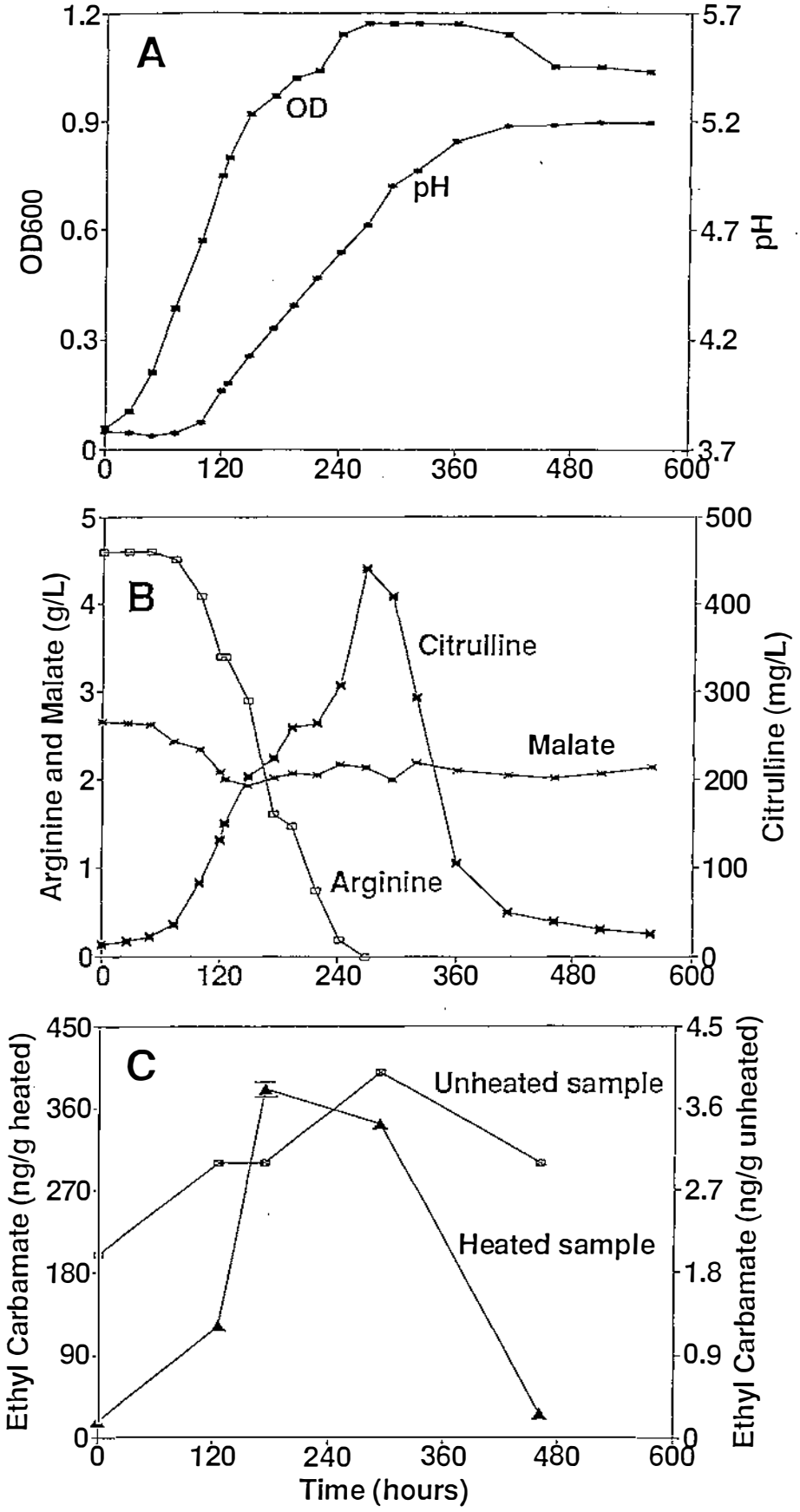

Fig. 7.3 Arginine and L-malic acid catabolism and metabolite formation during incubation (22°C) of *Lb. buchneri* CUC-3 in synthetic wine. Samples for measuring ethyl carbamate taken at the times indicated were treated as in Fig. 7.1. In graph C, the bars at 174 hours indicate the range of duplicate determinations.

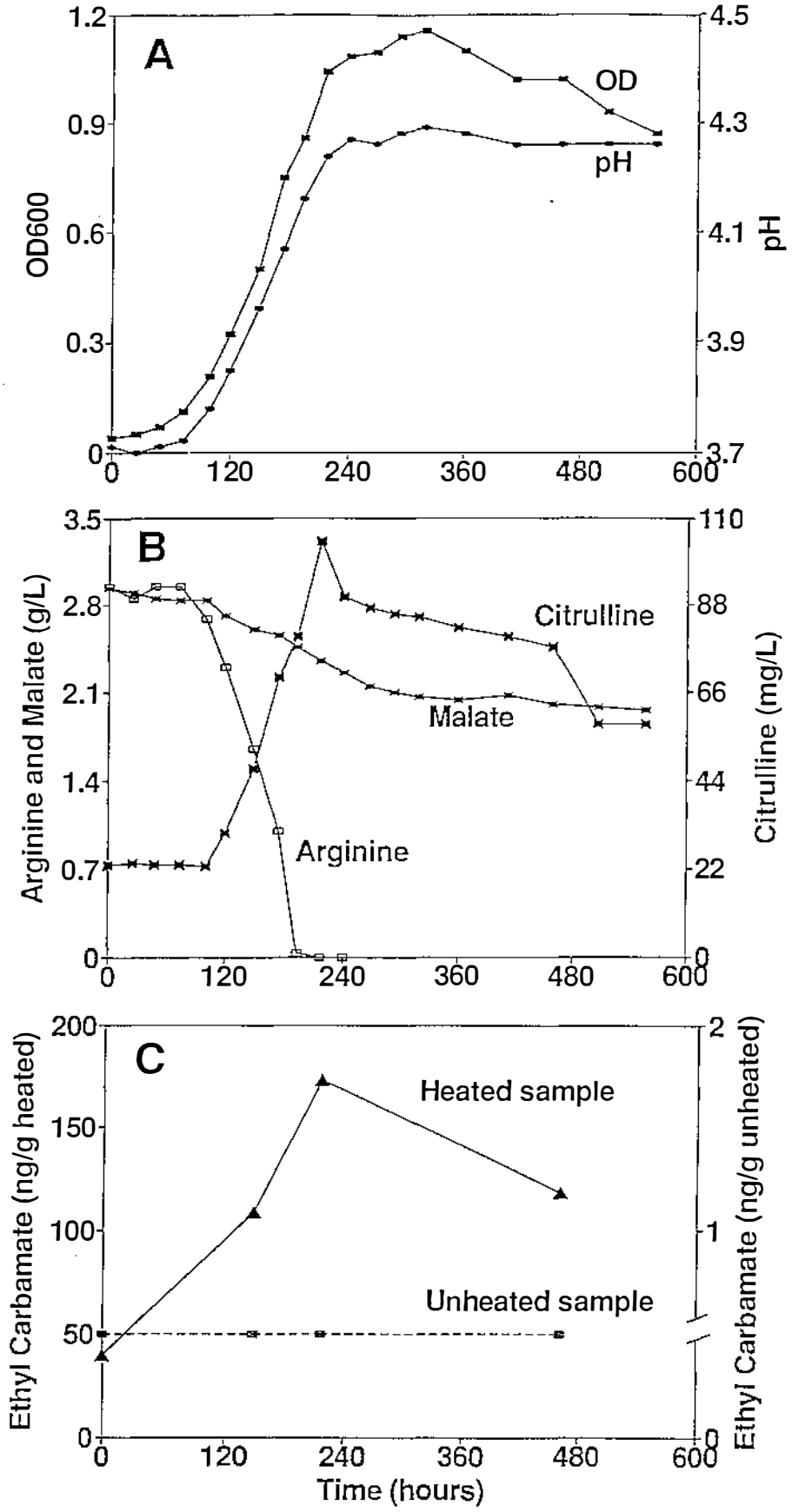

Fig. 7.4. Arginine and L-malic acid catabolism and metabolite formation during incubation (22°C) of *Lb. buchneri* CUC-3 in a white wine. Samples for measuring ethyl carbamate taken at the times indicated were treated as in Fig. 7.1. The dashed plot of the unheated samples in graph C indicates concentrations of ethyl carbamate below detectable values.

decreased after reaching a maximum, the concentrations of ethyl carbamate formed on subsequent heating also decreased (Figures 7.3B,C and 7.4B,C). The relationship between the citrulline concentration and the potential for ethyl carbamate formation is particularly evident for this strain. Thus, in all cases, there was a good correlation between the concentration of citrulline produced in the medium as a result of arginine degradation and the concentration of ethyl carbamate formed in heated samples.

7.3.4 Control experiments

Lc. oenos ML34 does not possess the complete enzyme system of the arginine deiminase pathway (Table 4.2). As shown in Table 7.1, this organism grew in both the synthetic wine and wine, although better in wine. L-malic acid was totally degraded in both media with the expected increase in pH. However, arginine was not catabolised and no additional citrulline was formed during malolactic fermentation. Trace amounts of ethyl carbamate were detected without heating before and after growth in both media. Upon heating, low concentrations of ethyl carbamate were detected, but very much lower than in the arginine-catabolising strains. However, the concentration did increase in wine after growth, even though there was no increase in the concentrations of citrulline (Table 7.1). Replication of this determination confirmed the result. It is unlikely that the ethyl carbamate

Table 7.1 Growth, metabolism and ethyl carbamate formation of *Lc. oenos* ML 34 in synthetic wine and wine

Parameters[a]	Synthetic Wine		Wine	
	Day 0	Day 36	Day 0	Day 30
$OD_{600\ nm}$	0.020	0.135	0.035	0.450
pH	3.76	3.88	3.70	4.01
L-malic acid	2.6	0	2.9	0
Arginine	4.8	4.8	2.95	2.95
Citrulline	12.5	10.0	23.5	21.0
Ethyl carbamate (sample heated)	24	25	19, 23	38, 40
Ethyl carbamate (sample unheated)	2	3	<1	<1

[a]Malic acid and arginine are in g/L; citrulline in mg/L; ethyl carbamate in ng/g.

came from carbamyl phosphate, since there was no increase in ethyl carbamate in unheated samples. Therefore, the ethyl carbamate must have been formed from some unknown precursor(s) that was produced during growth of this strain in wine. The identity of such a precursor is not clear at this stage.

The presence of the small amount of citrulline in the synthetic wine (Table 7.1) may be due to citrulline contamination from the high amount of arginine added. Analysis of the arginine used (Sigma) showed about a 0.02% citrulline contamination.

7.3.5 Percentage yields of ethyl carbamate from citrulline

Calculations of the percentage yields (mole/mole) of ethyl carbamate from citrulline in the samples analysed are presented in Table 7.2. It should be noted that the conversion of this precursor is not stoichiometric. In fact, the percentage yields are extremely low (<1%). These low yields, however, translate to high concentrations when considering the amounts expressed as ng/g (ppb). Also of interest is that the percentage yields of ethyl carbamate from citrulline (plus carbamate phosphate) were consistently higher in the wine than in the synthetic wine, for both strains inoculated. This is unexpected and cannot be attributed to the yield from carbamyl phosphate in the heated samples. This contribution would be insignificant given that less than 10 ng/g of ethyl carbamate were found in all the unheated samples (see Figures 7.1C-7.4C). The average percentage yield of ethyl carbamate from citrulline for all the synthetic wines was 0.23% and for the wines was 0.35%. The difference between these means is statistically significant ($p = 0.03$) by Student's t-distribution analysis.

