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ARGININE DEGRADATION BY  
MALOLACTIC WINE LACTIC ACID  
BACTERIA AND ITS OENOLOGICAL  
AND TOXICOLOGICAL IMPLICATIONS

A Thesis Presented in Partial Fulfilment of the Requirements for the  
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## ABSTRACT

Malolactic fermentation (MLF) in wines is a secondary fermentation carried out by lactic acid bacteria (LAB), mostly encouraged for decreasing acidity by degradation of malic acid and for modifying flavour. During MLF, wine LAB may also degrade arginine, leading to the formation of ATP, ammonia and citrulline, among others. This is of concern to the winemaker and the consumer alike because ammonia increases the pH and thus the risk of growth by spoilage micro-organisms, and citrulline is a precursor in the formation of carcinogenic ethyl carbamate (EC).

The degradation of arginine and the excretion of citrulline was studied with resting and growing cells of selected wine LAB in synthetic buffer and wine. All wine LAB tested degraded arginine and excreted citrulline, and also degraded citrulline as a sole amino acid. However, reutilization of excreted citrulline depended on the biomass condition. Arginine degradation and citrulline excretion rates had a linear, positive correlation. Arginine to citrulline conversion ratios ranged between 0.8 and 4.6% (w/w) and can be used to estimate the potential formation of citrulline from a given amount of arginine. Combining these ratios with literature data, an approximate arginine to EC conversion ratio of 0.006% (w/w) can be calculated. In *Lactobacillus buchneri*, arginine degradation occurred during growth and malic acid degradation, and this stimulated growth. In contrast, arginine degradation in oenococci occurred in late exponential or stationary phase and after malic acid degradation, and this did not stimulate growth.

Citrulline excretion results from an unfavourable equilibrium of the citrulline degrading reaction. The pH (3.5 - 6.5) did not have a direct effect on citrulline excretion. However, the excretion of citrulline was influenced by the growth phase in which arginine degradation occurred and can be partially regarded as an overflow metabolism caused by insufficient coupling of energy formed from arginine degradation to growth.

To control citrulline formation and pH increase in wine from bacterial arginine degradation, the results suggest carrying out MLF with pure oenococcal cultures and inhibiting bacterial metabolism after malic acid depletion. This is especially important in wines with high arginine concentrations and high pH values. Malic acid itself should be measured since pH increase or CO<sub>2</sub> formation may also result from arginine degradation. Alternatively, arginine negative *Oenococcus* strains could be isolated and used for MLF. The excessive addition of ammonia as yeast nutrient during alcoholic fermentation and storage of wines on yeast sediments (lees) increase the potential for citrulline formation. A simple enzymatic assay for the determination of arginine was developed and this method enables a fast "risk-assessment" of must and wines.

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## ABBREVIATIONS

All abbreviations and units used in this thesis and not specified in this list are standard SI-units. Note that the anion names of acids have been used synonymously.

ADI.....	arginine deiminase
AF.....	alcoholic fermentation
$A_{\lambda}$ .....	absorbance at specified wavelength ( $\lambda$ ) in nanometers
Arg, arginine.....	L-arginine (174.25 g mol <sup>-1</sup> )
$c$ .....	concentration
cfu.....	colony forming unit (cp. 2.4.3.2)
Citr, citrulline.....	L-citrulline (175.25 g mol <sup>-1</sup> )
CK.....	carbamate kinase
Ctrl.....	Control (experimental control)
CV(%).....	coefficient of variation or relative standard error of the mean (=SE/mean $\times$ 100)
DAP.....	di-ammonium hydrogen orthophosphate (a common yeast nutrient)
dry wine.....	wine with no or low sugar concentration (0-8 g l <sup>-1</sup> of reducing sugars)
EC.....	ethyl carbamate (urethane)
FMOC.....	9-fluorenylmethyl chloroformate (for amino acid derivatization)
Fructose.....	D(-)-fructose
Galactose.....	D(+)-galactose
$g$ .....	acceleration equivalent to the earth's gravity (9.806 m s <sup>-2</sup> )
Gl-DH.....	glutamate dehydrogenase
Glucose.....	D(+)-glucose
HPLC.....	high performance liquid chromatography
ID.....	inner diameter (HPLC column parameter)
$\alpha$ -KG.....	$\alpha$ -ketoglutaric acid
LAB.....	lactic acid bacteria
<i>L. buchneri</i> .....	<i>Lactobacillus buchneri</i>
Malic acid or malate.....	L(-)-malic acid
Mannitol.....	D-mannitol
MLF.....	malolactic fermentation
$n$ .....	number of measurements
OD $_{\lambda}$ .....	optical density at specified wavelength ( $\lambda$ ) in nanometers
<i>O. oeni</i> .....	<i>Oenococcus oeni</i>
OPA/3-MPA.....	o-phthaldialdehyde/3-mercaptopropionic acid (for amino acid derivatization)
Orn, ornithine.....	L-ornithine (132.16 g mol <sup>-1</sup> )
OTC.....	ornithine transcarbamylase

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(abbreviations, cont'd)

PC .....	<i>Saccharomyces bayanus</i> Première Cuvée
PCR.....	polymerase chain reaction
PVPP.....	polyvinylpyrrolidone
<i>r</i> .....	coefficient of correlation
Rce .....	resting cell experiment (cp. 2.3.2.1)
Rhamnose .....	L(+)-rhamnose
Ribose .....	D(-)-ribose
RP-HPLC.....	Reverse Phase HPLC
<i>S. (cerevisiae or bayanus)</i> .....	<i>Saccharomyces cerevisiae</i> or <i>bayanus</i>
SD .....	standard deviation
SE.....	standard error (= $SD/\sqrt{n}$ )
TEA .....	triethanolamine
Trehalose .....	D(+)-trehalose
TRIS.....	2-amino-2-(hydroxymethyl)-1,3-propanediol-hydrochloride
U .....	enzymatic unit (enzyme quantity leading to formation of 1 $\mu\text{mol product min}^{-1}$ )
UV .....	ultraviolet (referring to the wavelength range of a spectrophotometer)
VIS.....	visible (referring to the wavelength range of a spectrophotometer)
v/v .....	volume/volume
w/v .....	weight/volume
w/w .....	weight/weight

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## **PREFACE**

Please pay attention to Figure 1 inside the back cover of this thesis. It contains a graphical representation of arginine degradation pathways in heterofermentative LAB and their consequences. The arginine deiminase pathway is essential for this thesis and Figure 1 allows the reader to view this pathway in relation to arginine degradation by yeasts and the formation of biogenic amines.

Whereas the experimental conditions of every experiment are outlined in the respective chapters, the detailed methods and media used are described in the general Materials & Methods section, simplifying the search for a specific method or medium.

To increase the readability of the thesis and to avoid unnecessary length of the main part, some figures have been cited in the text only and shown in the Appendix. This is consistent with the move of some scientific journals in providing "Supplementary Information" to facilitate the assessment of the work without increasing the length of manuscripts.

All chapters are intended to be independent and are fully discussed; cross-references are made where appropriate. Chapter 11 contains a synopsis that examines this work in the wider contexts of oenology and microbial metabolism.

## CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

### 1.1. Malolactic fermentation - a secondary fermentation in the production of table wines

The production of any alcoholic beverage, including white and red table wine, requires the conversion of sugars to alcohol by the metabolism of yeasts, a process known as alcoholic fermentation (AF). In the production of some white and most red wines, a secondary fermentation usually takes place that is called malolactic fermentation (Davis et al. 1985; Lonvaud-Funel 1999). The name has its origin in the conversion of malic acid to lactic acid and CO<sub>2</sub>, and contrary to AF by yeast, malolactic fermentation (MLF) is carried out by wine lactic acid bacteria (LAB) of the genera *Lactobacillus*, *Pediococcus* and *Oenococcus* (Wibowo et al. 1985; van Vuuren and Dicks 1993). There are several species within the wine lactobacilli and pediococci, but only one species, so far, is designated for *Oenococcus*, *O. oeni*, previously *Leuconostoc oenos* (Dicks et al. 1995). By converting the dicarboxylic malic acid into monocarboxylic lactic acid, the pH of wines increases and wines become softer and less acidic to the palate. This effect is the main interest in MLF, especially in high-acid wines from colder winemaking regions, but other important conversions take place during MLF, which have an effect on wine composition and quality.

The metabolism of wine LAB has a great effect on flavour compounds, such as diacetyl (Davis et al. 1985; Henick-Kling and Acree 1998; Nielsen and Richelieu 1999) and acetaldehyde (Osborne et al. 2000). Moreover, MLF can help in extracting flavour active substances from oak (De Revel et al. 1999). The metabolism of certain substances (acetaldehyde, citrate) by wine LAB can have an effect on wine colour and the usage of SO<sub>2</sub>, an important wine antimicrobial and antioxidant, too (Vetsch and Lüthi 1964; Osborne et al. 2000).

MLF leads to changes of the microbial stability of wine, as well. Whereas realization of MLF greatly decreases the risk of LAB growth in bottled wine by depleting malic acid and nutrients (Kunkee 1967; Rankine 1972), it is clear that the increase in pH from malic acid conversion leads to a greater danger of spoilage by wine micro-organisms that are growth inhibited at lower pHs. Costello et al. (1983) observed growth of lactobacilli and pediococci after MLF by *O. oeni* and Wibowo et al. (1988) showed that wines that had been sterile filtered after completion of MLF by *O. oeni* were once more able to support growth of the same micro-organism on re-inoculation.

MLF may occur spontaneously by LAB naturally present in grape must and wine. Although natural MLF in grape must is possible, usually, numbers of wine LAB decrease during AF and MLF takes place afterwards. However, it may be induced by addition of commercial starter bacteria, too. In this case, MLF can be carried out by *Lactobacillus plantarum* in must prior to AF (Prahl et al. 1988; Pilone 1995), but MLF is generally induced after AF with the preferred organism, *O. oeni*.

The preference given to strains of *O. oeni* is based on its natural adaptation to grow and to carry out MLF in low pH wines, and because of its metabolic characteristics that are considered favourable for wine quality. Accordingly, Costello et al. (1983) stated that in wines at pH 3.29, the only species isolated was *O. oeni* and Davis et al. (1986a) reported that *O. oeni* was the main species to conduct MLF in red wines at pH values ranging from 3 to 4, and the only one isolated in wines below pH 3.5. With reference to the metabolic characteristics, *O. oeni* is a preferred organism since many aspects of microbial wine spoilage are related to growth and metabolism of *Lactobacillus* or *Pediococcus*, especially in unprotected wines with pH values above 3.5 (see Amerine et al. (1980) for synopsis). Amongst others, these wine depreciations include the formation of health concerning substances such as biogenic amines (Moreno-Arribas and Lonvaud-Funel 1999; Moreno-Arribas et al. 2000); or sensory depreciation, as is the case with the excessive formation of lactic and acetic acids from heterofermentative sugar metabolism (Lonvaud-Funel 1999); ropiness caused by polysaccharide formation of pediococci (Lonvaud-Funel and Joyeux 1988; Canal Llauberes et al. 1989); or the degradation of tartaric acid (Radler and Yannissis 1972). Lactobacilli equally have been associated with the occurrence of sluggish or stuck AFs (Edwards et al. 1999). The risk of wine quality depreciation by *O. oeni*, however, cannot be denied. For example, it has been shown recently that *O. oeni* produce histamine (Lonvaud-Funel and Joyeux 1994; Coton et al. 1998), which is known for its negative effect on human health (Bardocz 1995; Silla-Santos 1996), and under certain circumstances, excessive formation of acetic acid has been observed (Lafon-Lafourcade et al. 1980). Equally, mousiness has been related to oenococci as well as to lactobacilli (Costello et al. 1996).

Finally, the degradation of the amino acid arginine by some wine LAB has several implications for the chemical composition and quality of wines, too. It is the aim of this thesis to investigate the arginine metabolism of wine LAB and its oenological implications.

## 1.2. Importance of arginine during the vine growing and winemaking process

Arginine is a basic amino acid of considerable importance for human nutrition and health (Wu and Morris, Jr. 1998; Efron and Barbul 1999) and is present in significant amounts in many agricultural products. In the growing of vines and the making of wines, it is of particular interest. There is evidence that arginine serves as the main form of nitrogen storage in vines and it accounts for 30-76% of the free amino acids in roots and above ground woody storage tissues (Kliewer and Cook 1971). As a consequence, the arginine concentration has been suggested for assessing the nitrogen status of vines (Kliewer and Cook 1974; Juhasz et al. 1984; Bath 1993) and the maturity of grapes (Kliewer 1968; Bath et al. 1991). Therefore, it is not surprising that arginine is the major amino acid found in grape musts of many grape varieties around the world (Rodriguez et al. 1988; Sponholz 1991; Capela and Bakker 1991; Henschke and Jiranek 1993; Spayd and Andersen-Bagge 1996).