This result agrees with the findings of others in suggesting that wines may contain unknown precursors that contribute to ethyl carbamate formation (Tegmo-Larsson and Henick-Kling, 1990; Sponholz, 1992; Stevens and Ough, 1993), or that wines contain factors which facilitate the ethanolysis of citrulline to form ethyl carbamate with heat treament.

Table 7.2 Percentage yields of ethyl carbamate from citrulline in synthetic wine and wine

Medium/Strain	Ethyl Carbamate Yield from Citrulline[a] (% mole/mole)	
	Mean	SD
Synthetic Wine/CUC-3	0.213	0.083 (5)[b]
Synthetic Wine/OENO	0.261	0.183 (4)
Wine/CUC-3	0.357	0.068 (4)
Wine/OENO	0.352	0.046 (4)

[a]The very small contribution from carbamyl phosphate in wine and synthetic wine were insignificant and not subtracted (see text).
[b]Numbers in parentheses are the numbers of determinations.

7.4 Discussion

This research has provided evidence that certain wine LAB do have the potential to produce ethyl carbamate precursors. In synthetic wine as well as in wine, two strains of wine LAB, *Lc. oenos* OENO and *Lb. buchneri* CUC-3, catabolised arginine and excreted citrulline (Figures 7.1B-4B), which is known to form ethyl carbamate on reaction with ethanol (Ough *et al*, 1988). Upon heating, significant amounts of ethyl carbamate were formed in both media, although only very small amounts were formed without heat treatment (Figures 7.1C-7.4C). The amount of ethyl carbamate formed in heated samples correlated well with the extent of arginine degradation and content of citrulline in the medium, providing good evidence for the role of citrulline as an ethyl carbamate precursor.

In the current study, the use of a totally chemically defined synthetic wine precluded the presence of other unknown ethyl carbamate precursors. Furthermore, the use of a laboratory-vinified wine confirmed results obtained using the synthetic wine. Relatively high concentrations of arginine were used in the synthetic wine (5 g/L) and wine (3 g/L) so that the potential for ethyl carbamate precursor formation would be exaggerated. These concentrations are higher than those normally found in wines, though it is

possible to find wines with arginine concentrations over 2 g/L (Sponholz, 1991).

There is very little information on the effect of wine LAB on ethyl carbamate formation in wine. Tegmo-Larsson *et al.* (1989) found no increases in the concentration of ethyl carbamate over controls in Chardonnay wines after malolactic fermentation (MLF). However, the fate of arginine was not assessed, nor the ability of the inoculated strains to catabolise arginine. They used five strains of *Lc. oenos*: ML34, MCW, X-3, OENO and PSU-1. Data in Table 4.2 show that strains ML34 and PSU-1 do not possess the complete enzyme system required for arginine degradation and, thus, it is not surprising that there was no increased formation of ethyl carbamate after MLF by these two strains. The ability of strain X-3 to catabolise arginine is not known. Although strain MCW contains the three enzymes of the arginine deiminase pathway, it is not known whether this strain excretes citrulline from the catabolism of arginine. Strain OENO has been shown to degrade arginine and excrete citrulline in this research and previously (Chapter 5). Indeed, there is a good correlation between ethyl carbamate formation and citrulline excretion from arginine degradation by strain OENO (Figures 7.1B,C and 7.2B,C). The inability of Tegmo-Larsson *et al.* (1989) to show increased formation of ethyl carbamate in Chardonnay wines inoculated with strain OENO could be attributed to a very low concenrtation of arginine present. The level of arginine in the wines used in their experiment was not mentioned.

Reports are also available showing increased formation of ethyl carbamate in wines after MLF (Sponholz *et al.*, 1991; Sponholz, 1991,1992). In these studies, it was assumed that arginine was transformed into ornithine and urea by way of the urea cycle in wine LAB. Urea would then react with ethanol to form ethyl carbamate, since urea contains a carbamyl group (Ough *et al.*, 1988). However, earlier work (Chapter 5) has proved that the urea cycle is not present in wine LAB, while the arginine deiminase pathway has been shown to be active in some heterofermentative wine LAB strains (Chapters 3-5). Interestingly, Sponholz (1992) also showed initial increases and subsequent decreases in the concentrations of ethyl carbamate in relation to arginine degradation by strains of *Lb. brevis* and *Lb. buchneri*. But the increases and subsequent decreases were attributed to urea formation from arginine and the subsequent transformation of urea to ammonia and

carbon dioxide in these strains. By comparison with Figures 7.3B,C and 7.4B,C that show a good correlation between ethyl carbamate formation and citrulline excretion and reutilisation after the exhaustion of arginine in *Lb. buchneri* CUC-3, the so-called urea reported by Sponholz (1992) was likely to be citrulline.

Citrulline and carbamyl phosphate are two ethyl carbamate precursors. The reaction of citrulline with ethanol to form ethyl carbamate needs eleveted temperature, while the ethanolysis of carbamyl phosphate to form ethyl carbamate is a spontaneous chemical reaction that does not require an elevated temperature (Ough *et al.* 1988). It is good news for the winemakers that very low levels of ethyl carbamate (<10 ng/g) were formed without heat treatment. This also indicates that little carbamyl phosphate was excreted. This compound was not analysed because it is unstable and the method of determination (differential phosphate analysis) is not suitable for the medium used (see Section 5.4).

Carbamyl phosphate exists in two forms, the monoanion or dianion, depending on pH. The monoanion form predominates at pH 2-4 while the dianion predominates at pH 6-8 (Allen and Jones, 1964). The monoanion carbamyl phosphate decomposes to NH_3, CO_2 and phosphate, but the dianion carbamyl phosphate decomposes to phosphate and cyanate (Jones, 1962; Allen and Jones, 1964; MacKenzie *et al.* 1990). It is proposed that ethyl carbamate formation from carbamyl phosphate and ethanol proceeds via the decomposition of the dianion form involving cyanate as an intermediate (Jones, 1963; MacKenzie *et al.*, 1990). Therefore, little formation of ethyl carbamate from carbamyl phosphate and ethanol would occur at low pH, since little cyanate can be formed. This may account for the low concentrations of ethyl carbamate found in the unheated samples, particularly during the early stages of fermentation when the pH was low. The trend of ethyl carbamate to increase with time in unheated samples (Figures 7.1C-7.3C) could be attributable to the increasing pH, since more cyanate can be formed from carbamyl phosphate at higher pH. It is possible that small amounts of carbamyl phosphate were indeed excreted, but decomposed to NH_3, CO_2 and phosphate due to low pH. The possibility of carbamyl phosphate excretion is supported by the trend of ethyl carbamate concentration to increase in the synthetic wine (Figures 7.1C and 7.3C). On the other hand, carbamyl phosphate, if excreted by bacteria or yeasts, may

have little potential to form significant amounts of ethyl carbamate because of the low pH found in wine.

Low pH is, however, conducive to the formation of ethyl carbamate from citrulline and ethanol, because the ethanolysis of citrulline is an acid-catalysed reaction (Ough *et al*. 1988). This may explain the higher concentrations of ethyl carbamate formed after subsequent heating samples during the early stages of fermentation in the synthetic wine when the pH was relatively low (Figures 7.1C and 7.3C). These higher concentrations of ethyl carbamate were confirmed by repeating the analysis. The potential for ethyl carbamate formation from citrulline and ethanol should not be underestimated even without heat treatment. Heat treatment accelerates the process of ethyl carbamate formation, but this reaction also occurs slowly at low to normal wine storage temperatures (Tegmo-Larsson and Spittler, 1990; Stevens and Ough, 1993). Therefore, the presence of citrulline is of concern to the oenologists.

This research work has several oenological implications. Wine LAB strains with the complete enzyme system for arginine catabolism may be more competitive in growth, since they can gain extra energy (ATP) from arginine degradation (see Section 6.3). However, these organisms may not be desirable in that ethyl carbamate precursors, citrulline and possibly carbamyl phosphate, can also be produced from arginine and excreted. Fortunately, not all wine LAB strains have the complete enzyme system for arginine degradation, as revealed in the survey (Table 4.2). Strains lacking some or all of the enzymes should be the choice as starter cultures, since these strains cannot degrade arginine and, therefore, would not produce ethyl carbamate precursors from arginine. However, those strains possessing ADI only, or ADI and OTC, or ADI and CK only, specifically should be avoided. These strains potentially can excrete citrulline and/or carbamyl phosphate (see Section 4.4) with consequent ethyl carbamate formation, although these phenotypes of wine LAB were not found in the survey (Table 4.2).

It may still be possible, however, to use strains with the complete enzyme system for arginine catabolism to conduct MLF provided L-malic acid degradation is closely monitored and bacterial growth is terminated prior to the commencement of arginine degradation. As seen in Figures 7.1B and 7.2B, L-malic acid was completely degraded by strain OENO before the start

of arginine degradation. For bacteria such as strain CUC-3, timing of the termination of bacterial growth is even more important. As shown in Figures 7.3B and 7.4B, the citrulline concentration is maximal when the arginine concentration is essentially zero. If bacterial growth (metabolism) is stopped at this point, high concentrations of citrulline will remain in the wine. This may result in large amounts of ethyl carbamate being formed upon exposure to high temperatures or even over long-term normal wine storage temperature, as mentioned before. On the other hand, since citrulline is reassimilated after arginine exhaustion, bacterial metabolism should be continued to a point when the citrulline concentration becomes low. The results demonstrate that the timing of analysis is also extremely important when investigating ethyl carbamate formation using strains that recatabolise citrulline. In this instance, a study involving only analysis before and after growth would give very misleading information. For example, there were no significant increases in ethyl carbamate concentrations upon heating after growth of strain CUC-3 to the late stationary phase in the synthetic wine, compared with the initial concentration found upon heating (Figure 7.3C). The time-course study is much more meaningful in this case.

The research also suggests that spontaneous MLF should be discouraged, since the characteristics of the undefined indigenous wine LAB are not known. Therefore, there is a risk of arginine being catabolised and citrulline being excreted. This may result in the formation of ethyl carbamate under the appropriate conditions.

Chapter 8

Correlation of Ammonia and Citrulline Formation from Arginine with the Occurrence of Arginine Deiminase Pathway Enzymes in Wine Lactic Acid Bacteria

8.1 Introduction

Previous work (Chapter 5) has shown the excretion of citrulline into the medium as a result of metabolism of arginine by wine lactic acid bacteria (LAB). This finding is oenologically significant in that large amounts of ethyl carbamate can be formed from this citrulline upon subsequent heating or after long-term storage of wine at low to normal temperatures (see Chapter 7). Obviously then, wine LAB are potential producers of ethyl carbamate precursors in wine if arginine is catabolised. Hence, it would be useful to have available a simple test to detect arginine degradation, citrulline excretion and the occurrence of the arginine deiminase pathway enzymes in wine LAB, rather than performing expensive and time-consuming enzyme assays on cell-free extracts.

Pilone *et al.* (1991) used a heterofermentation-arginine (HFA) broth to detect the formation of ammonia from arginine as well as to examine other biochemical characteristics of wine LAB. In this investigation, ammonia released into the medium was detected with Nessler's reagent. This method is rapid and easy to perform and, thus, may be useful in correlating the formation of ammonia from arginine with the occurrence of the arginine deiminase pathway enzymes (Table 4.2). The current experiment was carried out to verify this and to investigate whether citrulline excretion is correlated with the occurrence of these enzymes.

8.2 Materials and methods

8.2.1 Lactic acid bacterial strains

Strains of wine LAB in the genus *Leuconostoc*, *Pediococcus* and *Lactobacillus* were used. The original source of these strains is given in Table 4.1.

8.2.2 HFA Media

The test medium used was heterofermentation-arginine (HFA) broth (Pilone *et al.*, 1991). The HFA broth (pH 5.5) contained the following ingredients per litre: 5 g each of tryptone, yeast extract, peptone and glucose; 20 g fructose; 6 g L-arginine; 1 mL 5% (w/w) aqueous solution of Tween 80; and 200 mL vegetable juice serum. The vegetable juice serum was prepared as described earlier (Section 3.2.2). The HFA broth was sterilised at 121°C for 15 minutes.

8.2.3 Culturing procedures

Wine LAB strains were first subcultured at pH 5.5 in VJMRS broth (Section 3.2.2). A loopful of the subculture was then inoculated, in duplicate, into 10 mL HFA broth in universal bottles. All cultures were incubated at 30°C for four weeks and analysed for ammonia using Nessler's reagent. Cultures were then stored at -13°C for additional chemical analysis.

8.2.4 Analyses

Methods for the determination of arginine, citrulline, ammonia (by enzymatic method) and urea have been described in Section 3.2.4. It should be noted that the brownish-yellow colour of HFA broth interfered with the colourimetric determination of citrulline by the method of Archibald (1944). Instead, citrulline was analysed using an Amino Acid Analyser as described in Section 3.2.4. Single determinations were made for each compound.

At the end of incubation, the culture broths were tested with Nessler's reagent (BDH) for the presence of ammonia, as described by Pilone *et al.* (1991). One mL of culture was placed in a well of a white tile spot-plate and 1 to 2 drops of reagent added. Immediate formation of a brick-orange colour (positive reaction) indicated the presence of ammonia.

8.3 Results

Table 8.1 shows arginine consumption, formation of ammonia detected with Nessler's reagent or determined using enzyme test kits, and activities of the arginine deiminase pathway enzymes. It should be pointed out that the uninoculated HFA broth contained 32.7 mM arginine and 4.1 mM ammonia. This low level of ammonia tested negative with Nessler's reagent.

Table 8.1 Correlation of ammonia formation from arginine in HFA broth with the occurrence of arginine deiminase pathway enzymes

Strain	Arginine Consumed[a] (mM)	Ammonia Formed[a] Enzyme Assay (mM)	Ammonia Formed[a] Nessler's Test	Enzyme Activity[b] ADI	Enzyme Activity[b] OTC	Enzyme Activity[b] CK
Group I						
Lc. oenos (heterofermentative)						
OENO	32.7, 32.7	54.1, 57.0	+, +	8.8	32.2	18.1
2008	32.7, 14.6	51.6, 24.7	+, +	12.0	40.5	18.6
2043	32.7, 32.6	49.6, 45.3	+, +	11.7	47.0	15.4
2035	32.7, 32.6	53.6, 45.5	+, +	13.2	51.2	19.3
DSIR-C	32.7, 32.7	55.4, 54.4	+, +	8.9	30.7	18.9
Ey2d	32.7, 19.1	55.4, 33.1	+, +	10.6	33.2	15.6
Lactobacillus sp. (heterofermentative)						
CUC-3	32.7, 32.7	55.9, 56.5	+, +	67	217	41.5
EQ	32.7, 32.7	57.9, 55.2	+, +	89	215	30.8
MHP	32.7, 32.7	53.6, 47.8	+, +	92	177	27.6
250	32.7, 32.7	54.6, 52.3	+, +	76	229	20.8
Group II						
Lc. oenos						
122	0, 2.9	1.5, 1.8	-, -	10.5	34.9	19.2
252	4.0, 0.6	1.6, 1.1	-, -	9.1	28.8	15.0
MCW	2.3, 1.2	1.6, 1.7	-, -	10.0	30.1	17.1
Er1a	1.2, 0	1.3, 1.5	-, -	8.8	30.2	18.1
MB1	1.2, 1.2	1.3, 1.1	-, -	12.6	35.3	27.3
MB2	0, 0	1.5, 1.5	-, -	12.5	32.3	18.3
2001	1.2, 0.6	1.3, 1.7	-, -	13.7	42.5	22.6
DSIR-A	2.9, 0.6	1.6, 1.7	-, -	11.2	35.5	21.5
DSIR-B	0.7, 0.7	1.4, 1.6	-, -	8.4	31.0	20.4
INO	1.2, 2.9	1.6, 1.7	-, -	2.4	5.1	4.6
Lactobacillus sp. (heterofermentative)						
ML30	1.2, 0.6	1.9, 1.7	-, -	0	2.2	3.4
Group III						
Lc. oenos						
ML34	0.6, 0	0.7, 0.5	-, -	0	0	0.8
PSU-1	0, 0	0.8, 0.6	-, -	0	0	0.2
L181	0, 0	0.3, 0.5	-, -	0	0	0
MBO	0, 0	1.5, 0.4	-, -	0	0	0
MB3	0, 0	0.6, 0.5	-, -	0	0.2	0
Lactobacillus sp. (homofermentative)						
CUC-1	0, 0.6	0.6, 0.5	-, -	0	0	0
CH2	0, 0.6	0, 0	-, -	0	0	0
49	0, 0	0.7, 0.4	-, -	0	0	0
Pediococcus sp. (homofermentative)						
44.40	0, 0.6	0.6, 0.5	-, -	0	0	0
272	0.6, 0	0.6, 0.6	-, -	0	0	0
93	0.6, 0	0.6, 0.5	-, -	0	0	0
C5	0.6, 0.5	0.2, 0.5	-, -	0	0	0
CUC-4	0.6, 0.6	0.6, 0.5	-, -	0	0	0

[a]Initial arginine and ammonia, 32.7 mM and 4.1 mM, respectively.
[b]Data from Table 4.2; cells were cultured in TJBAG broth, Chapter 4.

Wine LAB can be categorised into three groups based on the extent of arginine consumption, the amount of ammonia formed and the activities of the arginine deiminase pathway enzymes.

Strains in Group I possess the three enzymes of the arginine deiminase pathway (Table 8.1). These strains degraded all of the initial arginine, except for one of the two duplicate fermentations of strains 2008 and Ey2d. Substantial amounts of ammonia were produced and released into the media by these strains, as determined using enzyme test kits. This ammonia was also detected with Nessler's reagent. In this group of strains, there is a good correlation between ammonia detection with Nessler's reagent, arginine degradation and the activities of the arginine deiminase pathway enzymes.

For the leuconostocs in Group II (Table 8.1), the activity of all three enzymes involved in arginine catabolism was comparable to that in Group I. For the *Lb. brevis* ML30, the activity of OTC and CK was much lower and there was no detectable ADI. Surprisingly, the leuconostocs in this group catabolised arginine only slightly in contrast with those in Group I, even though the enzyme activities were similar, except for the lower activities in strain INO. In some fermentations, arginine consumption was too small to be measured, but the ammonia formed could still be determined using the enzymatic method. This is probably because the enzymatic method of ammonia determination is more sensitive than the chemical method of arginine analysis. Strain ML30 also degraded small amounts of arginine and formed small amounts of ammonia, even though this strain did not show any detectable ADI activity, as mentioned above. Although ammonia formation by the strains in Group II could be detected by the enzymatic method, ammonia was not detectable with the less sensitive Nessler's reagent test.

The heterofermentative leuconostocs in Group III lack ADI, and/or OTC or CK (Table 8.1). The homofermenters in this group do not possess any of the enzymes required for arginine catabolism (Table 8.1). Therefore, the strains in Group III would not be expected to degrade arginine. This agrees with the results for arginine consumption found in Table 8.1. The very small "positive" arginine consumptions reported in some fermentations in this group were probably analytical errors. Although arginine was not catabolised by Group

III strains, very small amounts of ammonia were consistently found by the enzymatic assay in the fermentation broths, except for the fermentations with strain CH2. The relatively higher level of ammonia (1.5 mM) in one replicate fermentation with strain MBO seemed to be an anomaly, since this strain did not degrade arginine and its cell-free extracts did not show any activity for the arginine deiminase pathway enzymes (Table 8.1). The ammonia consistently found in the fermentation broths of Group III strains may have arisen from unkown sources such as deamination of other amino acids, rather than arginine or citrulline. This is because these strains lack the enzymes for the degradation of the two amino acids, as mentioned above, and citrulline was not detectable in the uninoculated HFA broth. The formation of small amounts of ammonia from unknown sources may also take place with strains in Groups I and II, but it would be obscured by the larger amounts of ammonia formed from arginine. In all cases, the fermentation broths of Group III strains tested ammonia-negative with Nessler's reagent. One of the two duplicate fermentations of all strains was also analysed for urea using enzyme test kits from Boehringer Mannheim. Urea was not found in any of the samples after growth of these wine LAB.

Due to the cost of analysis, only selected samples were analysed for the excretion of citrulline using an amino acid analyzer. The results are presented in Table 8.2. These samples were selected as representative of the range of the ability of the inoculated strains to degrade arginine and of the levels of the arginine deiminase pathway enzymes (Table 8.1). As seen in Table 8.2, the strains chosen represented heterofermenters possessing the complete enzyme system (strains OENO, 2008, Ey2d, EQ and CUC-3 from Group I), or having part of the enzyme system (strain ML30 from Group II and strains ML34 and PSU-1 from Group III), and one homofermenter (strain CH2 from Group III).

Citrulline in various amounts (Table 8.2) was excreted into the broth by heterofermentative strains OENO, 2008, Ey2d and EQ from Group I. These strains also completely degraded arginine. Citrulline was not detectable in the broth inoculated with strain CUC-3, also from Group I, even though arginine was totally catabolised by this strain. Strains ML34, PSU-1 and CH2 of Group III did not degrade arginine, nor excrete citrulline, since they lack some or all of the enzymes of the arginine deiminase pathway (Table 8.1).

Table 8.2 Correlation of citrulline excretion with arginine degradation by wine lactic acid bacteria cultured in HFA broth

Strains	Arginine Consumed[a] (mM)	Citrulline Formed[b] (mM)
Group I		
Lc. oenos		
OENO	32.7	1.3
2008	32.7	1.9
Ey2d	32.7	2.2
Lb. brevis EQ	32.7	2.6
Lb. buchneri CUC-3	32.7	0
Group II		
Lb. brevis ML30	1.2	0
Group III		
Lc. oenos		
ML34	0	0
PSU-1	0	0
Lb. plantarum CH2	0	0

[a]Arginine concentration in uninoculated HFA broth was 32.7 mM.
[b]Citrulline in the uninoculated HFA broth was not detectable.

Strain ML30 of Group II degraded a small amount of arginine, but no detectable amount of citrulline was found in the broth.

8.4 Discussion

This study has found a good correlation between arginine degradation and detection of ammonia with Nessler's reagent, as well as by enzymatic assay, in some heterofermentative wine LAB possessing the three enzymes of the arginine deiminase pathway (strains in Group I). These strains catabolised arginine extensively and, accordingly, released substantial amounts of ammonia into the fermentation broths that were easily detected with Nessler's reagent (Table 8.1). Therefore, a positive ammonia test with Nessler's reagent could be used to demonstrate arginine degradation by these strains in HFA broth and, therefore, this test would be a simple screening test for wine LAB (see below).