The nitrogen status of the vines and the resulting arginine concentration in the grapes have important implications for the winemaking process. Low arginine concentrations in grape musts may lead to poor fermentation kinetics (Salmon and Barre 1998) and sluggish AFs (Löhnertz and Rauhut 1997), or cause sensory imperfections in resultant wines (Müller et al. 1998; Fischer et al. 2000). Yeast degradation of arginine may lead to the formation of urea, which can again be reutilized by the yeast later during AF (Ough et al. 1990; An and Ough 1993). Remaining urea in the wine after AF is of concern because it has been shown to be a precursor in the formation of carcinogenic ethyl carbamate (EC) (Tegmo-Larsson and Henick-Kling 1990a; Kodama et al. 1994). But even though arginine is in the group of amino acids that are first utilized by yeast during AF (Bisson 1991; Boulton et al. 1995), high arginine concentrations ( $> 2 \text{ g l}^{-1}$ ) can still be found in wines (Mayer et al. 1973; Sponholz 1991).

## 1.3. Arginine degradation by heterofermentative wine lactic acid bacteria

Arginine remaining in the wine after AF can serve as a substrate for some wine LAB. According to several authors, it is essential for the growth of *O. oeni* (Garvie 1967; Fourcassie et al. 1992). In fact, for some time it has been known that arginine was degraded during MLF, producing ornithine (Mayer et al. 1973; Kuensch et al. 1975; Temperli and Kuensch 1976; Pilone et al. 1991). Initially it was suggested that the degradation by wine LAB occurred as in yeast by the enzyme arginase, yielding urea apart from ornithine (Kuensch et al. 1974; Sponholz et al. 1991). Significant studies on arginine metabolism in

wine LAB and the pathways involved have been summarized in a recent review article (Liu and Pilone 1998). Specifically, it was shown that arginine is degraded only by heterofermentative wine LAB, i.e. *O. oeni* and heterofermentative lactobacilli, whereas no homofermentative wine lactobacilli and pediococci were able to do so (Pilone et al. 1991; Edwards and Jensen 1992; Edwards et al. 1993; Liu et al. 1994; Liu et al. 1995a; Liu et al. 1995b). Urea is not produced during arginine degradation by heterofermentative wine LAB; in fact, arginase activity is not detectable in these micro-organisms (Liu et al. 1996). Instead, activities of the arginine deiminase pathway enzymes arginine deiminase (ADI), ornithine transcarbamylase (OTC), and carbamoylphosphate kinase (CK) were detected (Liu et al. 1995b; Liu et al. 1996). Figure 1 (see folding page inside back cover) contains a graphical representation of the ADI pathway, its enzymes and metabolites.

## 1.4. The arginine deiminase pathway

The arginine deiminase (ADI) pathway was first discovered by Hills (1940) who described the conversion of arginine to ornithine in *Streptococcus* and *Staphylococcus*. The initial name, arginine dihydrolase pathway, although still sporadically used in recent publications, should be abandoned because it originates in the wrongful assumption that one mole of arginine and two moles of water would react in a single enzymatic step to form ornithine, two moles of ammonia and one mole of CO<sub>2</sub>, whereas citrulline was deliberately excluded as an intermediate (Hills 1940). The ADI pathway has since been described in many micro-organisms and is best studied in *Mycoplasma*, *Streptococcus*, *Pseudomonas*, *Clostridium* and *Serratia*. It is not the aim of this introduction to review the knowledge about the ADI pathway and its enzymes in these bacteria and other non-wine LAB and the interested reader is referred to comprehensive review articles available (Abdelal 1979; Cunin et al. 1986). However, references to pertinent literature will be given where necessary with respect to arginine metabolism in wine LAB.

### 1.4.1. Enzymes of the ADI pathway

The enzymes of the ADI pathway have been isolated, purified and characterized in many micro-organisms. Work on all ADI enzymes of a micro-organism closely related to wine LAB (the heterofermentative dairy LAB *L. buchneri* NCDO<sub>110</sub>) has been published by Manca de Nadra et al. (1988). The molecular weight of the enzymes after partial or complete purification was reported as 199 kDa, 162 kDa and 97 kDa for ADI, OTC and CK, respectively, and the optimum pH values as pH 6.0 (Manca de Nadra et al. 1984a), pH 8.5 (Manca de Nadra et al. 1984b) and pH 5.4 (Manca de Nadra et al. 1986; Manca de Nadra et al. 1987), respectively. In many micro-organisms, two OTC enzymes are present, one with a catabolic and one with an anabolic function (Manca de Nadra et al. 1988; Liu and Pilone 1998). However, according to Amoroso et al. (1993), arginine is essential for growth of several *O. oeni* strains, and Garvie (Garvie 1967) and Fourcassie et al. (1992) also reported

arginine to be essential for *O. oeni*. It is therefore likely that there is no anabolic function of the OTC, at least in *O. oeni*.

Whereas a detailed characterization of the enzymes is not useful for this work, it is interesting to note that in *L. buchneri* NCDO<sub>110</sub>, both ADI and OTC have sulfhydryl groups in the active centre, an observation made for ADI (Shibatani et al. 1975; Park et al. 1984) and OTC (Marshall and Cohen 1972; Ahmad et al. 1986) from many other organisms. The arginine-ornithine exchange system reported for arginine uptake in dairy LAB (cp. 1.4.2) has been reported to have sulfhydryl groups in its active centre, as well (Konings et al. 1989). Although few arginine analogues are available that are not active on OTC (Lusty et al. 1979; Ahmad et al. 1986; Laliberté and Hellebust 1990), the similarity of the groups active in the enzymes ADI and OTC totally negates the approach of using specific inhibitors to control arginine degradation. But, the success of this approach would be rather doubtful, anyway, given the significance of functioning arginine metabolism in animal nutrition and stringent laws on additions to grape musts and wine.

#### 1.4.2. Transport of substrates

In membrane vesicles (Driessen et al. 1987) and resting cells (Poolman et al. 1987) of *Streptococcus lactis* (now *Lactococcus lactis*), an inducible arginine-ornithine exchange system was found and the occurrence of this system was confirmed for several other streptococci. The exchange system is energy independent and apart from the 1:1 arginine-ornithine exchange, lysine is transported by the system, as well (Thompson 1987; Konings et al. 1989). Liu and Pilone (1998) proposed the existence of an arginine-citrulline antiport and a citrulline-ornithine antiport in wine LAB based on results from citrulline excretion by *O. oeni* and *L. buchneri* and citrulline reutilization by *L. buchneri*. However, no citrulline uptake or citrulline-ornithine exchange could be found in streptococci (Poolman et al. 1987).

#### 1.4.3. Genetics and regulation of ADI enzymes

While the genes coding for the ADI pathway enzymes of numerous Gram positive and Gram negative micro-organisms have been cloned and sequenced several years ago, only recently has the first genetic analysis of ADI pathway enzymes of an *O. oeni* strain been carried out (Tonon et al. 2001). Cloning of the ADI pathway genes led to isolating the arcABC cluster, consisting of arcA (ADI), arcB (OTC), arcC (CK) and several open reading frames (ORF) upstream of arcA. Sequence homologies of arcA and arcB were high compared with other Gram positive bacteria (> 50%), especially the LAB *L. sake*. For arcC, homologies were generally high, even compared with archaeobacteria or protozoa, suggesting a very conserved sequence. Concerning the ORFs, one of them, orf229, codes for a protein (ORF229p) that has similarities with proteins involved in transcription activation.

By using total RNA isolation and RT-PCR, Tonon et al. (2001) could also show that the three arc genes and orf229 were expressed constitutively in the absence of arginine in the growth medium. Addition of arginine further stimulated the transcription of the genes, whereas addition of arginine in the absence of fermentable sugars decreased the translation of the arc genes, markedly the expression of orf229. However, these findings do not agree with results from other studies at the enzymatic level. Liu et al. (1996) showed that none of the ADI pathway enzymes of an *Oenococcus* and a *Lactobacillus* were constitutive and all three enzymes were induced simultaneously in the presence of arginine, confirming results from a dairy *L. buchneri* (Manca de Nadra et al. 1986) and *S. faecalis* (Simon and Stalon 1982). In *Lactococcus* spp., ADI and OTC were found to be inducible and only CK was present constitutively (Crow and Thomas 1982; Konings et al. 1989). The expression of the arc genes and the translation of the enzymes, therefore, need to be studied further.

The expression of ADI enzymes and the arginine-ornithine exchanger in *Streptococcus* was repressed by glucose and induced by arginine (Poolman et al. 1987) and Liu and Pilone (1998) reviewed the repression of the formation of ADI enzymes by sugars for several bacteria including *Lactobacillus* spp. and *Streptococcus* spp. It is reported that at the level of enzymatic activity, some sugars, or more generally factors that increase the energetic status of the cells, also have an inhibitory effect on CK or OTC as described below (1.4.4).

#### 1.4.4. Physiology of the ADI pathway

In section 1.5, the oenological consequences of arginine degradation by wine LAB is described. These consist mainly of pH increase, the formation of energy and the excretion of citrulline. In order to understand these phenomena, it is useful to consider which physiological or enzymatic conditions lead to the degradation of arginine and what the consequences at a cellular level are.

The formation of ammonia during arginine degradation and the conservation of energy by the CK catalysed reaction of the ADI pathway seem to be logical for micro-organisms growing in acidic conditions and gaining energy only from anaerobic metabolism. It has been shown that the ADI system acts to relieve acid stress in *Streptococcus* and *Pseudomonas* (Marquis et al. 1987) and it is generally recognized as a mechanism for protecting oral streptococci from acidic conditions (Curran et al. 1998). The role of the ADI pathway in providing energy for growth has equally been described for many micro-organisms, including *Streptococcus* (Bauchop and Elsdon 1960; Crow and Thomas 1982), *Pseudomonas* (Marquis et al. 1987) and especially *Mycoplasma hominis*, which has a very reduced genome where the ADI pathway allows the generation of energy in a system consisting only of 3 enzymes (Schimke et al. 1966). Also, Manca de Nadra et al. (1988) suggested that ATP formation from arginine degradation increased growth of *L. buchneri*

and Liu confirmed this for a wine strain of the same species (Liu 1993; Liu and Pilone 1996).

However, the excretion of citrulline is less clear from a physiological point of view, since it prevents formation of ATP and yields only half the amount of ammonia than complete arginine degradation. Liu and Pilone (1998) suggested that excretion of citrulline is characteristic of micro-organisms that degrade arginine by the ADI pathway, and in fact, citrulline excretion from arginine degradation by this pathway has been observed in several micro-organisms. Cohen and Marshall (1962) stated that the excretion of citrulline is attributable to the thermodynamic balance of the OTC catalyzed reaction which lies overwhelmingly on the side of citrulline and not ornithine and carbamoyl phosphate ( $K = 1 \times 10^5 \pm 9 \times 10^3$  for the OTC equilibrium with citrulline as product). They argued further, that the continued formation of ornithine could only occur if the products of the reaction (carbamoyl phosphate and ornithine) were continually removed from the reaction site and this was confirmed from studies made by Legrain and Stalon (1976). Cohen and Marshall (1962) showed, too, that citrulline was not exchanged against arginine or ornithine in resting cells and suggested that this would keep intracellular citrulline concentrations high enough to drive the unfavourable OTC reaction.

Thompson et al. (1990) stated that during sugar degradation, arginine catabolism was inhibited and suggested that OTC was directly inhibited by ATP from the CK reaction and other ATP forming reactions; in short, conditions that enhance the energetic status of bacteria as the degradation of primary energy sources (Poolman et al. 1987; Konings et al. 1989). Indeed, Liu et al. (1995b) showed that ammonia formation from arginine degradation by wine LAB was inhibited in the presence of fructose. In fact, fructose is supposed to increase the energetic status and growth of wine LAB by being reduced to mannitol, supporting the regeneration of reduction equivalents, and thus allowing the generation of another ATP from glucose degradation (Salou et al. 1994).

It may be possible that the reasons for citrulline excretion by LAB accommodate several of these arguments and investigations about these are still necessary. From the data available in the literature, it is not possible to deduce conclusions for wine LAB. Also, there is no satisfactory explanation for the repeated observation of Liu et al. (1994; 1996) that *L. buchneri* CUC-3 was able to reutilize previously excreted citrulline after complete degradation of arginine, whereas *O. oeni* was not able to do so.

## 1.5. Consequences of arginine degradation

The degradation of arginine via the ADI pathway in heterofermentative wine LAB and its implications are shown in Figure 1 (see inside back cover), where comparisons are made

with the degradation of arginine via arginase in yeasts and the degradation of arginine in the formation of biogenic amines. Complete degradation of one mol of arginine by the ADI pathway leads to the formation of one mole of ornithine, ATP and carbon dioxide, and two moles of ammonia. This conversion may have an advantageous sensory aspect in wine since arginine, which imparts a bitter taste (Eggenberger 1988), disappears. Also, ornithine is produced, which has been shown to be inhibitory to growth of the yeast *Hansenula minuta* (Mayer et al. 1973; Kuensch et al. 1974), and could thus increase microbiological stability. However, most consequences of the ADI pathway are less desirable and will be discussed subsequently.