This correlation, however, was not found in other leuconostoc strains that show similar activities for enzymes of the arginine deiminase pathway (strains in Group II). Very small amounts of ammonia could be detected in the fermentation broths by the sensitive enzymatic assay, while the broths tested negative with Nessler's reagent. This correlates with the very small amounts of arginine degraded (Table 8.1). However, the amounts of ammonia formed by this group of strains as determined by enzymatic assay, although small, were two to three times greater than that formed by strains from Group III, which were completely unable to degrade arginine since they lacked the arginine deiminase pathway enzymes. Evidently, the ammonia test with Nessler's reagent is too insensitive to detect small amounts of arginine by strains of Group II in HFA broth.

The reason(s) for the inability of the leuconostoc strains in Group II to catabolise arginine extensively in HFA broth is not known. These strains have been shown to contain the three enzymes of the arginine deiminase pathway (Table 4.2) using extracts prepared from cells cultured in TJBAG broth (Section 3.2.2). Furthermore, the specific activities of the three enzymes in these leuconostocs are similar to those in Group I, except for strain INO (Table 8.1). A comparison of the composition of the two broths shows that the HFA broth used for the experiments in this chapter contained 20 g/L fructose, whereas this sugar was not added to the TJBAG broth. The arginine level was similar in both media, although small amounts of minerals were added to TJBAG broth. It is not known whether the high concentration of fructose in HFA broth inhibited (repressed) the catabolism of arginine in the leuconostocs of Group II.

Several other apparent anomalies are revealed by inspection of the data in Table 8.1, as discussed below.

Lb. brevis ML30 showed no detectable activity for ADI and had weak activity for OTC and CK (Table 8.1). Therefore, this strain was not expected to degrade arginine, since the key enzyme (ADI) was not detectable. Unexpectedly, this strain did degrade a small amount of arginine and released some ammonia which was detectable by enzymatic assay, but not by Nessler's reagent (Table 8.1). This suggests that there is very low ADI activity in this strain but it is too weak to be measurable by the assay procedure described in Chapter 3.

Strain ML30 is distinctly different from other strains of *Lb. brevis* in that all other known strains produce large amounts of ammonia from arginine. Examples include strains 250 and EQ (Table 8.1) and four other strains of *Lb. brevis* (Edwards *et al.*, 1993). The activities of ADI, OTC and CK of strain ML30 also differ greatly from those of other heterofermentative lactobacilli including *Lb. brevis* 250 and EQ (Table 8.1). This raises a question about the identity of this strain. Is ML30 really a strain of *Lb. brevis*? The questionable identity of this strain is supported by a recent report demonstrating that the lactate dehydrogenase (LDH) pattern of strain ML30 does not match that of other known *Lb. brevis* strains (Edwards *et al.*, 1993).

Citrulline was not found in the broth inoculated with *Lb. buchneri* CUC-3 (Table 8.2), although this strain has been shown to excrete citrulline (Chapters 5 and 6). However, since strain CUC-3 has also been shown to recatabolise citrulline after the exhaustion of arginine (Figures 5.4 and 7.3), the citrulline released into the broth would have been completely degraded by this strain by the time of sampling for analysis. This again underlines the importance of the timing of analysis when dealing with strains capable of reassimilating and utilising citrulline, as discussed in Section 7.4. For the same reason, it is not known whether other strains examined in this investigation reutilised citrulline after the depletion of arginine during incubation. For example, this could well be the case for *Lb. brevis* EQ, since two lactobacilli (strains CUC-3 and 250) have been shown to recatabolise citrulline after the disappearance of arginine (Figures 5.3-5.6).
Consequently, the determination of citrulline is not a suitable way to assess arginine degradation.

Citrulline is a precursor of the carcinogenic compound, ethyl carbamate, found in wine and other alcoholic beverages (Ough *et al.*, 1988). The excretion of citrulline during arginine degradation has been demonstrated previously with heterofermentative strains OENO, 250 and CUC-3 (Chapters 5 and 7). The excretion of citrulline was also found in the current survey with several other heterofermentative strains capable of extensive arginine utilisation (Table 8.2). This suggests that the excretion of citrulline from arginine could be characteristic of all heterofermentative wine LAB able to catabolise arginine. If so, the screening of wine LAB becomes of paramount importance for selecting starter cultures to conduct malolactic fermentation

in wine in view of the potential for ethyl carbamate formation, as discussed in Section 7.4.

As discussed above, Group I strains should not be selected as starter cultures because of the possibility of citrulline excretion. The suitability of strains in Group II as starter cultures remains to be determined, although these strains utilised only small amounts of arginine (on average 1.24 mM or 216 mg/L, Table 8.1). Assuming that 10% of the degraded arginine (1.24 mM) was excreted as citrulline, as demonstrated with strain OENO (Chapter 5), this would result in an accumulation of 0.124 mM (22 mg/L) citrulline in the medium. This amount of citrulline would translate to 39 µg/L (ppb) potential ethyl carbamate in a wine with about 10% ethanol, using a conversion rate of 0.35% (Chapter 7), and assuming conversion on prolonged storage would be similar to that produced on heating. This potential concentration of ethyl carbamate is well above the recommended USA limit of 15 ppb in table wines. Therefore, Group II strains should be evaluated for the excretion of citrulline from arginine degradation before being employed as starter cultures. From the standpoint of citrulline formation, strains in Group III should be "safe" to use for conducting malolactic fermentation in wine, since they do not utilise arginine.

The traditional ammonia test with Nessler's reagent is simple to perform, but suffers from poor sensitivity when the arginine concentration is low (Pilone *et al.* 1991). This is because the brownish-yellow colour of the HFA broth interferes with the interpretation of the colour formed by the reaction of Nessler's reagent with low ammonia concentrations. To increase the sensitivity of the test, Pilone *et al.* (1991) increased the arginine concentration in the growth medium from the traditional 3 g/L (Garvie, 1967) to 6 g/L. This allowed for a higher concentration of excreted ammonia from the arginine deiminase pathway. This test could be helpful in the initial screening of potential wine LAB strains for use as starter cultures to conduct malolactic fermentation. Heterofermentative wine LAB strains that produce a positive reaction with Nessler's reagent after growth in HFA broth should be avoided, since they definitely degrade arginine and, therefore, have the potential to excrete the ethyl carbamate precursor, citrulline.
Heterofermentative wine LAB strains that show a negative reaction after growth in HFA broth would need further testing by a more sensitive method.

The enzymatic method used in this study is clearly more sensitive than the Nessler's test for the determination of ammonia. This enzyme assay clearly showed that almost all the wine LAB tested produced ammonia, but the amounts formed varied between strains (Table 8.1). With Nessler's test, however, only strains in Group I give a positive reaction for ammonia (Table 8.1). One problem that can be encountered when using the enzymatic method is the difficulty in interpretation of the small amounts of ammonia formed, as by strains in Group II and Group III (Table 8.1), particularly when needing to subtract ammonia concentrations initially present in the uninoculated medium from the amounts found after growth. In this case, data for arginine degradation would be preferred. Although the enzymatic method is sensitive, performing the assay is tedious and not as simple as the Nessler's test. Measurement of pH increases that accompany arginine degradation, as demonstrated previously (Chapters 5 and 7), perhaps would be another simple means of assessing arginine degradation.

In addition to the excretion of ammonia and citrulline, the possibility of urea formation by these wine LAB was also explored in this investigation. No urea was found in any of these fermentations, and this agrees with previous results found for strains OENO, 250 and CUC-3 (Chapter 5). Again, this provides further evidence that wine LAB do not metabolise arginine to urea by way of the urea cycle, as suggested by Kuensch *et al*. (1974) and Sponholz (1991) and discussed in detail already in Section 5.4.

Chapter 9

Regulation of Enzyme Synthesis in the Arginine Deiminase Pathway of Selected Wine Lactic Acid Bacteria

9.1 Introduction

Previous chapters in this thesis have not addressed the question as to whether the three enzymes of the arginine deiminase pathway (ADI pathway), arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase CK), are constitutive or inducible in some wine lactic acid bacteria (LAB). A few previous studies have indicated that these enzymes are inducible by arginine in other LAB (Simon *et al.*, 1982; Manca de Nadra *et al.*, 1986a,b). Some preliminary experiments described in Chapter 4 have shown that in several strains of *Leuconostoc oenos*, the activities of the arginine deiminase pathway enzymes were low during early growth, but were high between the late exponential and early stationary phases. This suggests that these enzymes may be inducible in wine LAB. This chapter presents the results of a more precise study of the ability of arginine to induce changes in the level of activity of the three enzymes of the arginine deiminase pathway in strains of heterofermentative *Lc. oenos*, *Lactobacillus brevis* and *Lb. buchneri*, and also in strains of homofermentative *Lb. delbrueckii* and *Pediococcus sp.*

9.2 Materials and methods

9.2.1 Lactic acid bacterial strains

Both homofermentative and heterofermentative strains of wine LAB were used. The homofermentative strains were *Lactobacillus delbrueckii* CUC-1 and *Pediococcus sp.* CUC-4. The heterofermentative strains included *Leuconostoc oenos* OENO, *Lactobacillus brevis* 250 and *Lactobacillus buchneri* CUC-3. The original source of these strains is listed in Table 4.1.

9.2.2 Media composition and preparation

Two types of medium were employed, a synthetic and a complex medium. The synthetic medium was used to compare enzyme activities after growth with or without arginine, while the complex medium was used for observing changes in enzyme activity during growth in the presence of arginine. This

complex medium was needed to provide sufficient cell mass to enable enzyme activity measurements to be made at various stages during growth.

The composition and preparation of the synthetic medium have been described in Section 5.2.2. For the purpose of this study, glucose (5 g/L) was the carbon and energy source, with or without the addition of arginine (5 g/L). In one experiment with *Lb. brevis* 250, both glucose and fructose (5 g/L each) were present in the medium containing arginine. The synthetic medium was adjusted to pH 3.5 when arginine was present, and to pH 5.5 when arginine was absent for the reasons given in Section 9.3.1.

Two versions of the complex medium were prepared. Medium A (pH 5.5) was the basal medium (Section 3.2.2) supplemented with glucose (5 g/L) and was sterilised by autoclaving (121°C/15 min). Medium B was autoclaved double-strength basal medium (pH 4.5) mixed with an equal volume of sterile-filtered (0.4 μM pore-size membrane) arginine (20 g/L) and glucose (10 g/L) solution (pH 4.5). Medium A and B were used in studies on the time-course of enzyme induction. Addition of medium B to an equal volume of culture growing in medium A yielded a medium with a final concentration of 5 g/L arginine and 2.5 g/L glucose.

9.2.3 Culturing procedures

For experiments using the synthetic medium, strains were first subcultured in VJMRS broth (Section 3.2.2), followed by subculturing in the synthetic medium with or without arginine. This second subculture was then used to inoculate (1% v/v) the experimental synthetic medium with or without arginine (same as the second subculture medium). The cultures were incubated at 30°C, and growth was monitored as changes in cell mass (optical density, OD). Cultures were harvested between the late log and early stationary phases when enzyme activities were high, as previously demonstrated (Section 4.3.1). Cells were harvested by centrifugation as described in Section 3.2.2 and stored at -70°C until used for measurement of enzyme activity.

Time-course induction experiments were conducted in the complex medium. Strains precultured in VJMRS broth (Section 3.2.2) were first inoculated (0.5% v/v) into medium A (Section 9.2.2) and allowed to grow (30°C) until mid- to late log phase. During incubation, 5-10 mL samples were taken

periodically for measurements of growth (OD) and pH. At appropriate times during growth, as indicated by OD and pH changes, two large-size samples (200-250 mL) were also taken for harvesting cells for measurement of enzyme activities. Immediately after taking the second large sample, 500 mL medium B (Section 9.2.2) was added into the remaining culture (approximately 500 mL at about pH 4.0-4.3), so that the final concentration of arginine would be about 5 g/L and the final volume about 1L. Incubation was continued, and OD, pH and arginine levels were monitored. Several samples were also taken during this incubation period for harvesting cells for measurement of enzyme activities as described above.

9.2.4 Measurement of enzyme activities

The activities of the ADI pathway enzymes were measured using assays verified in Chapter 3.

As a control to distinguish specific responses of the arginine deiminase pathway enzymes from general overall changes in protein synthesis, the activity of glucose-6-phosphate dehydrogenase (G6P-DH) was also measured using the method of Garvie and Farrow (1980). G6P-DH was assayed in the crude extracts prepared for arginine deiminase pathway enzymes. The reaction mixture contained 1.5 mL triethanolamine buffer (0.2 M, pH 7.6); 0.1 mL sodium glucose-6-phosphate (10.0 mg/mL); 0.1 mL $NADP^+$ (12.5 mg/mL) and 0.2 mL $MgCl_2$ (0.1 M). The volume was made up to 3.0 mL with the addition of 1.1 mL deionised water. The enzymic reaction was started by adding 0.1-0.3 mL of an appropriately diluted solution of cell-free extract. The reduction of coenzyme was followed (37°C) at 340 nm on a Gilford Spectrophotometer.

9.2.5 Determination of cell mass, pH and arginine

Methods for the determination of cell mass (OD), pH and arginine have been described in Section 3.2.4.

9.3 Results

9.3.1 Activities of arginine deiminase pathway enzymes from wine LAB strains grown with or without arginine

Tables 9.1-9.3 show the activities of the ADI pathway enzymes in extracts of *Lb. buchneri* CUC-3, *Lc. oenos* OENO and *Lb. brevis* 250 cultured in a synthetic medium with or without the presence of arginine. An initial pH of 3.5 was used when arginine was present. This low initial pH level allowed for

some pH increase (because of the formation of ammonia from arginine degradation, as demonstrated earlier, Figures 5.1-5.6), before becoming too high and inhibiting growth. An initial pH of 5.5 was used when arginine was absent. This high initial pH level allowed for some pH decrease because of acid formation from fermentation of glucose before becoming too low and inhibiting growth.

As shown in Table 9.1, in strain CUC-3, the activities of all three enzymes from cells grown with arginine were very much higher than those from cells cultured in the absence of arginine. Enzyme activities were increased by 103- to 168-fold. With strain OENO (Table 9.2), relatively high activities of all three enzymes were achieved from cells cultured in the presence of arginine. When this strain was cultured in the absence of arginine, however, the activity of ADI was undetectable. The activities of OTC and CK for this strain were increased by 391- and 50-fold, respectively, when grown with arginine, compared with those grown in the absence of arginine (Table 9.2). With strain 250 (Table 9.3), the activities of the three enzymes were increased by 19- to 60-fold when cultured in the presence of arginine, compared with those in its absence. It should be noted that in the experiment with strain 250, the carbohydrate in the medium was a mixture of glucose and fructose. The mixture of sugars was used in this case because the experiment was also part of another experiment with this strain (described in Sections 5.2.2 and 5.3.2). These results clearly show that all three of the arginine deiminase pathway enzymes are inducible by arginine.

In some experiments, the homofermentative strains *Lb. delbruekii* CUC-1 and *Pd. sp.* CUC-4 were cultured in the synthetic medium (pH 4.6) containing 1 g/L glucose (instead of 5 g/L) and 5 g/L arginine (see Sections 5.2.2 and 5.3.3). Cells were harvested and assayed for the activities of the arginine deiminase pathway enzymes. However, no activities for any of the three enzymes could be detected in extracts from these two strains (data not shown). This agrees with and explains the earlier finding that arginine was not degraded by these two strains in this synthetic medium (Section 5.3.3).

Table 9.1 Specific activities of arginine deiminase pathway enzymes from *Lb. buchneri* CUC-3 cultured in a synthetic medium with or without arginine[a]

Enzyme	Specific Activities[b]		
	+arg	-arg	+arg/-arg
ADI (x100)	101	0.60	168
OTC	258	2.50	103
CK	27	0.24	113

[a]Glucose (5 g/L) as a carbohydrate source.
[b]Results are the averages of duplicate measurements in two separate extracts of one culture. Specific activities are expressed as μmole citrulline/min/mg protein for ADI and OTC and as μmole ATP/min/mg protein for CK.

Table 9.2 Specific activities of arginine deiminase pathway enzymes from *Lc. oenos* OENO cultured in a synthetic medium with and without L-arginine[a]

Enzyme	Specific Activities[b]		
	+arg	-arg	+arg/-arg
ADI (x100)	10	ND[c]	NA[c]
OTC	43	0.11	391
CK	23	0.46	50

[a]Glucose (5 g/L) as a carbohydrate source.
[b]Results are the averages of duplicate measurements in two separate extracts of one culture. Specific activities are expressed as μmole citrulline/min/mg protein for ADI and OTC and as μmole ATP/min/mg protein for CK.
[c]ND, none detected; NA, not applicable.

Table 9.3 Specific activities of arginine deiminase pathway enzymes from *Lb. brevis* 250 cultured in a synthetic medium with and without L-arginine[a]

Enzyme	Specific Activities[b]		
	+arg	-arg	+arg/-arg
ADI (x100)	21	0.45	47
OTC	51	0.85	60
CK	3.5	0.18	19

[a]Glucose and fructose (5 g/L each) as carbohydrate sources.
[b]Results are the averages of duplicate measurements in two separate extracts of one culture. Specific activities are expressed as µmole citrulline/min/mg protein for ADI and OTC and as µmole ATP/min/mg protein for CK.

9.3.2 Time-course induction of the arginine deiminase pathway enzymes

In order to study the induction of the arginine deiminase pathway enzymes by arginine in more detail, time-course experiments were carried out in which changes in enzyme activity were followed after addition of arginine to growing cultures. As described in Section 9.2.3, cultures were grown to mid- to late log phase on a medium containing glucose but without addition of arginine (medium A). Medium B containing arginine at twice the required final concentration was then added in a volume equal to that of the culture, and samples were taken at various times to determine cell mass (OD), pH and arginine concentration, and to measure enzyme activities.

Figures 9.1-9.3 illustrate growth, changes in medium pH, arginine degradation and activity of the arginine deiminase pathway enzymes in *Lb. brevis* 250, *Lb. buchneri* CUC-3 and *Lc. oenos* OENO growing in complex media before and after arginine addition. All strains had achieved good growth, as indicated by OD readings, and the medium pH had decreased considerably at the time of addition of arginine.

The addition of the arginine solution (medium B, Sections 9.2.2 and 9.2.3) diluted the cell density and thereby reduced the OD reading by

approximately 50% (Figures 9.1-9.3). This addition also slightly raised the pH of the culture medium, because the pH of medium B (pH 4.5) was higher than the pH of the culture (pH 4.0-4.3). After arginine addition, growth continued and arginine was almost completely degraded by all strains by the late log phase. The pH of the "arginine-spiked" medium declined initially, but subsequently increased. This increase in medium pH correlated well with the degradation of arginine, presumably due to the release of ammonia.

The activities of the arginine deiminase pathway enzymes in extracts from cells harvested before the addition of arginine (Figures 9.1-9.3) were relatively high, compared with the activities of these enzymes from cells cultured in the synthetic medium without added arginine (Tables 9.1-9.3). This is because the basal complex medium contained some arginine (0.5 g/L), which presumably originated from yeast extract, peptone and tryptone present in the medium, whereas the synthetic medium contained none. The enzyme activities seemed to be decreasing in the lactobacilli by the time arginine was added. These decreases, however, did not occur with *Lc. oenos* OENO. This is probably because some of the basal level of arginine was still present when samples were taken for strain OENO.

The activities of all three enzymes in extracts increased significantly during growth after the addition of arginine (Figures 9.1-9.3). These increases correlated well with the degradation of arginine. Enzyme activities in extracts of strain 250 (Figure 9.1) reached a maximum when arginine was nearly depleted in the medium, while those in extracts of strain CUC-3 (Figure 9.2) continued to rise slightly after the exhaustion of arginine. High enzyme activities in extracts of strain OENO (Figure 9.3) coincided with the complete degradation of arginine. On the whole, ADI activities were increased by 6- to 8-fold, OTC 4- to 9-fold and CK 4- to 8-fold. Although these increases were small compared to the differences in synthetic media between cultures grown with and without arginine, the increases in activities of the arginine deiminase pathway enzymes are a specific response to the added arginine, since the activities of glucose-6-P dehydrogenase (which is not involved in arginine metabolism) showed no increase following arginine addition.

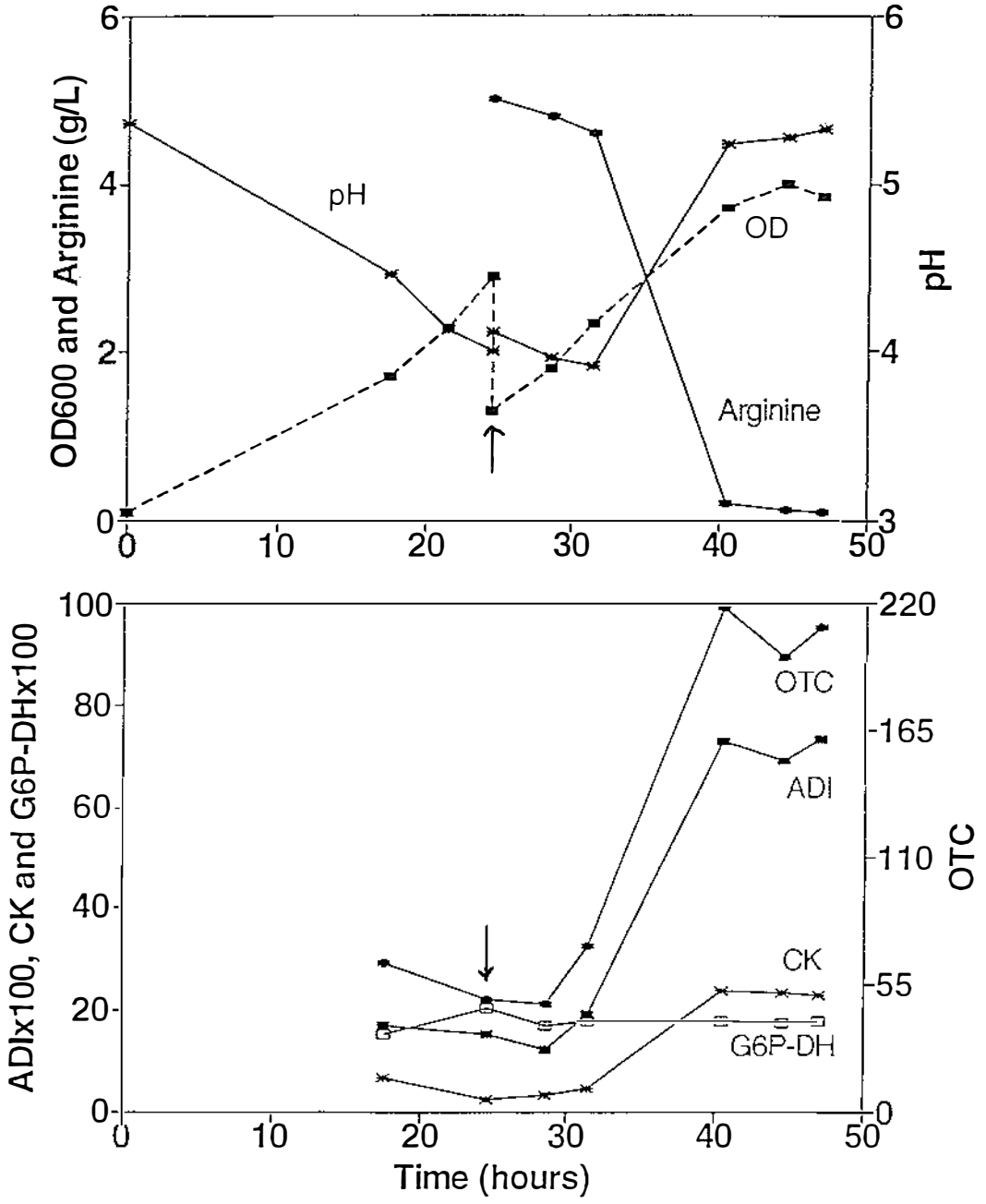

Fig. 9.1 Growth, arginine catabolism, pH and activities of the arginine deiminase pathway enzymes during incubation (30°C) of *Lb. brevis* 250 in a complex medium. Arrow indicates time of arginine addition. Enzyme activities at each point are the averages of duplicate measurements in one extract from a single culture.