### **1.5.1. Increase of pH**

Complete degradation of one mole of arginine by the ADI pathway leads to the formation of two moles of ammonia (Figure 1, inside back cover), which may contribute to the increase in pH. Apart from colour changes that red wines will undergo with increasing pH values (Ribéreau-Gayon et al. 1998b), the microbiological stability of wines will be greatly affected. An increased pH, especially if it occurs in addition to the pH increase from malolactic conversion, will encourage growth of potential spoilage micro-organisms like lactobacilli and pediococci (Wibowo et al. 1985). Additionally, a higher pH will decrease the concentration of molecular sulphur dioxide in wine, the most active antimicrobial form of SO<sub>2</sub>, and thus requiring the addition of extra SO<sub>2</sub> (Boulton et al. 1995). In addition to the increase in pH, potential spoilage by yeasts is enhanced with the availability of ammonia, a readily assimilable nitrogen source.

### **1.5.2. Energetic implications**

During the degradation of citrulline by OTC, carbamoyl phosphate is formed, an intermediate with a highly energetic bond. Degraded by CK, carbamoyl phosphate will lead to generation of ATP. This formation provides bacteria with an additional substrate level phosphorylation site and could thus contribute to an increase in the energetic status. If ATP formation is effectively coupled to anabolic reactions, it may enhance growth, and this potential advantage could be of concern if present in spoilage bacteria.

### **1.5.3. Formation of citrulline, an ethyl carbamate precursor**

During the degradation of arginine by heterofermentative wine LAB, citrulline may be excreted in varying amounts. For several wine LAB studied in wines and synthetic media, it was found that between 4 and 28% (% mole/mole  $\approx$  % w/w) of the initial arginine available was excreted as citrulline (Kuensch et al. 1974; Liu et al. 1994; Liu et al. 1996). The excretion of citrulline is problematic for several reasons: citrulline may be degraded by spoilage bacteria present in wine to enhance growth (cp. 1.5.2) and cause increase in pH

(cp. 1.5.1); but mainly the formation of citrulline in wine is undesirable as it is a precursor in the formation of carcinogenic ethyl carbamate.

### 1.5.3.1. Ethyl carbamate and its mode of action

Ethyl carbamate (EC) is a pluripotent carcinogen with respect to tumour induction in different species, organs and stages of development of animals (Zimmerli and Schlatter 1991), including nonhuman primates (Thorgeirsson et al. 1994). It is regarded as an initiator of cancer by itself, but also as a co-carcinogen and specifically as a promoter of radiation-induced cancer (Svensson 1988). EC was formerly used as a hypnotic, but because of the carcinogenic potential, its application is now limited to anaesthetize laboratory animals. EC is not a final mutagen, itself, even in the presence of a microsomal activation systems (Guengerich and Kim 1991). During the bioactivation of EC, a cytochrome P-450 enzyme (P-450 2E1) is involved in the formation of the terminal mutagens, namely vinyl carbamate and its oxidation product epoxyethyl carbamate (Stoewsand et al. 1996). There is evidence that the final mutagenic effect is based on the alkylation of proteins and DNA by these two substances (Svensson 1988; Guengerich and Kim 1991).

The formation of EC from precursors occurs by different pathways. In the past, diethyl dicarbonate (DEDC; also called diethyl pyrocarbonate, DEPC), a cold sterilization substance used in the beverage industry until the early 70s, was found to form EC in the presence of ammonia ions (Löfroth and Gejvall 1971; Ough 1976a). DEPC has since been replaced by dimethyl pyrocarbonate (DMDC). Although there were reports (Ough 1976a; Rankine 1987) indicating that the EC formation potential of DEPC had been greatly exaggerated in the work done by Löfroth and Gejvall (1971), it is clear that DEPC has the potential to increase EC concentrations (Fischer 1972; Ough 1976b), and DEPC has never been permitted as an additive in some European countries (Zimmerli and Schlatter 1991). In order to reduce the carcinogenic burden already upon human beings, deliberate additions of carcinogenic precursors should be avoided where feasible.

This is especially true because EC is naturally present in most fermented food stuffs and beverages including cheese, yoghurt, bread, soy sauce, beer and other alcoholic beverages (Ough 1976a; Canas et al. 1989; Dennis et al. 1989; Battaglia et al. 1990; Zimmerli and Schlatter 1991). Table 1.1 summarizes the EC content of different food items and beverages. Based on the data available, it can be assumed that the main source of the daily intake of EC is bread and this burden seems unavoidable. However, apart from smoking, only the consumption of alcoholic beverages can increase this burden substantially.

Considering the mean EC concentrations found, daily consumption of 200-300 ml of table wine or 30 ml of stone-fruit brandies may increase the daily intake 5-60 fold (Schlatter and Lutz 1990; Zimmerli and Schlatter 1991).

**Table 1.1** EC concentrations found in some foods and beverages according to Zimmerli and Schlatter (1991).

Food Item/ Beverage	Range ( $\mu\text{g kg}^{-1}$ or $\text{l}^{-1}$ )	Mean ( $\mu\text{g kg}^{-1}$ or $\text{l}^{-1}$ )
Stone-fruit brandies	100-20 000	2 000
Sake, rice wine	10-900	130
Bourbon whiskies	< 10-350	40-90
Table wines	< 1-110	10-15
Sweet wines	10-250	50
Soy sauce	< 1-95	18
Bread	< 1-15	2-7

Ethyl carbamate formation in stone-fruit brandies and table wines involves different precursors. In stone fruit brandies, the precursor seems to be hydrogen cyanide and light plays a role in the EC formation. In table wines, two major precursors have been identified, both containing a carbamoyl group. These are urea and citrulline, although a minor role may be attributable to carbamoyl phosphate (Ough et al. 1988a; Ough et al. 1988b; Ough et al. 1990; Trioli and Colagrande 1990; Sponholz et al. 1991; Ough 1991; Liu et al. 1994; Kodama et al. 1994). In spite of the different precursors found, it has been suggested that the ultimate reactant to form EC with ethanol is undissociated cyanic acid ( $\text{HNCO}$ ), which is formed by oxidation or dissociation of the above precursors (Zimmerli and Schlatter 1991).

Since the reaction of precursors with ethanol to form EC is slow, the ability of potential precursors to form EC has been studied often using accelerated tests with heated wine samples. This technique, however, may not only accelerate the formation of EC from known precursors, but also encourage other reactions that would not proceed at lower temperatures. This aspect has been neglected in the literature, although upon heating, amongst many other reactions, amino acids and aldehyde groups of carbohydrates can react to form the EC precursor hydrogen cyanide (Lehmann and Zinsmeister 1979). Also, Ough et al. (1990) reported that accelerated storage, while giving valuable information for correlating possible precursors with EC, overestimated EC formation and was therefore unsuitable to estimate EC values in wine after prolonged storage. The application of accelerated storage and the lack of consideration of some precursors might explain some of the inconsistent results about the nature of precursors in wine and their potential to form EC. For example, Tegmo-

Larsson and Henick-Kling (1990a) excluded any correlation between urea or citrulline and EC and suggested that no EC precursors were formed during alcoholic fermentation, since EC formation from heated samples was the same in musts and the wines produced from them (Tegmo-Larsson and Henick-Kling 1990b). Equally, some workers suggested that urea was a more potent EC producer than citrulline (Ough et al. 1988a; Trioli and Colagrande 1990) even in long term experiments without heating (Ough 1991). However, the precursor role of citrulline has been demonstrated clearly by Liu et al. (1994) and Ough (1991), and Sponholz et al. (1991) showed that on a molar basis citrulline equalled the EC formation potential of urea. Besides, in a recent long term study on the formation of EC in table wines, it was found that 20 mg l<sup>-1</sup> of citrulline would react to yield 30 µg l<sup>-1</sup> of EC after 3 years of storage at 15°C and at this temperature citrulline equalled the EC formation potential of urea on a molar basis (R. Morenzoni, Personal Communication). The citrulline to EC conversion ratio (w/w) calculated from this data was 0.15% and this corresponds well with values calculated by Liu et al. (1994) from accelerated tests (0.11 - 0.18% (w/w)). Sponholz (1991) showed that German wines after MLF always contained more EC than those without MLF, and that EC itself is stable and not destroyed even under prolonged heating. Thus, it can be assumed that the formation of citrulline by wine LAB definitely increases the pool of EC precursors in wine.

### 1.5.3.2. Risk assessment and limits for EC

Canada is the only country worldwide, to have a legal limit for EC (Conacher and Page 1986). By using several approaches, a tolerable daily intake of 300 ng kg body weight<sup>-1</sup> was calculated and this led to a limit of 30 µg l<sup>-1</sup> of EC in table wines, considering the average weight and wine consumption of Canadians. However, Schlatter and Lutz (1990) proposed a tolerable daily intake of 20-80 ng kg body weight<sup>-1</sup> which would result in an EC limit of 2-8 µg l<sup>-1</sup> in wine. In the U.S.A., the Food and Drug Administration (FDA) reached an agreement with the wine industry in 1988 that the weighted average of EC in table wines containing 14% alcohol or less by volume is not to exceed 15 µg l<sup>-1</sup>, starting with wines from the 1988 harvest crush (Anonymous 1988). Statistical data for U.S.A. wines showing the results of efforts to reduce EC confirms that a general EC limit of 15 µg l<sup>-1</sup> in table wines is realistic (Foulke 1993). Regarding the prevailing limits in Canada (Conacher and Page 1986) and the Swiss recommendations (Schlatter and Lutz 1990), the 15 µg l<sup>-1</sup> limit seems to be reasonable from a toxicological point of view, as well. However, there is a discrepancy between average values found in American wines and wines imported into the U.S.A. (Foulke 1993) and as a consequence, the FDA recommended that wine importers begin on-going testing programmes (Anonymous 1996). Europe has been reluctant so far to introduce limits. In Germany, the federal health department (BGA) has argued that no limits have been set so far because the formation pathways of EC are complex and not completely elucidated, and no process to eliminate EC is available (Diemair 1989). Since formation of

EC may take place after the sale and depend on storage conditions that are out of control of the producer, legal questions could arise, too. The same report states average EC concentrations of  $13.4 \mu\text{g l}^{-1}$  for German white wines and  $21.6 \mu\text{g l}^{-1}$  for German reds. In Canada, routine testing of wines has been carried out since the legal limit for EC was introduced (Conacher and Page 1986). Karumanchiri et al. (1997) of the Liquor Control Board of Ontario reported that from over 16,000 wines tested between 1994 and 1995, 209 had EC concentrations between 20 and  $30 \mu\text{g l}^{-1}$  and 49 wines had EC levels over  $30 \mu\text{g l}^{-1}$ . Several alcoholic beverages, some of them imported, had to be withdrawn from sale (Conacher and Page 1986). Regarding the European inactivity on the one hand and the preoccupation of the Canadian authorities and the U.S.A. FDA on the other, it is clear that EC limits, apart from any toxicological aspect, have the effect of commercial import barriers.

### 1.5.3.3. Prevention of EC precursor formation

It has been specified that the formation of EC precursors urea and citrulline by yeasts and wine LAB, respectively, depends on the presence of arginine in musts and wine. In turn, the concentration of arginine in grape musts depends on the production of this compound in the vineyard and nitrogenous compounds like arginine, increase proportionally with the fertilization of vineyards. In a long term vineyard nitrogen fertilization experiment ( $100 \text{ kg N ha}^{-1} \text{ year}^{-1}$ ), Bertrand et al. (1991) showed that nitrogen fertilization increased total nitrogen (+ 50 %) and arginine (+ 150%) concentrations in must compared with must from a non-fertilized control vineyard. After AF, arginine concentrations were still significantly increased in wine (+ 100%) compared with wine vinified from must of the control vineyard. Ough et al. (1989) went further and showed that there is a direct relationship between vineyard fertilization and the formation of EC, itself (Ough et al. 1989). Therefore, it is clear that methods to decrease EC formation potential in wines have to start at the nitrogen status of the vineyards, as suggested in the EC preventative action manual of Butzke and Bisson (1997). It is necessary to balance vine nitrogenation in order to achieve amino acid concentrations, particularly arginine concentrations, that meet yeast growth requirements (Spayd et al. 1995) but are not so high as to cause urea or arginine residues in wines after AF. It has been found that in musts containing less than  $400\text{--}500 \text{ mg l}^{-1}$  of arginine, no urea or arginine residues are usually found after completion of yeast AF. However, when arginine concentrations are higher, particularly over  $1 \text{ g l}^{-1}$ , considerable urea and arginine residues are found in the wine, constituting a potential source for EC formation (R. Morenzoni, Personal Communication).