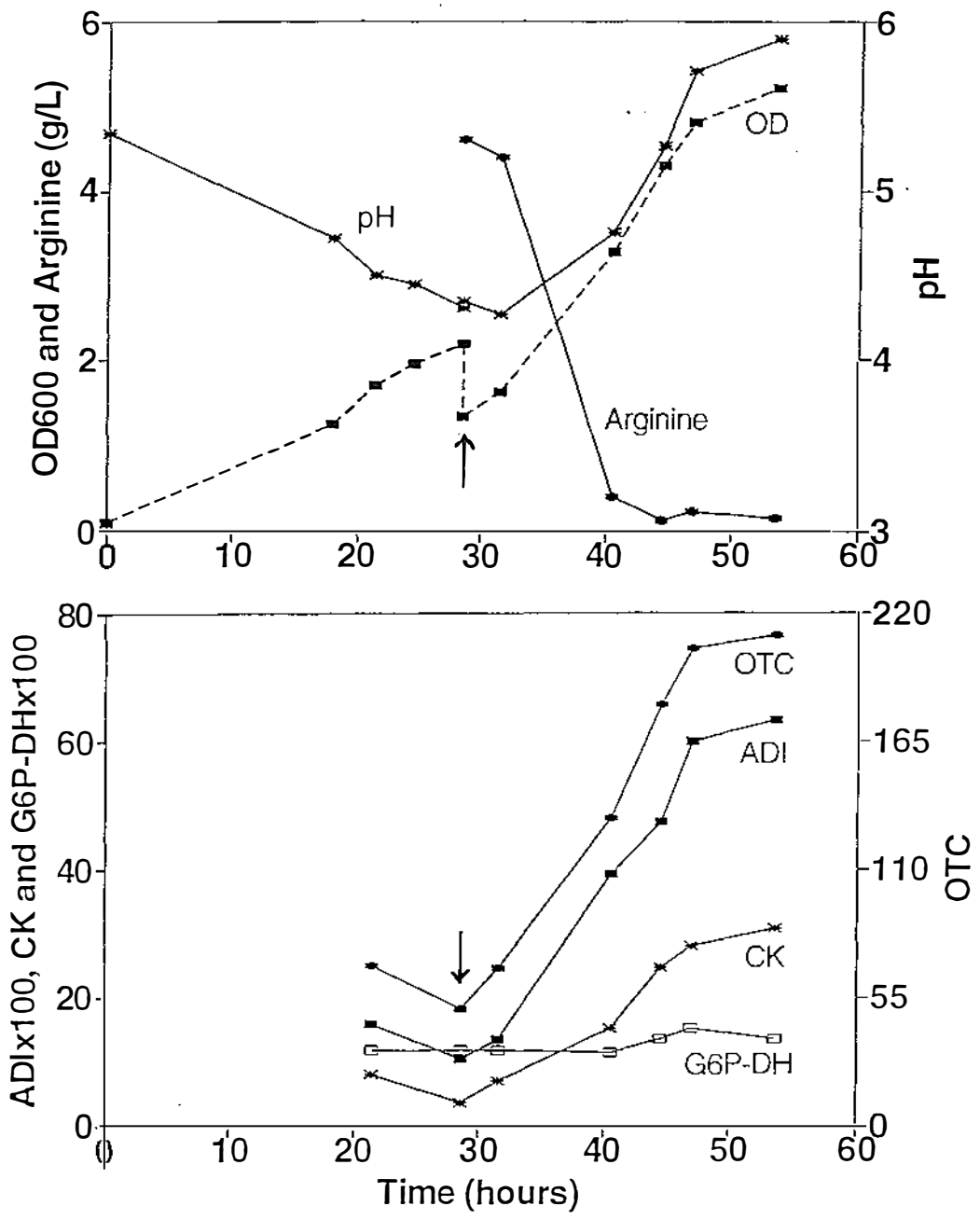

Fig. 9.2 Growth, arginine catabolism, pH and activities of the arginine deiminase pathway enzymes during incubation (30°C) of *Lb. buchneri* CUC-3 in a complex medium. Arrow indicates time of arginine addition. Enzyme activities at each point are the averages of duplicate measurements in one extract from a single culture.

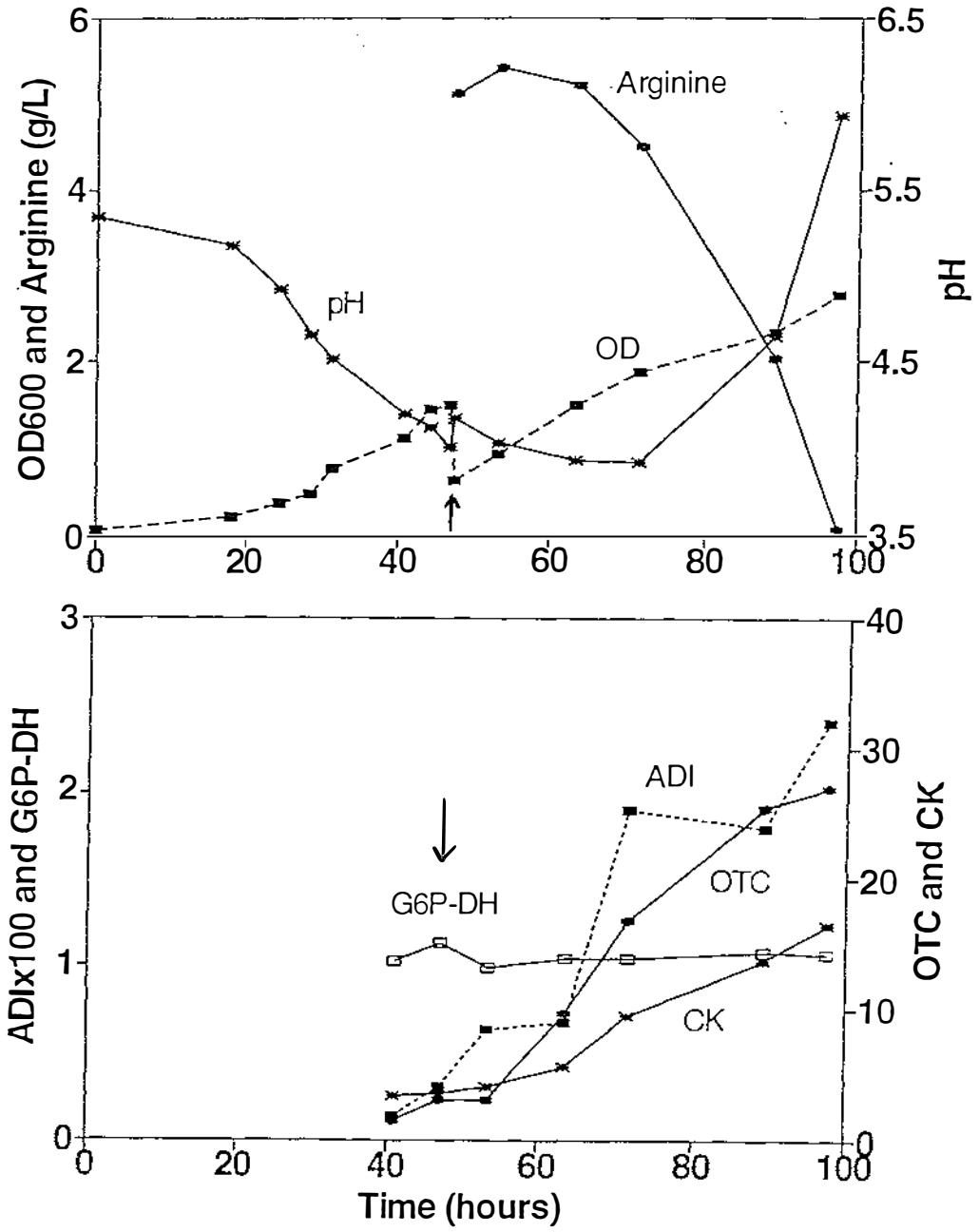

Fig. 9.3 Growth, arginine catabolism, pH and activities of the arginine deiminase pathway enzymes during incubation (30°C) of *Lc. oenos* OENO in a complex medium. Arrow indicates arginine addition. Enzyme activities at each point are the averages of duplicate measurements in two separate extracts from a single culture.

9.4 Discussion

The influence of addition of arginine on the activity of the ADI pathway enzymes in some wine LAB has been ascertained in the current investigation. This study has shown that in several heterofermentative wine LAB, the ADI pathway enzymes are strongly induced by arginine (Tables 9.1-9.3). All three enzymes are induced simultaneously and the time-course of induction correlates well with that of arginine degradation (Figures 9.1-9.3). Enzyme activities in extracts increased during the log phase and reached a maximum between the end of log and early stationary phases, when arginine was exhausted or nearly depleted. These results confirm the earlier observations that enzyme activities are low during early stages of growth in the presence of arginine (Figures 4.1-4.4).

The induction of the ADI enzymes by arginine has been demonstrated in other microorganisms. The simultaneous induction of ADI, OTC and CK by arginine has been demonstrated in *Streptococcus faecalis* ATCC 11700 (Simon *et al*, 1982), and *Lactobacillus buchneri* NCDO 110 (Manca de Nadra *et al*., 1986a). Manca de Nadra *et al*.(1986b) also reported the induction of ADI and OTC by arginine in *Lb. leichmannii* ATCC 4797, but the induction of CK was not mentioned. However, Crow and Thomas (1982) demonstrated the inducibility of ADI and OTC in lactococci, but CK was shown to be constitutive. Interestingly, Mercenier *et al*. (1980) showed the strong induction of the three enzymes in *Pseudomonas aeruginosa* PAO by a shift from aerobic growth conditions to very low oxygen tension. In this case, arginine was not essential for this induction, although the maximum enzyme levels were increased twofold by the presence of arginine. It is not known if oxygen has any effect on the synthesis of the ADI pathway enzymes in wine LAB. Oenologically, this may not be important, since wine is usually an anaerobic environment.

The homofermentative wine LAB examined did not contain the arginine deiminase pathway enzymes, as shown in the survey results (Table 4.2), and seem indeed unable to catabolise arginine (Figure 5.8). Glucose is known to repress the activity of the ADI pathway enzymes in other homofermentative LAB, such as some lactococci (Crow and Thomas, 1982), *Str. faecalis* ATCC 11700 (Simon *et al*., 1982), a *Lb. plantarum* fish isolate (Jonsson *et al*., 1983), *Lb. leichmannii* ATCC 4797 (Manca de Nadra *et al*., 1986b), and a *Lb. sake* meat isolate (Montel and Champomier, 1987). The

synthesis of the ADI pathway enzymes could not be induced in the homofermentative wine LAB studied here even under conditions of low glucose concentrations (1 g/L).

The experiments presented in this chapter represent a limited study on the regulation of enzyme synthesis in the ADI pathway in wine LAB strains. Other aspects of regulation of enzyme synthesis should be further investigated, and could include the effects of sugar type on enzyme synthesis, since the nature of the carbohydrate source has been shown to have a marked effect on activity of the ADI pathway enzymes (Crow and Thomas, 1982; Manca de Nadra *et al.*, 1986a,b).

The time-course studies of arginine catabolism described in Chapter 5 have shown that glucose does not inhibit arginine utilisation. This suggests that glucose is not likely to affect the synthesis of the ADI pathway enzymes. However, the time-course studies also showed the sequential utilisation of fructose and arginine (Figures 5.6 and 5.7). This suggests that fructose inhibits the utilisation of arginine and, therefore, may repress the synthesis of the ADI enzymes. Since the activities of the ADI pathway enzymes were not measured during the metabolism of fructose and arginine in these experiments (Chapter 5), it is not known if the synthesis of the ADI pathway enzymes occurred during the metabolism of fructose. It is possible that synthesis of the ADI pathway enzymes does not occur during the extensive metabolism of fructose, because arginine, the inducer, is not transported into the cell (Section 5.4). Further work is needed to confirm the possible repression or reduction of enzyme synthesis by fructose. If fructose represses the activities of the three enzymes, this may explain the lower activities of these enzymes in *Lb. brevis* 250 cultured in the synthetic medium containing fructose and arginine (Table 9.3), compared with the activities found in the survey (Table 4.2).

Chapter 10

General Discussion

10.1 The pathway of arginine catabolism in wine lactic acid bacteria

This research has confirmed that wine lactic acid bacteria (LAB) able to catabolise (L-)arginine do so exclusively by means of the arginine deiminase pathway (ADI pathway) shown in Figure 2.1. This pathway is also referred to as the arginine dihydrolase pathway in the older literature. These wine LAB do not metabolise arginine via the urea cycle (described in Figure 5.9 and below), as suggested by Kuensch *et al.* (1974), Sponholz *et al.* (1991) and Sponholz (1991, 1992). These conclusions are supported by several lines of evidence found in this research, as discussed below.

a) The presence of the ADI pathway enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK), has been demonstrated in cell-free extracts of wine LAB capable of degrading arginine (Chapter 3). The activities of the three enzymes from several strains possessing the ADI pathway ranged from 0.002 to 0.92 μmol/min/mg protein for ADI; 5.1 to 229 μmol/min/mg protein for OTC and 4.6 to 41.5 μmol/min/mg protein for CK (Chapter 4).

In the experiments described in Chapter 3, however, only cell-free extracts were used to establish the conditions appropriate for assays of the ADI pathway enzymes from wine LAB. The three enzymes have not been purified and hence, their biochemical properties such as molecular weights and kinetic parameters are still not known. In contrast, the ADI pathway enzymes from *Lb. buchneri* NCDO110 isolated from dairy products have been partially purified and biochemically characterised (Manca de Nadra *et al.*, 1984a,b; 1986c; 1987; 1988). The biochemical properties of ADI pathway enzymes from other sources also have been studied and are discussed in a review by Abdelal (1979).

b) The stoichiometric conversion of arginine to ornithine and ammonia (one mole of arginine converted to one mole of ornithine and two moles of ammonia) is consistent with the ADI pathway. Although this stoichiometry

can be consistent with the urea cycle described in Figure 5.9 and below,

$$\text{arginine} \xrightarrow{\textit{arginase}} \text{ornithine} + \text{urea} \xrightarrow{\textit{urease}} CO_2 + 2\,NH_3$$

the presence of arginase and urease has not been assayed in wine LAB. But no urea was detected in the media in which wine LAB were grown (Chapters 5 and 8), though all urea could be hydrolysed if urease is not rate-limiting.

c) The production of energy (ATP) from arginine degradation for growth has been implicated (Chapter 6). This is consistent with the ADI pathway, since ATP is generated from arginine catabolism through this pathway. Similarly, coupling of energy from arginine catabolism for growth has been reported for other LAB possessing the ADI pathway (Crow and Thomas, 1982; Jonsson *et al.*, 1983). Metabolism of arginine using the arginase reaction would not provide ATP to support growth.

Wine LAB strains surveyed in Chapter 4 exhibit various activities of the ADI pathway enzymes. However, strains possessing ADI, but lacking OTC, CK or both, were not found, nor were any strains found which showed large differences in the relative activities of the three enzymes. These strains possibly could be generated through mutation studies, as achieved with other microorganisms. Haas *et al*. (1979) isolated mutants of *Ps. aeruginosa* affected in catabolic OTC (*arcB*) that excrete citrulline. In addition, Simon *et al*. (1982) isolated four groups of *Str. faecalis* mutants displaying various activities of ADI pathway enzymes, and these mutants varied in their ability to catabolise arginine and produce metabolites. For example, a group of mutants found by Simon *et al*. (1982), which have low activities of catabolic OTC, converted arginine stoichiometrically to citrulline. The isolation of wine LAB mutants deficient in one or more of the ADI pathway enzymes, or showing large differences in the relative activities of the three enzymes, would produce useful information on arginine metabolism and the accumulation of metabolites.

The three enzymes of the ADI pathway are generally induced simultaneously by arginine, for example, in *Str. faecalis* (Simon *et al*., 1982). This is also true of the three wine LAB tested: *Lc. oenos* OENO, *Lb. brevis* 250 and *Lb. buchneri* CUC-3 (Chapter 9). In *Lactococcus lactis* ML3, arginine also stimulates the arginine-ornithine exchange (antiporter) activity as well as the

ADI pathway activity (Poolman *et al.*, 1987), indicating that the arginine uptake system is also inducible. The arginine transport system in wine LAB may also be inducible by arginine.

Factors affecting the catabolism of arginine are ill-defined for wine LAB. Although glucose does not affect arginine utilisation, the observation that utilisation of fructose and arginine is sequential (Chapter 5) suggests that fructose may repress the degradation of arginine. The influence of different sugars on the activities of the ADI pathway enzymes has been reported for other LAB (Crow and Thomas, 1982; Manca de Nadra *et al.*, 1986a,b). It is not known whether the nature of sugars (pentoses, other hexoses and disaccharides) and other factors (oxygen and pH) would affect the activities of the ADI pathway enzymes in wine LAB. Previous work (Liu, 1990) shows little degradation of arginine at low pH (<pH 3.4), but extensive utilisation of this amino acid at higher pH (>pH 4) in two strains of *Lc. oenos*. Further work is needed to confirm this. It would also be interesting to see how these conditions affect the excretion of citrulline. Definition of conditions affecting arginine utilisation and citrulline excretion would be of use in controlling malolactic fermentation in wine, because this will help minimise the formation of the ethyl carbamate precursor, citrulline.

10.2 Excretion of citrulline during arginine catabolism by wine lactic acid bacteria

This research (Chapters 5, 7 and 8) has demonstrated the excretion of some citrulline from arginine catabolism by both leuconostocs and lactobacilli isolated from wine and the reutilisation of the excreted citrulline by the lactobacilli. The excretion of citrulline as a result of arginine catabolism has also been found in *Mycoplasma sp.* (Smith, 1955; Schimke and Barile, 1963; Fenske and Kenny, 1976) and in *Spiroplasma citri* (Townsend, 1976), but none of these microorganisms degraded the citrulline released.

The reason(s) for citrulline excretion and reutilisation is not known. Cunin *et al.* (1986) suggested that repression of OTC or CK by an excess of energy sources other than arginine may result in excretion of citrulline. This suggestion, however, is not consistent with the excretion of citrulline found in the time-course study of arginine catabolism (Figures 5.2-5.4) where the concentration of the energy source (1 g/L glucose) was not considered excessive. It is reported that pseudomonads possessing the ADI pathway convert 5-15% of the available arginine into citrulline and 85-90% into

ornithine under low oxygen tension, but produce no measurable levels of the two metabolites under high oxygen tension (Stalon and Mercenier, 1984). This suggests that oxygen plays a role in regulating arginine metabolism in these organisms. In other bacteria, overproduction of intermediary metabolites from carbon substrates is a widespread manifestation, and the excretion of specific metabolites appears to play a key role in uncoupling catabolism from anabolism under particular conditions (Tempest and Neijssel, 1992). That is, the catabolism of carbon substrates is diverted to metabolite formation, rather than to energy formation and growth. If this mechanism provides an explanation for citrulline excretion, the reutilisation of the released citrulline after the depletion of arginine and glucose (Figures 5.3 and 5.4) remains unexplained.

10.3 Relationship between the utilisation of arginine and citrulline and the possible uptake system of arginine and citrulline

While some bacteria such as *Ps. aeruginosa* strains (Mercenier *et al.*, 1980a; Rahman *et al.*, 1980) and *Ps. putida* P2 (Fan *et al.*, 1972) can utilise arginine as a sole carbon and nitrogen source, wine LAB (Liu, 1990) and other bacteria (Bauchop and Elsden, 1960; Deibel, 1964; Jonsson *et al.*, 1983) cannot grow on arginine as a sole carbon and energy source. The biochemical reasons for the requirement of glucose for arginine utilisation are not understood. It may be that arginine transport initially depends on the presence of glucose, for example, for the production of ATP or a proton-motive force, or that glucose metabolism produces the energy for induction of the ADI pathway enzymes. It was observed (Figures 5.1 and 5.2) that *Lc. oenos* OENO continued to catabolise arginine after the depletion of glucose, indicating that once the ADI pathway is induced and functioning, the metabolism of arginine is no longer dependent on glucose. This continued utilisation of arginine after the disappearance of glucose may be related to the arginine transport system. Using membrane vesicles of galactose and arginine-grown cells of *Lactococcus lactis* ML3, it has been shown that arginine is transported in antiport with ornithine (arginine-ornithine exchange) and metabolic energy was not required for the antiport process (Driessen *et al.*,1987; Poolman *et al.*,1987; Konings *et al.*, 1991). As high intracellular concentrations of ornithine accumulate after some degradation of arginine, the ornithine gradient then drives the uptake of arginine without further expense of metabolic energy. This exchange process can continue even after the exhaustion of glucose as long as arginine is present. If this hypothesis is tenable for wine LAB, it may explain the observations

mentioned above.

The utilisation of citrulline as a sole source of carbon and nitrogen is mainly restricted to species of *Pseudomonas* (Stalon and Mercenier, 1984). However, the use of citrulline as an energy source has not been reported for any microorganisms. This research has indicated that citrulline is catabolised to produce energy in *Lb. buchneri* CUC-3 in the presence of glucose (Chapter 6). Presumably citrulline alone (like arginine) cannot be utilised by this strain as a sole source of energy for growth. However, it was noted that citrulline was reassimilated and degraded by both *Lb. buchneri* CUC-3 and *Lb. brevis* 250 after the disappearance of arginine and glucose (Figures 5.3 and 5.4). By analogy with the arginine-ornithine exchange system in lactococci (Driessen *et al.*, 1987), a citrulline-ornithine antiporter may be functional in the wine lactobacilli, and citrulline uptake may be driven by the ornithine gradient after the exhaustion of arginine and glucose. However, the existence of a citrulline-ornithine antiporter system in wine LAB remains to be established.