Investigations indicated that the amount of urea excreted and reabsorbed by yeasts primarily depends on the remaining arginine level in the medium and it was shown that yeasts possess different abilities to produce and reutilize urea (Ough et al. 1990; An and Ough 1993),

opening the possibility of selecting appropriate starters to further reduce the risk of EC precursor formation (Butzke and Bisson 1997). Additionally, the use of urea as cheap yeast nutrient has been forbidden in many countries (Ingledew et al. 1987; U.S.Federal Register 1990) and a method has been described to remove residual urea in wines with an acid stable urease (Ough and Trioli 1988). This method has been successfully applied in removing urea in wines although the application of the enzyme may be limited by inhibitory substances like fluoride and malic acid (Trioli and Ough 1989; Famuyiwa and Ough 1991). The importance of the storage temperature and fortification with ethanol on EC formation has been emphasized, equally (Butzke and Bisson 1997). There is, however, no method for the removal of residual arginine, which can serve as a substrate to heterofermentative wine LAB. Additionally, not much is known about the degradation of arginine and excretion of citrulline by these micro-organisms, including the preferred species, *O. oeni*. Thus, after AF, natural or induced MLF may lead to uncontrolled citrulline production, and so far, no method for the removal of citrulline has been described, either.

Some wines have been reported to have arginine levels as high as 2-5 g l<sup>-1</sup> after AF (Mayer et al. 1973; Sponholz 1991; Capela and Bakker 1991; Lehtonen 1996) and this could give rise to significant citrulline concentrations in wine after MLF. In fact, high citrulline concentrations (> 70 mg l<sup>-1</sup>) have been observed in wines (Ough 1991). Using the conversion ratios presented in section 1.5.3.2, it becomes evident that citrulline concentrations of only 10-20 mg l<sup>-1</sup> have the potential to form enough EC to exceed existing voluntary or legal limits. If the lowest amount of citrulline excreted from initial arginine given in the literature is considered (4.2%, (Kuensch et al. 1974)), approximately 250-500 mg l<sup>-1</sup> of arginine would have to be present in the wine after AF to cause the citrulline concentrations mentioned (10-20 mg l<sup>-1</sup>). Even if the arginine and citrulline concentrations cited by Ough (1991) were exceptional, the addition of a smaller amount of citrulline to an already existing EC precursor pool could easily tip the scales. This consideration and the high EC levels still measured recently in many wines (cp. 1.5.3.2 and Karumanchiri et al. (1997)) demonstrate the need for studying the metabolism of arginine by wine LAB to further control the formation of EC precursors in wine.

## 1.6. Summary and objectives of the thesis

This introduction has shown that the amino acid arginine may be present in considerable concentrations in grape musts and wines (1.2 and 1.5.3.3) where it can serve as a substrate for heterofermentative wine LAB (1.3 and 1.4). The degradation of arginine by these micro-organisms occurs by the ADI pathway (Figure 1, inside back cover) and leads to the formation of ammonia, ATP and citrulline, amongst others (1.4). The formation of these compounds has oenological and toxicological implications (1.5): the increase of pH by

formation of ammonia (1.5.1) and the generation of ATP (1.5.2) may affect the microbiological stability of wines, and citrulline is a precursor of carcinogenic ethyl carbamate (1.5.3).

It was the aim of this thesis project to investigate the metabolism of arginine and its intermediate citrulline by wine LAB to enhance understanding of the ADI pathway and its kinetics, to judge the impact of this pathway on winemaking, and in the best case, to control the excretion of citrulline from arginine degradation. Equally, the effect of arginine and citrulline on growth of wine LAB was studied to assess the energetic consequences of their degradation.

In order to estimate the impact of arginine degradation by wine LAB on winemaking, the distribution of the ability to degrade arginine, and to excrete and reutilize citrulline among wine LAB needed to be assessed. Therefore, a survey was carried out where most commercially available malolactic bacteria were investigated along with other heterofermentative wine LAB (Chapter 4).

Following this, the kinetics of arginine degradation and citrulline excretion under different conditions were targeted to identify factors that affect the metabolism of arginine and citrulline. These experiments were carried out with resting cells in a tartaric acid buffer (Chapter 5) and with growing cells in wine (Chapter 6).

In Chapter 7, the impact of some common winemaking techniques on the arginine metabolism of two commercial *O. oeni* strains was considered under practical conditions.

The investigation of winemaking techniques was then extended to the application of simultaneous alcoholic and malolactic fermentations (Chapter 8), an experiment with a wider focus that also covered interests outside the scope of this thesis.

In Chapter 9, the effect of arginine and citrulline on growth of several wine LAB was thoroughly studied to allow an evaluation of the potential energetic advantages that LAB may gain from arginine or citrulline degradation in wine.

In Chapter 10, experiments were conducted to investigate the effect of a wide pH range on the metabolism of arginine by a *Lactobacillus*.

## CHAPTER 2. GENERAL MATERIALS AND METHODS

### 2.1. General

#### 2.1.1. Statistical analysis and precision of analytical methods

Statistics are not reported when the biological variation within a treatment was small and the difference among treatment means was large (greater than 3 standard deviations). Where statistics are reported, standard parameters are given (standard deviation, SD; standard error, SE; coefficient of variation, CV). Significance tests were carried out either with the student t-test or by analysis of variation (ANOVA) at the specified level of significance. The correlation coefficient  $r$  is reported for calibrations of analytical methods. Strictly speaking, this is statistically incorrect because a requirement for the calculation of  $r$  is random sampling and the independence of data. However, since the scientific community generally attaches importance to this value to evaluate the "goodness of fit", it is reported in this thesis. Unless otherwise stated, the precision of all analytical methods used was  $< 2\%$  CV.

#### 2.1.2. Water

Water purified by a Milli-Q reagent water system (Millipore; Bedford, MA, U.S.A.) and having a resistance of at least  $18 \text{ M}\Omega \text{ cm}^{-1}$  was used throughout the work. For RP-HPLC, the Milli-Q water was further purified by filtration through activated carbon followed by redistillation.

#### 2.1.3. Sterilization of media

Media were sterilized by autoclaving or sterile filtering. Autoclaving was carried out by heating media and glassware at  $121 \text{ }^\circ\text{C}$  and  $1000 \text{ hPa}$  for 15 minutes. Sterile filtrations were carried out by passing media through nitrocellulose filters ( $0.22 \text{ }\mu\text{m}$  pore size,  $47 \text{ mm}$  diameter; MFS, Dublin, CA, U.S.A.) in a filter holder type SM 16510 (Sartorius, Göttingen, Germany) into previously sterilized glassware.

#### 2.1.4. Origin of chemicals and media

Unless otherwise stated, all ingredients for growth media were supplied by Difco (Detroit, MI, U.S.A.) and all chemicals by Sigma ([www.sigma-aldrich.com](http://www.sigma-aldrich.com)).

#### 2.1.5. Glass or polypropylene containers

Unless otherwise stated, all glassware used was from Schott (Schott Duran, Mainz, Germany). Disposable polypropylene tubes were used throughout the work and are identified by their capacity:  $1.7 \text{ ml}$  microcentrifuge tubes were from AxyGen (type MCT-175-C; Union City, CA, U.S.A.), and sterile  $15$  or  $50 \text{ ml}$  tubes were from Becton Dickinson (type Falcon "Blue Max", Lincoln Park, NJ, U.S.A.).

### **2.1.6. Solid media**

Agar plates and agar slants were prepared by adding 15 g l<sup>-1</sup> of agar to the respective media before autoclaving.

### **2.1.7. Yeast growth inhibition**

Pimaricin (natamycin) was added to some media to inhibit yeast growth. It was prepared as follows: 50 mg of Delvocid (containing 50% pimaricin, Gist-Brocades, Netherlands) were dissolved in 5 ml DMSO (dimethyl sulfoxide) in a sterile capped test tube to give a 5 mg ml<sup>-1</sup> solution. This solution was added to media at 1% (v/v) to give a final concentration of 50 mg l<sup>-1</sup>.

## **2.2. Micro-organisms, growth media and buffers**

### **2.2.1. Micro-organisms**

All wine LAB used (Table 4.1, p.30) were heterofermentative and taken from the Wine Microbiology Laboratory Culture Collection of the Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand. Some newer commercial strains were provided by Lallemand, Inc. (Montréal, Canada) and Chr. Hansen (Copenhagen, Denmark).

### **2.2.2. Growth media and buffers**

#### **2.2.2.1. Mineral solution**

Mineral solution was prepared by dissolving 20 g MgSO<sub>4</sub> × 7 H<sub>2</sub>O and 5 g MnSO<sub>2</sub> × 4 H<sub>2</sub>O in 100 ml of water. The solution was stored at -18 °C.

#### **2.2.2.2. Tween 80 solution**

Tween 80 Solution (5%) was prepared by dissolving 5 g Tween 80 (Difco) in 100 ml. The solution was stored at -18 °C.

#### **2.2.2.3. VJ (vegetable juice medium)**

VJ (Liu et al. 1995b) contained per litre of water: 5 g Bacto Tryptone, 5 g Bacto Peptone, 5 g yeast extract, 200 ml vegetable serum, 1 ml mineral solution (2.2.2.1) and 1 ml 5% Tween 80 solution (2.2.2.2). Vegetable serum was prepared by filtering commercial tomato juice (Campbell's V8 Tomato Juice, Campbell, Australia) through Whatman No. 1 filter with diatomaceous earth (Kenite, Diatomite 3000, Witco, Greenwich, CT, U.S.A.) as filter aid. After dissolving the constituents, VJ was adjusted to pH 4.5 with concentrated H<sub>3</sub>PO<sub>4</sub>, and autoclaved.

#### **2.2.2.4. VJG and VJAG (vegetable juice glucose medium and vegetable juice arginine glucose medium)**

VJG and VJAG were prepared in the same way as VJ but with added glucose (5 g l<sup>-1</sup>), or glucose and arginine (both at 5 g l<sup>-1</sup>), respectively.

#### **2.2.2.5. AMRS (apple-MRS medium)**

AMRS is a modification of MRS medium (de Man et al. 1960) for the growth of wine LAB. It contained per litre of water: 52 g MRS broth (Oxoid, Hampshire, U.K.) and 50 ml of a commercial apple juice concentrate (Fresh-Up, Old Fashioned Apple Juice Concentrate, Frucor Beverages, Auckland, New Zealand). The medium was adjusted to pH 4.5 with NaOH or H<sub>3</sub>PO<sub>4</sub> and autoclaved.

#### **2.2.2.6. YM (Bacto yeast maltose broth)**

YM medium was prepared by adding 21 g of Difco YM to 1 litre of water followed by autoclaving.

#### **2.2.2.7. NaHT (Sodium hydrogentartrate) washing buffer**

50 mM NaHT washing buffer was prepared by dissolving 7.5 g tartaric acid, 5 ml mineral solution (2.2.2.1) and 1 ml 5% Tween 80 solution (2.2.2.2) per litre of water and adjusting to pH 4 with 5N NaOH.

#### **2.2.2.8. NaHT buffer for resting cell experiments**

Buffer for resting cell experiments was prepared as NaHT washing buffer (2.2.2.7) but with only 1 ml mineral solution (2.2.2.1) per litre of water. Higher concentrations of minerals in the buffer may delay lysis of resting wine LAB but will also lead to interferences with the arginine determination by the Staron-Allard or Sakaguchi methods because of Mg(OH)<sub>2</sub> precipitation. However, when arginine is analyzed by HPLC or enzymatically, the formula for the washing buffer can be applied to resting cell buffer, too.

#### **2.2.2.9. P<sub>2</sub>T<sub>2</sub>-buffer (piperazine-phosphate-TRIS-tartaric acid buffer)**

P<sub>2</sub>T<sub>2</sub>-buffer was used for a special resting cell experiment that consisted in examining bacteria over a wide pH range (Chapter 10). P<sub>2</sub>T<sub>2</sub>-buffer was prepared by adding the following substances and adjusting to 200 ml of water (final concentration in parentheses): 3.88 g piperazine × 6 H<sub>2</sub>O (100 mM), 3 g tartaric acid (100 mM), 1.81 g TRIS (75 mM), and 2.06 g NaH<sub>2</sub>PO<sub>4</sub> × 1 H<sub>2</sub>O (75 mM). The pH of this solution was approximately 6.1 and portions were adjusted to pH values between 3.5 and 9.5 by addition of 5 M HCl or 5 M NaOH. The added volumes of acid or base were recorded and batches with lower additions were compensated for by addition of water.

### 2.2.2.10. Grape musts, grape juices and wines

Base wines used as LAB growth medium were either prepared from fresh white grape must provided by commercial wineries (Chapter 7 and Chapter 8), or from a white retail grape juice. If wines were made from grape must, the preparation of the wines and its chemical composition is described in the respective chapters. To produce base wine from retail grape juice, a natural, pure, white grape juice without preservatives (Grapetise, Pacific Beverages, Australia) was adjusted with sucrose to a total soluble content of 17 Brix (1.0696 g ml<sup>-1</sup> specific gravity) and fermented without any further modification. Unless otherwise stated, AF was always carried out at 18°C after inoculation with 2% (v/v) of *S. bayanus* strain Première Cuvée (PC), pre-grown in the same grape juice with 5 g l<sup>-1</sup> yeast extract added (pH 4.5). *S. bayanus* PC was used because it is a low urea producer (Ough et al. 1988b; Liu et al. 1994) and urea interferes with the measurement of citrulline by the Archibald method (cp. 2.4.4.2). After completion of AF, wines were "racked-off" the yeast sediment (decanted), "cold-settled" overnight (sedimentation at 4°C) and filtered through cellulose pads (Ekwip D9, Revesby, Australia). The chemical composition of the base wines prepared by this protocol and the additions made to them in order to obtain the final wines used in experiments are described in the "Experimental Conditions" section of respective chapters.

## 2.3. Culture conditions

### 2.3.1. Maintenance conditions

#### 2.3.1.1. Yeast

For maintenance of wine yeasts, a loopful of a liquid yeast culture was spread over YM agar slants, which were incubated at 27°C until growth was observed and then kept at 4°C. The slants were prepared by cooling the 25 ml glass bottles containing 10 ml freshly autoclaved YM agar on an inclined surface.

#### 2.3.1.2. Wine LAB

For short term maintenance of wine LAB (<1 month), a loopful of liquid medium containing the bacteria was suspended in 5 ml AMRS medium and stored at 4°C without previous incubation. For long term maintenance (>1 month), bacteria from liquid cultures were introduced into 10 ml AMRS agar medium by way of a needle and also immediately stored at 4°C. Prior to use, both types of cultures were incubated at 27°C until growth was observed.

## 2.3.2. Growth conditions

### 2.3.2.1. Resting cell experiments (rces)

Bacterial cells used in rces were prepared by cultivation in VJAG (2.2.2.3) at 30°C to the late-log/early stationary phase (as assessed by cell sedimentation), followed by centrifugation at 5,000 *g* for 10 min at 15°C. The cell sediment was then washed twice with NaHT washing buffer (2.2.2.7). Next, cell pellets were resuspended in appropriate amounts of NaHT rce-buffer (2.2.2.8) and pipetted into glass vials (10-25 ml volume) containing small amounts of highly concentrated aqueous solutions of several substrates, depending on the experiment. The substrates included (concentration of stock solutions in parentheses) glucose and fructose (50% w/v), and ribose, citrulline and arginine (10% w/v). The final substrate concentrations adjusted are specified in the respective chapters. Cell dry weights in rces were adjusted to 2-10 g l<sup>-1</sup> depending on the required speed of degradation. The glass vials were placed in a water bath (25°C) and stirred gently with a submersible water driven magnetic stirrer (Cole Parmer; Vernon Hills, IL, U.S.A.). Samples were taken periodically, centrifuged (10,000 *g* for 5 min) and the supernatant frozen (-18°C) until analyzed.

### 2.3.2.2. Induction of MLF in wine

To induce MLF, unless otherwise stated, wine LAB were inoculated into the final, sterile filtered wine either directly using commercial freeze dried cultures (treated according to manufacturers directions), or after pre-growth in a complex medium. In the latter case, 2% (v/v) bacteria, pregrown in 50% grape juice with 5 g l<sup>-1</sup> yeast extract added (pH 3.6) were inoculated, leading to initial populations of 7.8 × 10<sup>5</sup> to 1.8 × 10<sup>6</sup> cfu ml<sup>-1</sup>. All MLF were carried out at 18-20°C in a temperature controlled incubator (type 190F, Contherm Scientific Inc., Lower Hutt, New Zealand) and were protected from oxidation by flushing the headspace of bottles with CO<sub>2</sub> upon inoculation and taking samples.

## 2.4. Analytical methods

### 2.4.1. Analysis of musts and wines

Soluble solids in grape musts were measured with a handheld refractometer (Atago; Japan) and readings were temperature compensated. Progress of AFs was monitored with submersible hydrometers (Kessler Instruments; Westbury, NY, U.S.A.). The end of AF and assessment of dryness ("dry" typically is used for a wine with low sugar concentration, i.e. 0-8 g l<sup>-1</sup>) was carried out with a colorimetric test for reducing sugars (Clinitest, Miles Inc.; Elkhart, IN, U.S.A.). SO<sub>2</sub> was measured by the aspiration/oxidation method or by the iodine titrimetric procedure of Ripper as modified by Amerine and Ough (1974).

## 2.4.2. General analytical apparatus

### 2.4.2.1. pH determination

The pH was determined with a pH meter (Orion 50T digital ionalyzer; Cambridge, MA, U.S.A.), calibrated daily with standard buffer solutions (pH 4.01 and pH 7) from Mallinckrodt Baker (Paris, Kentucky, U.S.A.).

### 2.4.2.2. Spectrophotometric measurements

Unless otherwise stated, all spectrophotometric measurements were made with a standard VIS spectrophotometer (Pharmacia LKB Biochrom 80-2088-64, Cambridge, UK).

## 2.4.3. Cell enumeration and biomass estimation

Most of the growth related data in this thesis has been expressed as optical density (OD) readings obtained using a spectrophotometer. This measure was favoured over the determination of colony forming units (viable cell counts, cp. 2.4.3.2) because bacterial growth by formation of long chains (as in the case of *Oenococcus*) is underestimated when measuring cfu, unless techniques are applied to break up the bacterial chains. The determination of the OD gives a better assessment of the total bacterial biomass. For some yield studies, the dry weight of cultures was determined directly, or OD readings were transformed into cell dry weight according to Liu (1993).

### 2.4.3.1. Direct cell counts

A Neubauer counting chamber was used to enumerate yeast cells microscopically. Budding cells were counted as single cells when the daughter cell was less than 50% of the size of the mother cell.

### 2.4.3.2. Viable cell counts

In some cases, viable bacterial cell counts were carried out by spreading 100  $\mu$ l of diluted culture samples onto AMRS (2.2.2.5) agar plates (2.1.6) containing pimarin (2.1.7). The formation of colonies was quantified after 5 days incubation at 27°C. Sterile Bacto Peptone (Difco, cp. 2.1.4) water (0.1%) was used to dilute culture samples.

### 2.4.3.3. Dry weight determination

The dry weight of cultures in rces was determined by pipetting 1.5 ml of culture into a preweighed 1.7 ml microcentrifuge tube. The supernatant was decanted after centrifugation (10,000 g for 10 min) and the reaction tube dried overnight at 100°C. The difference in weight after cooling was corrected for weight loss of the tubes by subjecting empty tubes to the same procedure.

### 2.4.3.4. OD (optical density) measurements

Bacterial growth was measured by determination of the OD at either 600 or 750 nm. The wavelength was chosen where the background absorbance of the medium was lowest.

#### **2.4.3.5. Determination of cell condition**

The lysis of bacterial cells during rces was assessed by the release of cellular material according to the procedure of Garbay and Lonvaud-Funel (1990) with a modification: The absorbance of undiluted samples was followed at 260 nm.

### **2.4.4. Chemical or enzymatic analysis of metabolites**

#### **2.4.4.1. Arginine**

Arginine was determined colorimetrically by the Staron-Allard Method as described by Micklus and Stein (1973), enzymatically by a method described in Chapter 3, and by HPLC as described in 2.4.4.5. A detailed assessment of arginine determination is found in Chapter 3, as well.

#### **2.4.4.2. Citrulline**

Citrulline was determined colorimetrically using the method of Archibald (1944) as modified by Spector (1963), or by HPLC as described in 2.4.4.5.

#### **2.4.4.3. Ornithine**

Ornithine was determined by HPLC as described in 2.4.4.5.

#### **2.4.4.4. Glucose, fructose, malic acid, urea, ammonia**

Glucose, fructose, malic acid, urea and ammonia were determined using Boehringer Mannheim (1989) test kits (now Roche Molecular Biochemicals).

#### **2.4.4.5. Amino acids by HPLC**

A Shimadzu Class VP HPLC system was used, consisting of a DGU-14A degasser, a binary LC-10ADVP pump system, a SIL-10ADVP auto injector, a CTO-10AS column oven, a SPD-10AVP UV-VIS-detector, a RF-10AXL fluorescence detector and a SCL-10AVP system controller. The system was connected to a PC equipped with Class VP software (version 5), which was used for data collection and analysis. The primary amino acids arginine, citrulline, ornithine and norvaline (as internal standard) were determined after pre-column derivatization with OPA/3-MPA according to the method of Bartók et al. (1994) with one modification. The second derivatization step with FMOC to derivatize secondary amino acids was omitted. The chromatographic separations were performed through a Supelco 100x4.6 mm ID column filled with 3 µm Hypersil ODS (Shandon, Cheshire, England). A cartridge type zero-dead-volume guard column (Phenomenex Securityguard with 2 x3 mm ID ODS cartridges) was attached directly to the analytical column, preceded by a 5 µm in-line filter (Upchurch, Oak Harbour, WA, USA).

## CHAPTER 3. QUANTITATIVE DETERMINATION OF ARGININE AND CITRULLINE - DEVELOPMENT OF AN ENZYMATIC ASSAY FOR ARGININE

### 3.1. Introduction

Accurate quantitative determination of the  $\alpha$ -amino acids arginine and citrulline was essential for this project. Initially, no HPLC apparatus was available, and thus, colorimetric chemical methods were applied for the determination of both compounds. Arginine was determined by the method of Staron and Allard (Micklus and Stein 1973), although the Voges-Proskauer method (Micklus and Stein 1973) and the Sakaguchi reaction (Sakaguchi 1925) as modified by Gilboe and Williams (1956) were evaluated, as well. L-Citrulline was analyzed by the method of Archibald (1944) as modified by Spector and Jones (1963).

Unfortunately, colorimetric methods can suffer from lack of specificity when not preceded by cumbersome separation steps. Ornithine, ammonia and citrulline were shown to interfere with the determination of arginine by the Voges-Proskauer method (Fig. 1, Appendix) and ornithine and ammonia with the determination by the Staron-Allard method (Fig. 2, Appendix). Glycine, TRIS (Gilboe and Williams 1956) and ammonia (Sakaguchi 1925) have been reported to interfere with the Sakaguchi method.

The colorimetric determination of citrulline was impracticable where high concentrations of D-ribose and D-fructose were encountered (Fig. 3, Appendix). Since citrulline determination by the Archibald method is based on the reaction of the carbamoyl-group, substances which carry a carbamoyl group, like urea, strongly interact with the method, too. Therefore, citrulline determination by the Archibald method was unfeasible where concurrent alcoholic fermentation by yeasts led to changing urea concentrations.

Generally, the determination with colorimetric methods of both arginine and citrulline was further complicated in samples originating from solutions that underwent colour changes over time at the wavelength of the chromophore. This happened, for example, in some wine fermentations as a result of partial oxidation of wine phenolics.

In the case of citrulline, analyses from problematic experiments had to be carried out with HPLC when it became available, since no immediate alternative was available. In the case of arginine, however, an alternative seemed to be practicable. To avoid interferences, an enzymatic method was strived for that was comparable with those described for many metabolites by Boehringer Mannheim (1989) and requiring only a standard laboratory spectrophotometer. One attempt to present such a method for arginine was done by Faby

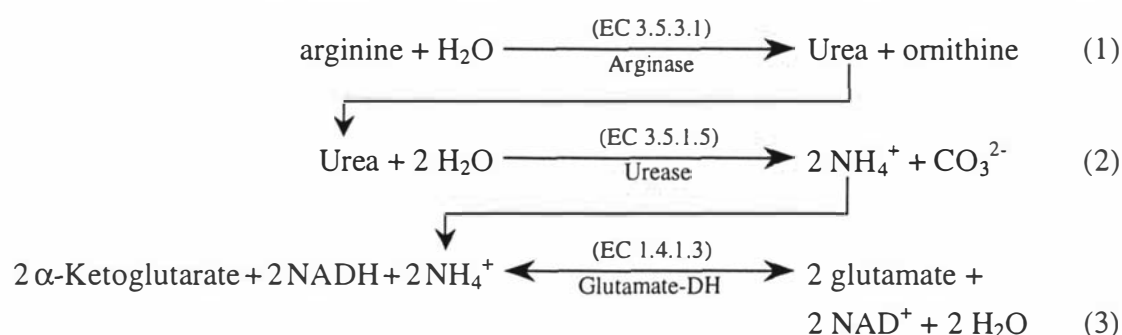
(1986). However, the method described is slow (60 min per sample) and is not suitable if urea or ammonia are present in the sample.

After evaluation of several possible enzyme systems, a simple and cheap enzymatic end-point method for the quantitative routine determination of arginine with the enzymes arginase, urease and glutamate dehydrogenase was developed and evaluated.

## 3.2. Experimental conditions

### 3.2.1. Principle

The determination of arginine is made possible by the following enzymatic reactions:



The decrease in concentration of NADH, as measured by the change of extinction at 340 nm, is proportional to the amount of arginine originally present. Two moles of ammonia (i.e. 2 moles of NAD) are formed from one mole arginine.