Only the lactobacilli were able to reutilise excreted citrulline. The reason for the inability of *Lc. oenos* OENO to take up excreted citrulline is not known, but may be due to the impermeability of the leuconostoc cells to citrulline. The inability to degrade citrulline (presumably due to lack of permeability) has also been reported in other micro-organisms possessing the ADI pathway, for example, mycoplasmata (Schimke and Barile, 1963; Schimke *et al.*, 1966) and in a *Lb. plantarum* fish isolate (Jonsson *et al.*, 1983). It has also been suggested that the rate of citrulline uptake in *Ps. aeruginosa* may be the rate-limiting step for its utilisation (Rahman *et al.*, 1980).

10.4 Oenological implications of the present studies

The results of this research have significant implications in the wine industry. The excretion of citrulline from arginine is oenologically important, as this can result in the formation of carcinogenic ethyl carbamate (Chapter 7). Since the experiments were conducted under conditions different from the winery situations, further trials should be carried out on a large scale, preferably in a winery, using several strains to confirm the results. It would also be important to establish whether the high ethyl carbamate levels found in the present studies upon heating of wine in which arginine has been degraded by wine LAB are also produced on prolonged storage of similar

wine at normal storage temperatures. The influence of pH on arginine degradation needs to be examined in more detail, since the wine fermentations used in this research had relatively high initial pH levels (pH 3.7). In a synthetic medium, low pH had a profound influence on arginine catabolism (Liu, 1990). Also, the potentially significant increase in pH that occurs during arginine degradation can be catastrophic for the biological stability of wine if the arginine level in wine is high, as high pH would encourage the growth of undesirable spoilage microorganisms. The limited information available indicates that ornithine has a pronounced inhibitory effect on wine yeasts, including wild yeasts (Kuensch *et al.*, 1974; Eggenberger, 1988). Therefore, the production of ornithine may impart a biological yeast control in wine. Other aspects with oenological implications which remain to be investigated include the effects of arginine degradation and metabolite formation on wine flavour, although Eggenberger (1988) regards the conversion of the bitter, musty arginine to ornithine as positive.

The selection of LAB starter cultures to conduct malolactic fermentation in wine becomes more important now than ever before because of the demonstration of the potential formation of ethyl carbamate precursor(s). Strains must be evaluated for their ability to excrete citrulline from arginine catabolism before being employed as starter cultures. Obviously, strains that release citrulline must not be selected. However, the excretion of citrulline is seemingly characteristic of strains possessing the three enzymes of the ADI pathway (Chapters 5, 7 and 8). Thus, these strains should not be chosen. Fortunately, not all strains possess the complete enzyme system for arginine degradation. For example, all homofermentative and some heterofermentative strains lack all or some of the enzymes for arginine catabolism (Chapter 4). This should be the basis for starter culture selection. But care must be taken not to select strains lacking OTC or CK or both, because these strains can release large amounts of citrulline (Chapter 4). As a general rule, arginine-degrading strains should not be selected as starter cultures. This entails the assessment of the ability of a strain to catabolise arginine. Performing enzyme assays for this is not always practical, since this is a complicated and time-consuming task. Monitoring the changes in the medium pH during growth in the presence of arginine, or testing ammonia with Nessler's reagent and/or with enzyme test kits may provide a simple inexpensive way of screening strains for arginine degradation, as discussed in Chapter 8.

Lastly, it must be stressed that the enzyme assay temperature (37°C) used for convenience in this research is above normal wine storage temperature (*ca* 18°C). Performing assays at 18°C is not practical because reaction rates would be much slower (very slow for ADI) at this temperature and, thus, this would be more time-consuming and prone to errors. Also, strains were cultured at 30°C in the absence of ethanol (Chapters 3,4,5,6,8,9) but at 22°C in the presence of ethanol (Chapter 7). This is justifiable since optimal growth of wine LAB occurs at about 30°C and 0-4% ethanol, but at 18-25°C and 10-14% ethanol (Asmundson and Kelly, 1990). Nevertheless, caution should be taken in the application of findings of this research to wine making. Perhaps further studies should be conducted to assess arginine catabolism at lower temperatures.

10.5 Suggestions for future work

This research project has generated several areas for future study of arginine metabolism. These areas of research have been indicated where appropriate in previous chapters and throughout this general discussion. Briefly, these include:

1) purification and biochemical characterisation of the enzymes of the arginine deiminase pathway;

2) study of the genetic reason(s) for the lack of the arginine deiminase pathway enzymes;

3) further work on regulation of the synthesis of the arginine deiminase pathway enzymes, especially repression by fructose;

4) study of other factors affecting arginine metabolism (pH, sugar type and concentration);

5) evaluation of strains for citrulline excretion under varying conditions (pH, sugar type and concentration);

6) studying the biochemical basis of citrulline excretion, reutilisation and non-utilisation;

7) characterisation of the transport systems of arginine and citrulline;

8) determining the oenological effects of arginine catabolism on sensory properties and biological stability of wine and potential formation of ethyl carbamate under winery conditions.

9) confirming absence of urea cycle enzymes (especially arginase).

Chapter 11

Summary and Conclusions

The presence of the arginine deiminase pathway (ADI pathway) in several wine lactic acid bacteria (LAB) was demonstrated by measurement of the activities of the ADI pathway enzymes, arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK), in cell-free extracts. ADI activity was assayed by measuring the rate of citrulline formation at the optimum pH of 5.8. OTC activity was also assayed by measuring the rate of citrulline formation in the direction of citrulline synthesis at the optimum pH, pH 8.0 for strains of *Lc. oenos* and pH 8.5 for strains of lactobacilli. CK activity was measured by coupling ATP formed from carbamyl phosphate and ADP to $NADP^+$ reduction via hexokinase and glucose-6-phosphate dehydrogenase at the optimum pH of 7.8.

A large number of commercial and laboratory strains of wine LAB were surveyed for the presence of ADI, OTC and CK activity. Homofermentative LAB examined did not show measurable activity for any of the three enzymes. However, there were large variations in ADI, OTC and CK activities among the heterofermentative LAB. Lactobacilli showed higher activity for these enzymes than leuconostocs, particularly for the enzymes ADI and OTC. However, not all the heterofermenters possessed the three enzymes. Some strains of *Lc. oenos* did not show any measurable activity for ADI, but showed very low activity for OTC or CK; while others showed no activities for any of the enzymes. The specific activities of ADI, OTC and CK were generally similar amongst *Lc. oenos* strains possessing all three enzymes.

Lc. oenos OENO, *Lb. buchneri* CUC-3 and *Lb. brevis* 250 transformed arginine to ammonia, ornithine and citrulline. Citrulline was reassimilated and catabolised after the exhaustion of arginine by the lactobacilli, but not by the leuconostoc strain. Glucose utilisation and malic acid degradation did not affect arginine catabolism, nor did the catabolism of arginine affect the utilisation of glucose and malic acid. However, when grown in a medium containing arginine and both fructose and glucose as sugar sources,

arginine degradation was delayed until nearly all of the fructose was metabolised. Arginine was stoichiometrically converted into two moles of ammonia and one mole of ornithine. A small proportion of the ADI pathway intermediate, citrulline, was excreted during the degradation of arginine, about 16% by strain OENO and 12% by strains CUC-3 and 250, before being recatabolised by the latter two strains.

Lc. oenos OENO and *Lb. buchneri* CUC-3 also degraded arginine and excreted citrulline in a synthetic wine and a laboratory-vinified white wine. Significant amounts of ethyl carbamate (urethane), a carcinogen, were found in the synthetic wine and wine samples after post-fermentation heat treatment. This formation of ethyl carbamate correlated well with arginine degradation and citrulline excretion by the wine LAB, demonstrating the potential of these bacteria to produce the ethyl carbamate precursor, citrulline. Only small amounts of ethyl carbamate (<10 ppb) were found in samples without subsequent heat treatment, suggesting that little carbamyl phosphate was excreted. Wine LAB strains capable of degrading arginine should not be used as starter cultures to induce malolactic fermentation (MLF) in wine, since excreted citrulline can react with ethanol even at normal wine storage temperatures to form the carcinogenic agent ethyl carbamate. In addition, spontaneous MLF should be discouraged, as this may result in the formation of ethyl carbamate precursors.

The cell yield (dry weight) of arginine-degrading wine LAB increased with increasing arginine consumption. Molar growth yields for arginine were 14.4 for *Lb. buchneri* CUC-3, 11.7 for *Lb. brevis* 250 and 4.4 for *Lc. oenos* OENO. The molar growth yield of citrulline was 4.5 for strain CUC-3. These results suggest that both arginine and citrulline are energy sources for these wine LAB, although there was a low energy coupling for strain OENO with arginine and for strain CUC-3 with citrulline.

Only very low activities of all three enzymes were detected in extracts from cells cultured in the absence of arginine, while high activities were measured in extracts from cells cultured in the presence of arginine. Specific activities of ADI, OTC and CK were increased from 19- to 391-fold in the presence of arginine. Activities for all three enzymes were undetectable in cell-free extracts of the homofermentative *Pd. sp.* CUC-4 and *Lb. delbruckii* CUC-1 grown in the presence of arginine (5 g/L) and low glucose level (1 g/L) in a

synthetic medium, indicating that the absence of the ADI pathway enzymes is probably not due simply to glucose repression of enzyme induction. Activities of the arginine deiminase pathway enzymes increased significantly during growth after the addition of arginine, again demonstrating their inducible nature. The activities of all three enzymes reached a maximum corresponding to the time at which arginine was depleted.

A good correlation was found between arginine degradation, ammonia production, ammonia test with Nessler's reagent and the activities of the arginine deiminase pathway enzymes in most wine LAB strains cultured in a complex broth (HFA) containing arginine (6 g/L). The ammonia test with Nessler's reagent is only useful in assessing arginine degradation for wine LAB strains that produce large amounts of ammonia. This test, however, has limitations for strains that form small amounts of ammonia because of the insensitivity of the test. Ammonia formed by weaker ammonia producers can be detected using enzyme test kits.

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List of Publications

The following papers published were derived from this research:

1. Liu, S-Q., Pritchard, G. G., Hardman, M. J. and Pilone, G. J. Citrulline production and ethyl carbamate (urethane) precursor formation from arginine degradation by wine lactic acid bacteria *Leuconostoc oenos* and *Lactobacillus buchneri*. American Journal of Enology and Viticulture. 45(2):in press.

2. Liu, S-Q., Pritchard, G. G., Hardman, M. J. and Pilone, G. J. Arginine metabolism in wine lactic acid bacteria and its oenological implications. New Zealand Microbiological Society Newsletter. pp20-25. September, 1993. NB: This was the NZMS Ph.D student prize-winning paper presented at the NZMS and NZ Society for Biochemistry and Molecular Biology held at Massey University, Palmerston North, New Zealand. 17-20 May, 1993.

3. Liu, S-Q. and Pilone, G. J. Arginine degradation by wine lactic acid bacteria and ethyl carbamate (urethane) formation in model wine and wine. *In*: Proceedings of the New Zealand Grape and Wine Symposium - Sustainability/Cabernet Sauvignon. D.T. Jordan (Ed.). Vol. 8. pp 118-125. Auckland. 3-5 November, 1993.

4. Pilone, G. J. and Liu, S-Q. Arginine metabolism by malolactic bacteria and its oenological implications. *In*: Proceedings of the New Zealand Grape and Wine Symposium - Profit: in the Market, in the Winery, in the Vineyard. Vol. 7. pp100-105. Christchurch. 3-6 November, 1992.