### 3.2.2. Chemicals

Arginine (Arg), triethanolamine (TEA), polyvinylpolypyrrolidone (PVPP),  $\alpha$ -ketoglutaric acid ( $\alpha$ -KG), urease (EC 3.5.1.5) and arginase (EC 3.5.3.1) were from Sigma. NADH, ADP and glutamate dehydrogenase (G1-DH, EC 1.4.1.3) were from Roche (previously Boehringer Mannheim). For the preparation of standards, anhydrous analytical grade arginine (dried for 24 h at 105°C) was used. A modified synthetic wine containing several amino acids, but without tannins (Liu et al. 1995a) and pure commercial grape juice (Grapetise, Pacific Beverages, Australia) were used as media. Clarification of grape juice was carried out with 10% (w/v) PVPP.

### 3.2.3. Instrumentation

A UV-VIS spectrophotometer (Cary 1, Varian Inc. Australia) was used for kinetic studies, whereas a simple laboratory spectrophotometer (300-900 nm; Pharmacia LKB Biochrom 80-2088-64, Cambridge, UK) was used for all other measurements. Disposable half-micro cuvettes (max. 2 ml) were used throughout the work.

### 3.2.4. Preparation of buffer and enzymes

Buffer was prepared by adding the following amounts of solutes per litre of water and adjusting to pH 8.5 with H<sub>3</sub>PO<sub>4</sub>: TEA, 32 g (0.21 M);  $\alpha$ -KG, 2.4 g (13 mM); NADH, 0.1 g (0.14 mM); ADP, 1 g (2.1 mM) (concentrations in final buffer in parentheses). At 4°C, buffer containing TEA and  $\alpha$ -KG was stable for at least 1 month, and buffer with added NADH and ADP was stable for at least 3 days. Gl-DH was used pure at a concentration of 200 U ml<sup>-1</sup>. Urease and arginase were dissolved in 50% glycerol (15 mg ml<sup>-1</sup> and 20 mg ml<sup>-1</sup>, respectively) to give concentrations of 1500 and 940 U ml<sup>-1</sup>, respectively. All enzyme solutions were stable for at least 3 months at 4°C. The maximum concentration of arginine allowed in the sample is 100 mg l<sup>-1</sup>.

### 3.2.5. Procedure and calculations

The assay procedure is described in Table 3.1 and the reactions were carried out at 25°C.

**Table 3.1** Enzymatic determination of arginine: addition of buffer, enzymes and substrate to the 2 ml cuvette.

Add to cuvette		Concentration in cuvette	
Buffer	1.4 ml	TEA	0.19 M
		$\alpha$ -KG	12 mM
		NADH	0.13 mM
		ADP	1.9 mM
Sample	0.1 ml	Arginine	max. 38 $\mu$ M
Gl-DH.	0.01 ml	Gl-DH	7.8 U ml <sup>-1</sup>
Urease	0.01 ml	Urease	9.8 U ml <sup>-1</sup>
Mix, wait for constant absorbance and read A <sub>1</sub>			
Arginase	0.01 ml	Arginase	6 U ml <sup>-1</sup>
Mix, wait for constant absorbance and read A <sub>2</sub>			

Under the conditions described, the reaction proceeds stoichiometrically and two moles of NAD are formed from one mole of arginine. The absorbance difference was calculated as  $\Delta A_{\text{arginine}} = \Delta A_1 - \Delta A_2$ . The absorbance difference of a blank assay ( $\Delta A_{\text{blank}}$ ) containing 0.1 ml of water instead of sample was subtracted from  $\Delta A_{\text{arginine}}$  for each measurement. The following formula was used to calculate arginine concentrations in samples:

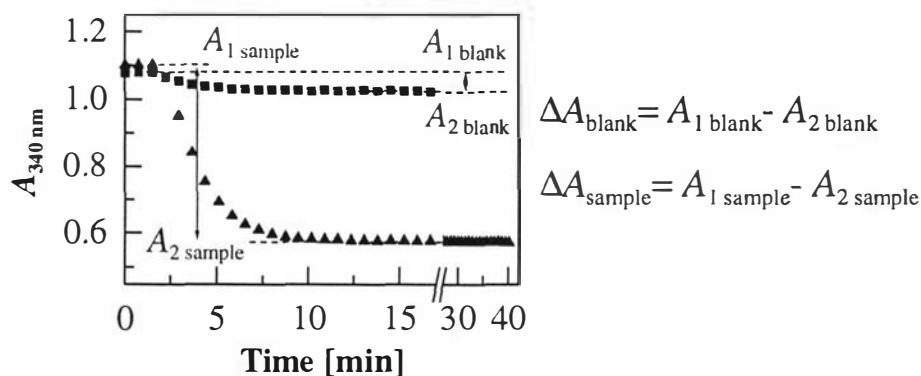
$$c_{\text{L-Arg}} [\text{mg l}^{-1}] = (\Delta A_{\text{arginine}} - \Delta A_{\text{blank}}) \times \frac{V \times \text{MW}}{v \times d \times \epsilon \times 2}$$

In this formula V = final volume in cuvette (1.53 ml), v = sample volume (0.1 ml), MW = molecular weight of the substance to be assayed (174.205 g mol<sup>-1</sup>), d = light path (1 cm) and  $\epsilon$  = absorption coefficient of NADH [6.3 l mmol<sup>-1</sup> cm<sup>-1</sup> at 340 nm]. It follows that

$$c_{\text{L-Arg}} [\text{mg l}^{-1}] = (\Delta A_{\text{arginine}} - \Delta A_{\text{blank}}) \times 211.53$$

### 3.3. Results

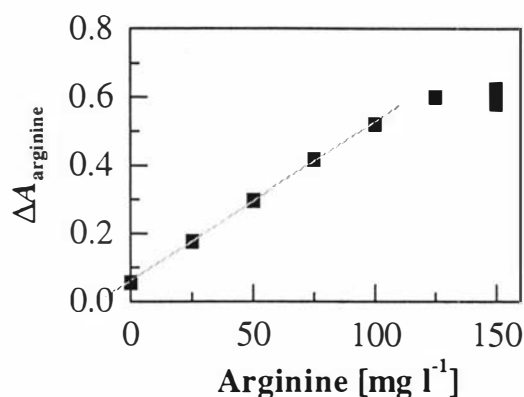
Figure 3.1 shows examples of absorbance time courses measured to determine arginine in synthetic wine samples with the enzymatic assay. At an arginine concentration of  $100 \text{ mg l}^{-1}$  in the sample, the final absorbance  $A_2$  reached after addition of arginase was obtained within 12 min. It was possible to reduce the duration of the assay to under 5 minutes by increasing the enzyme concentrations in the assay to  $10 \text{ U ml}^{-1}$  for glutamate dehydrogenase and to  $15 \text{ U ml}^{-1}$  and  $8 \text{ U ml}^{-1}$  for urease and arginase, respectively. The absorbance  $A_2$  was stable for at least 20 min after reaching the final value.



**Figure 3.1.** Enzymatic determination of arginine in synthetic wine. Time course of absorbances at 340 nm. Reaction started by arginase addition at  $t = 2$  min. Blank (■);  $100 \text{ mg l}^{-1}$  arginine sample (▲).

Accuracy and precision of the enzymatic method were determined by measuring the arginine concentration in several samples of synthetic wine, adjusted to arginine concentrations of 0 to  $150 \text{ mg l}^{-1}$ . The linear regression of a standard response curve generated with data from 5-fold determinations in the concentration range 0 –  $100 \text{ mg l}^{-1}$  of arginine had the equation  $c_{\text{L-Arg}} [\text{mg l}^{-1}] = [\Delta A_{340 \text{ nm}} - 0.059(\pm 0.002)] / 0.005(\pm 2.8 \times 10^{-5})$  (method of least squares, standard errors in brackets,  $n=25$ ,  $r^2=0.999$ ). Under the conditions described, arginine concentrations exceeding  $100 \text{ mg l}^{-1}$  in the sample led to a non-linear response curve and, therefore, a maximum arginine concentration of  $100 \text{ mg l}^{-1}$  in the sample was established (Figure 3.2). Table 3.2 shows the recovery and repeatability results of the enzymatic method from these determinations. The recovery of arginine in the synthetic wine samples ranged from 98.3 to 104.4% and the precision (%CV) ranged from 0.4 to 1.47%.

Evaluation of recovery and repeatability was further carried out using a commercial grape juice with unknown arginine concentration that was clarified with PVPP and spiked with an arginine standard solution of known concentration. The results of this evaluation are shown in Table 3.3. The arginine concentration in the juice was measured with 1:10 and 1:20 diluted juice samples.



**Figure 3.2** Standard response curve with data from arginine measurements in synthetic wine. Absorbance values were taken 12 minutes after addition of arginase. Each arginine concentration was measured 5 times.

The arginine concentration of a 1:10 diluted standard solution ( $0.5 \text{ g l}^{-1}$ ) of arginine was determined, as well. Measurement of a 1:1 mixture of both juice and arginine standard solution (1:10 diluted) showed that arginine recovery in the spiked juice ranged between 100 to 101.3% of the theoretical value calculated from the arginine concentrations determined in the grape juice and the arginine standard. The precision of the determinations with spiked grape juice was 0.6 (% CV).

**Table 3.2** Precision of the enzymatic method and recovery of arginine in synthetic wine: results of arginine determinations in synthetic wine, adjusted to arginine concentrations of 25 -  $100 \text{ mg l}^{-1}$ .

Arg added ( $\text{mg l}^{-1}$ )	n	Mean ( $\text{mg l}^{-1}$ )	%CV <sup>a</sup>	Recovery (%)	
				Min.	Max.
25	5	25.7	1.47	101.0	104.4
50	5	50.9	1.12	100.5	103.4
75	5	76.2	0.5	101.1	102.5
100	5	98.6	0.4	98.3	99.1

<sup>a</sup> coefficient of variation

**Table 3.3** Precision of the enzymatic method and recovery of arginine in grape juice (after PVPP clarification): results of arginine determinations in grape juice samples, an arginine standard solution and a 1:1 mixture of both grape juice and arginine standard.

Sample <sup>a</sup> (dilution factor)	Mean ( $\text{mg l}^{-1}$ )	%CV <sup>b</sup>	Recovery (%)	
			Min.	Max.
Grape juice (1:10)	58	0.67	-	-
Grape juice (1:20)	28.85	0.46	-	-
$0.5 \text{ g l}^{-1}$ arginine standard (1:10)	50.7	0.64	100.6	102.1
1:1 grape juice/standard (1:10)	54.63	0.6	100.0	101.37

<sup>a</sup>  $n = 5$  for all samples

<sup>b</sup> coefficient of variation

Unclarified samples of grape juice led to creeping absorbance decreases and reaction times of over 30 minutes. Increasing the enzyme concentrations in the assay alone did not lower reaction times significantly, but clarification of the juice with PVPP before the assay reduced the assay duration to 16 minutes.

### 3.4. Discussion

A coupled enzymatic method including arginase, urease and glutamate dehydrogenase (G1-DH) has been evaluated for the determination of arginine. Although the application of the three enzymes for the determination of arginine is not new, no simple and readily applicable method was described in the literature. Additionally, data about specificity and accuracy of methods using these enzymes was unavailable.

A synthetic wine and grape juice were chosen for the evaluation of the method because they contain several substances known for their inhibitory effects on arginase and G1-DH. Citric acid (Greenberg 1960) and several amino acids (Kaysen and Strecker 1973) including L-lysine, L-valine and especially L-proline, which is abundant in grape juices and wines, are known to inhibit arginase. Tannins in fruit juices and their products inhibit G1-DH (Boehringer Mannheim GmbH 1989).

The results show the suitability of the enzymatic method for the determination of arginine in both media used. Accuracy and precision were satisfactory compared to values found for many enzymatic determinations described by Bergmeyer (1974). Utilization of a micro assay leads to low material costs of approximately 0.43 US\$ per assay, including the cuvette. The method is rapid and since the absorbance at the end-point is stable for 20 minutes, numerous samples can be analyzed concurrently.

The time for analysing grape juice was increased compared to synthetic wine in spite of clarification with PVPP. However, the assay time could be considerably reduced by increasing the concentrations of the enzymes. However, increased enzyme concentrations alone could not replace clarification with PVPP. It should be pointed out that samples with different composition from those used here should be prepared according to instructions given by Boehringer Mannheim (1989) for the analysis of ammonia and urea.

The enzymes urease and glutamate-dehydrogenase are specific for their substrates urea and ammonia, respectively (Gutmann and Bergmeyer 1974). Arginase is not a highly specific enzyme (Greenberg 1960). For its activity, the guanidino group and the carboxyl group have to be present. The amino group of the  $\alpha$ -carbon atom can be substituted (Hunter 1938) or even replaced (e.g. by a hydroxyl group in arginnic acid) without loss of activity (Hunter and Woodward 1941). Also, a carbon atom in the carbon chain can be replaced by oxygen, since canavanine ( $\alpha$ -amino- $\gamma$ -guanidinoxy-*n*-butyric acid) is hydrolyzed by arginase.

However, the length of the carbon chain between the guanidino and the carboxyl groups is important since neither the 4 carbon  $\gamma$ -guanidinobutyric acid or the 6 carbon  $\epsilon$ -guanidinocaproic acid are hydrolyzed by arginine (Greenberg 1960). From the known arginine analogues, only canavanine is naturally present and could therefore lead to significant false-positive results. However, its occurrence is confined to some legumes: canavanine has only been found in the subfamily *Papilionoidae* of the *Leguminosae* family (Turner and Harborne 1967).

When added to pure buffer containing Gl-DH and urease, arginase causes a significant decrease in absorbance ( $\Delta A \approx 0.06$ ). This may originate from arginine or canavanine impurities in the utilized commercial urease which is prepared from the Jack Bean (*Canavalia ensiformis*, a member of the *Papilionoidae*). It is therefore important to determine the decrease in absorbance of a blank, as described in the methods, and to subtract it from the value of each measured sample, prior to calculation of arginine concentrations. As observed from the coefficients of variation at different arginine concentrations (Table 3.2), the precision of the method decreases with lower arginine concentrations. This has to be considered especially when the ratio of ammonia and urea to arginine in the sample is high, since dilution of the samples has to be carried out according to the concentrations of all three substrates.

Apart from the significance of arginine in winemaking and particularly in the formation of ethyl carbamate precursors (cp. section 1.2), its determination has importance in other areas, too. For example, arginine concentrations are used to assess peanut maturity (Valle et al. 1980) and are important in the germination behaviour of pine seedlings (Ramaiah et al. 1970). The simplicity of the method presented here enables analytical laboratories in agriculture and the food industry to carry out routine arginine determinations in-house, avoiding cost and delay of analysis by outside services. The results of this evaluation suggest the enzymatic assay should be considered as a preferred method over colorimetric methods for the manual determination of arginine in foodstuffs.

Recently, a spectrophotometric assay for arginine has been published by Austin and Butzke (2000). Their "ArgOPA"-method is based on the colorimetric determination of  $\alpha$ -amino acids with o-phthaldialdehyde in wine ("NOPA"-method by Dukes and Butzke (1998)). While the colorimetric determination itself is not specific for arginine, it seems to produce very good results after separation of arginine from other amino acids by a strong ion-exchange column. Its obvious benefits are the independence from ammonia and urea concentrations in the sample and its wide concentration range. However, the method was only tested with juice samples and the interference by wine colour remains unclear. A comparison of this method, the enzymatic method presented in this thesis and an HPLC method is highly desirable.

## CHAPTER 4. DEGRADATION OF ARGININE AND CITRULLINE IN WINE LACTIC ACID BACTERIA - A SURVEY

### 4.1. Introduction

In order to assess the distribution of the ability to degrade arginine and to excrete citrulline amongst heterofermentative wine lactic acid bacteria (LAB), a survey was carried out that included the majority of commercially available strains used for induction of malolactic fermentation (MLF). Earlier, Liu et al. (1994) found that a heterofermentative wine *Lactobacillus* strain was able to reutilize citrulline excreted from arginine degradation, whereas an *Oenococcus* strain did not. To study this observation further, the ability of wine LAB to degrade citrulline as a sole amino acid was included in this survey.

Wine LAB can be assessed for arginine degradation by measuring the formation of ammonia after growth in an arginine containing medium (Pilone et al. 1991). However, it has been shown subsequently that ammonia can be formed by wine LAB from the degradation of other amino acids, too (Granchi et al. 1998). Because of this, arginine degradation and excretion and reutilization of citrulline were assessed in this study by measuring the metabolites directly. Also, several samples were taken during experiments because a single measurement at the end of an incubation period could fail to detect citrulline excretion where citrulline was reutilized.

Since the growth of LAB in wine is slow (days/weeks), resting cell experiments (rces) were used that consisted in growing cells in a complex medium followed by the examination of highly concentrated cell suspensions in a synthetic wine mimicking buffer.

### 4.2. Experimental conditions

The wine LAB used are listed in Table 4.1. All strains of LAB used were heterofermentative since Liu et al. (1995b) did not detect the ADI pathway enzymes in any homofermentative wine LAB. The realization of rces is described under section 2.3.2.1. The analysis of substrates and the cell biomass is described under section 2.4. Initial concentrations of arginine were adjusted to 400-1000 mg l<sup>-1</sup> and were equal to the initial glucose concentrations adjusted.

**Table 4.1** Degradation of arginine and citrulline, and excretion of citrulline by strains of heterofermentative wine LAB.

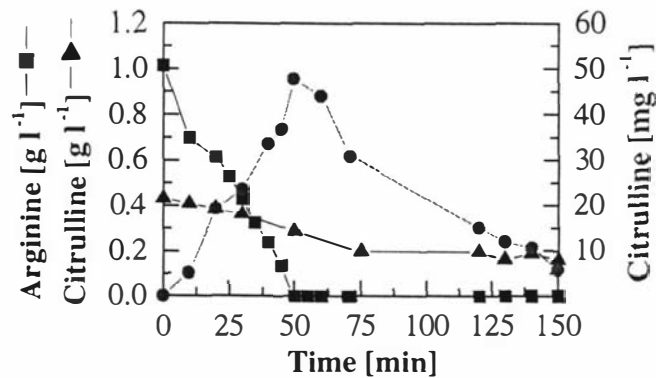
Strain	Commercial <sup>a</sup> Name	Reference	Arginine Degradation	Citrulline <sup>b</sup> Excretion	Citrulline Degradation
<i>Lactobacillus brevis</i>					
250		(Davis et al. 1986a)	+	+	+
Equilait		(Davis et al. 1985)	+	+	+
<i>Lactobacillus buchneri</i>					
CUC-3		(Pilone et al. 1966)	+	+	+
<i>Lactobacillus hilgardii</i>					
MHP	Microenos		+	+	+
<i>Oenococcus oeni</i>					
252		(Davis et al. 1986a)	+	+	+
Lo-121	Bitec-D1	(Krieger 1993)	+	+	+
Lo-111	Bitec-D1	(Krieger 1993)	+	+	+
2043	CIVC 2043	(Champagne et al. 1989)	+	+	+
EQ54	EQ54		+	+	+
DSIR-A	KIWI Culture		+	+	+
DSIR-C	KIWI Culture		+	+	+
DSIR-B	KIWI Culture		+	+	+
EQ34	Lalvin Inobacter		+	+	+
EQ33	Lalvin Inobacter		+	+	+
EQ59	Lalvin Inobacter		+	+	+
MCW	Lalvin MCW		+	+	+
2001	Lalvin MLF <sub>x</sub> 4	(Champagne et al. 1989)	+	+	+
2008	Lalvin MLF <sub>x</sub> 4		+	+	+
2035	Lalvin MLF <sub>x</sub> 4	(Champagne et al. 1989)	+	+	+
2006	Lalvin MLF <sub>x</sub> 4	(Champagne et al. 1989)	+	+	+
MTO1	Lalvin MTO1		+	+	+
Ey2d	Lalvin OSU	(Henick-Kling et al. 1989)	+	+	+
Erl a	Lalvin OSU	(Henick-Kling et al. 1989)	+	+	+
MB1	Microenos B1		+	+	+
MB2	Microenos B2		+	+	+
OENO	OENO		+	+	+
VFO	Viniflora Oenos	(Nielsen et al. 1996)	+	+	+

<sup>a</sup> Commercial name (strains may be part of a multistrain product but were examined separately in rces)

<sup>b</sup> Citrulline excretion from degradation of arginine

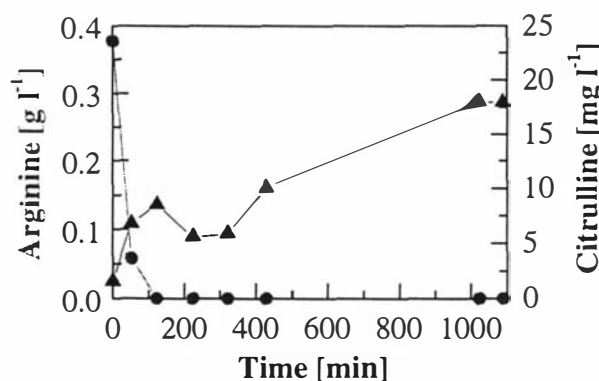
### 4.3. Results

Figure 4.1 shows an example of arginine and citrulline degradation by *Lactobacillus buchneri* strain CUC-3 in a resting cell experiment (rce). For the survey, this type of time course study was carried out for all strains listed in Table 4.1 with an initial arginine concentration of 400 mg l<sup>-1</sup>. All 27 strains tested degraded arginine as seen for strain CUC-3. Degradation rates were strain specific and clearly depended on the biomass concentration in the assay. All strains tested excreted citrulline from arginine degradation, the excretion rate being strain specific, as well. Figure 4.1 also shows degradation of citrulline in a separate rce, but using the same cell suspension at the same biomass concentration. Citrulline was degraded at a rate significantly slower than arginine and this was found for all 27 strains tested.



**Figure 4.1.** Degradation of arginine and citrulline by resting cells of *L. buchneri* CUC-3 at pH 3.6 in separate experiments. Both experiments were carried out with aliquots of one cell suspension: Arginine degradation: arginine (■), citrulline (●). Citrulline degradation: citrulline (▲).

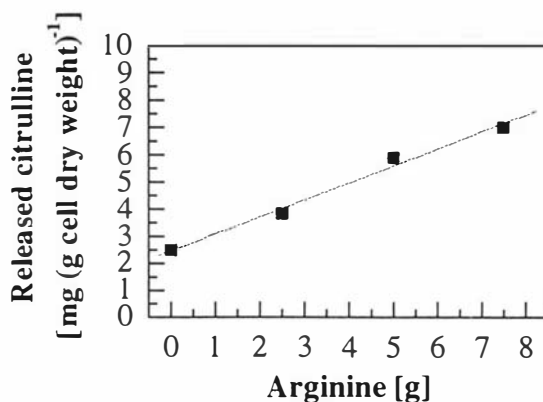
Although all strains degraded citrulline as a sole amino acid, excreted citrulline from arginine degradation was only found to be reutilized by a few strains after the depletion of arginine. This reutilization of citrulline is seen clearly for strain CUC-3 or Lo-111 (Figure 4.1 and Figure 4.2, respectively). With some of the strains, citrulline excretion from arginine degradation was followed by a temporary citrulline reutilization after arginine depletion; and then, surprisingly, a second increase in citrulline concentration occurred. This is documented, for example, in the rce for strain Lo-111 (Figure 4.2).



**Figure 4.2.** Arginine degradation and citrulline excretion by resting cells of *O. oeni* Lo-111 at pH 3.6. Arginine (●), citrulline (▲).

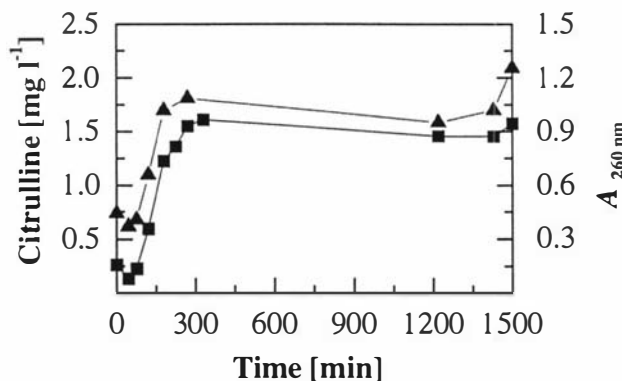
Because of this behaviour and the observation that in some of the rces the increase in the concentration of citrulline was higher than the decrease in the arginine concentration, the origin of the release of citrulline after depletion of arginine was investigated. For this, cells were first grown in VJAG medium, containing increasing concentrations of arginine; then, the cells were resuspended in rces buffer without addition of any substrate and the citrulline concentration measured after 24h. It was found that in rces without added arginine, citrulline

or glucose, the citrulline found after 24 h in the rce buffer was proportional to the amount of arginine initially added to the growth medium (Figure 4.3).



**Figure 4.3** Citrulline release by resting cells of *O. oeni* Lo-111 after 24 h of incubation in tartrate buffer (pH 3.6) without added arginine, citrulline, or glucose. The x-axis displays the amount of arginine added for the preparation of 1 litre VJAG broth (2.2.2.4). The data presented shows mean values from two separate experiments.

This indicated that the appearance of citrulline in the medium was related to cell lysis. To show this, the lysis of bacterial cells during rces was measured by the release of cellular material absorbing in the UV range (Figure 4.4). A similar method previously has been used for assessing the lysis of *O. oeni* strains (Garbay and Lonvaud-Funel 1990).



**Figure 4.4** Correlation between citrulline excretion and cell lysis by resting cells of *L. buchneri* CUC-3 in tartrate buffer (pH 3.6) without added arginine, citrulline, or glucose: Citrulline concentration (▲) and absorption at 260 nm (■). The data presented shows mean values from two separate experiments.

The ammonia concentration in the medium did not change during the release of citrulline in rces without added substrates. Therefore, it can be concluded that the cells did not accumulate arginine (or ornithine) to release citrulline after enzymatic conversion, but that citrulline itself was accumulated intracellularly during growth in arginine rich growth medium and released during lysis in rces.

## 4.4. Discussion

Resting cells of several heterofermentative wine lactic acid bacteria (LAB) were studied for their ability to excrete citrulline from arginine degradation and to degrade citrulline. All 27 strains tested degraded arginine and citrulline as sole amino acids in separate resting cell experiments (rces). It was also found that all 27 strains had the potential for citrulline excretion from arginine degradation. Hence, citrulline excretion appears to be characteristic of wine LAB able to degrade arginine. Citrulline degradation generally was slower than that of arginine in cell suspensions of the same batch. The reason for this difference may be related to differences in the uptake (cp. 1.4.2) or enzyme activities (cp. 1.4.4).

Both lactobacilli and oenococci were found to accumulate citrulline intracellularly during growth in arginine rich media. Accumulation of intracellular citrulline during degradation of arginine has been reported previously for milk LAB (Poolman et al. 1987) and is believed to be maintained by the cells to drive the OTC catalyzed reaction (Figure 1, inside back cover) that is thermodynamically unfavourable for citrulline degradation (cp. 1.4.4). The oenological relevance of intracellular citrulline accumulation is believed to be limited since the inoculation size for the application of commercial strains is small and would not introduce significant amounts of citrulline into the wine. However, higher amounts of citrulline may be introduced when wine LAB that are propagated in-house in arginine rich media are used for inoculation.

Liu et al. (1994) found that *L. buchneri* CUC-3 was able to reutilize citrulline after the depletion of arginine in wine and model wine, whereas strain MU2, an *O. oeni*, was not. Citrulline reutilization for strain CUC-3 was confirmed in rces in this research; however, some oenococci were found to reutilize citrulline as well. In rces, the reutilization was dependent on the lysis of the bacteria. Where lysis was delayed, reutilization of citrulline was observed. It was not possible to observe the ability to reutilize citrulline of all strains tested, since release of intracellular citrulline upon cell lysis may have obscured citrulline reutilization.

It is worth noting that the course of the rce with *L. buchneri* strain CUC-3 (Figure 4.1) was very similar to data obtained by Liu et al. (1994) for the same strain in wine and model wine. Whereas normal studies in wine with an initial inoculum of  $1-5 \times 10^6$  cfu ml<sup>-1</sup> will take over 5 days to finish, rces can be carried out in less than one day, including the analysis and the final evaluation of the data. Resting cell systems are not appropriate for studying metabolic activities that require growth for their induction and clear limitations were observed in this experiment by the lysis of the bacteria. However, rces proved useful for the rapid assessment of the metabolic activities of wine LAB under conditions similar to wine.

## CHAPTER 5. KINETICS OF ARGININE METABOLISM IN RESTING CELLS OF SELECTED WINE LACTIC ACID BACTERIA

### 5.1. Introduction

In Chapter 4 it was shown that all wine lactic acid bacteria (LAB) tested were able to excrete citrulline from arginine degradation and to degrade citrulline. In order to gain a better understanding of the arginine metabolism and to control citrulline excretion in wine, it was necessary to study further the kinetics of arginine degradation. Considering the good results obtained with resting cell experiments in Chapter 4, it was deemed useful to extend this type of experiment to study the arginine metabolism of selected wine LAB under several conditions. As representatives of lactobacilli and oenococci, *L. buchneri* CUC-3 and *O. oeni* strains Lo-111 and MCW were used.

Complete degradation of arginine leads to the formation of ATP (Figure 1, inside back cover). Following the pattern of enzymatic feedback inhibition by an end product (Gottschalk 1986), substrates that enhance the energetic status of LAB, such as fermentable sugars, could inhibit enzymes of the ADI pathway as suggested in some studies with non-wine LAB (Cohen and Marshall 1962; Legrain and Stalon 1976). Also, fructose has been shown to inhibit ammonia formation from arginine degradation by growing wine LAB in wine and synthetic medium (Liu et al. 1995b). However, nothing is known about the effect of pentoses on arginine degradation and the influence of sugars on the conversion of arginine to citrulline. According to the feedback inhibition model, high pH values could have a similar inhibitory effect on arginine metabolism, since arginine degradation increases the pH through the excretion of ammonia (Figure 1, inside back cover). Also, the initial arginine concentration itself might have an effect on the kinetics of citrulline excretion and could give insights into the arginine concentration needed to saturate the degradation pathway. Finally, ethanol, as an important factor for the growth and survival of LAB in wine (Wibowo et al. 1985), could have an effect on arginine degradation, too.

Because of the potential influence of these factors on the arginine metabolism in wine LAB, arginine degradation and citrulline excretion were investigated at several arginine and ethanol concentrations, pH values and with several sugars. Regarding the latter, it was assumed that in order to enhance the energetic status of a cell, a certain substrate had to be able to increase the growth rate of a growing culture compared to a control without addition of this substrate.

A pre-selection of potential substrates was made by comparing sugars present in musts and wines (Henick-Kling 1993; Bartowsky and Henschke 1995; Ribéreau-Gayon et al. 1998b) with those reported to be fermentable by wine LAB (Davis et al. 1988; Liu et al. 1995a). From this comparison, several sugars were chosen (Tab. 1, Appendix) and their effect on growth of one *Lactobacillus* and two oenococci was tested (see section 5.2 for experimental conditions). The results of this study (Tab. 1, Appendix) showed that only the addition of glucose, fructose or ribose resulted in an increase of the maximum growth rate ( $\mu_{\max}$ ) compared with a control with no sugar added. Thus, only these energy sources were considered further.

## 5.2. Experimental conditions

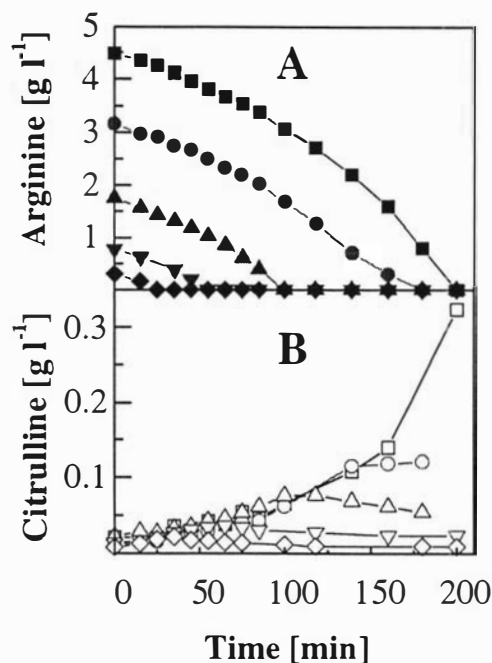
The protocol for resting cell experiments is described in section 2.3.2.1 and the analysis of substrates in section 2.4. For the pre-experiment, which investigated the effect of sugars on the growth of several wine LAB, bacteria were pre-grown in AMRS at pH 4.5 (2.2.2.5) and inoculated at 2% (v/v) into screw-cap spectrophotometer glass tubes (25 ml capacity, Pyrex 9826, U.S.A.) containing 10 ml of VJ medium (cp. 2.2.2.3), adjusted to pH 3.8, and different sugars added from stock solutions (10% (w/v)) to give 2 g l<sup>-1</sup>.

## 5.3. Results

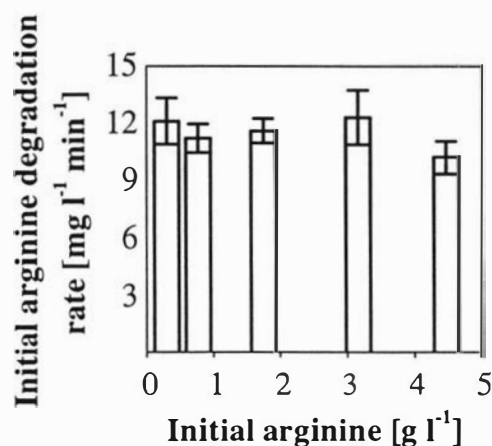
### 5.3.1. Effect of several arginine concentrations on arginine metabolism

Figure 5.1 A and B show citrulline excretion from arginine degradation by resting cells of *L. buchneri* CUC-3 at several initial arginine concentrations and pH 3.6. The arginine degradation pathway was already saturated at the lowest arginine concentration used, since the initial arginine degradation rate at 0.3 g l<sup>-1</sup> arginine was similar to those at higher arginine concentrations (Figure 5.2).

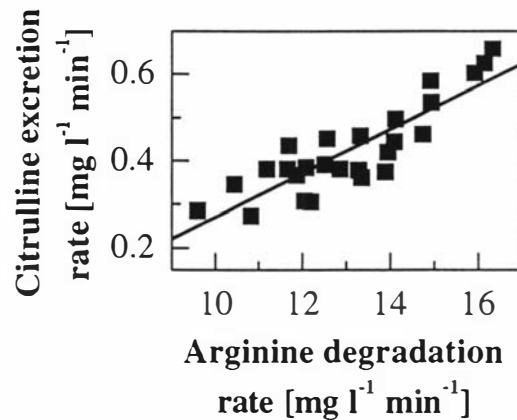
With the condition of a saturated degradation pathway met, all data from Fig. 1A and 1B could be used to investigate the correlation between the citrulline excretion and the arginine degradation rates. Figure 5.3 shows that there was indeed a good correlation. The slope of the function obtained through linear regression represents an arginine to citrulline conversion ratio of 0.05; that is, 5% (w/w) of the arginine degraded was excreted as citrulline.



**Figure 5.1** Arginine degradation and citrulline excretion by resting cells of *L. buchneri* CUC-3 at several initial arginine concentrations and  $4 \text{ g l}^{-1}$  of glucose (pH 3.6). Arginine (A, solid symbols) and citrulline (B, open symbols):  $4.5 \text{ g l}^{-1}$  arginine (■),  $3.2 \text{ g l}^{-1}$  (●),  $1.8 \text{ g l}^{-1}$  (▲),  $0.8 \text{ g l}^{-1}$  (▼),  $0.3 \text{ g l}^{-1}$  (◆).

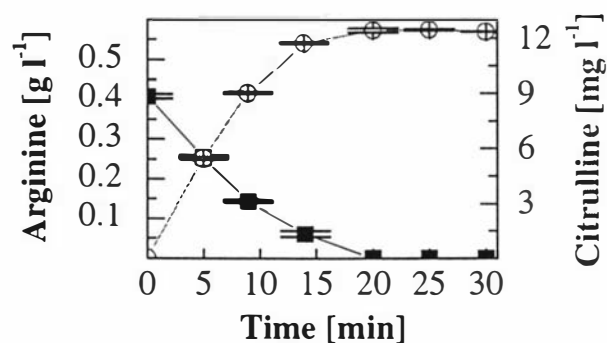


**Figure 5.2** Initial arginine degradation rates in resting cell experiments with *L. buchneri* CUC-3 and different arginine concentrations. Arginine degradation rates were calculated from linear regressions applied to data in Figure 5.1A. Error bars represent standard error of linear regressions.

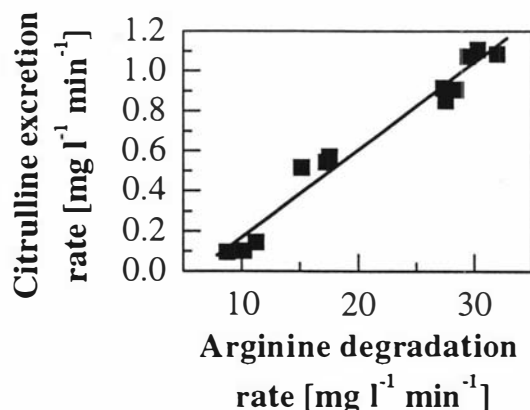


**Figure 5.3** Correlation of arginine degradation rates and citrulline excretion rates for *L. buchneri* CUC-3 calculated with data from Fig. 1A and 1B. Correlation coefficient  $r = 0.85$  ( $n = 28$ ). [Citrulline excretion rate] =  $-0.2(\pm 0.08) + 0.05(\pm 0.006) \times$  [arginine degradation rate]. Function obtained through linear regression (least squares method), standard error in parentheses.

Data from an experiment with resting cells of *O. oeni* Lo-111 (Figure 5.4) was used to examine the relationship between the arginine degradation rate and the citrulline excretion rate in oenococci, the common wine LAB used for inducing malolactic fermentation (MLF). A good linear relationship was found here, too (Figure 5.5). The arginine to citrulline conversion ratio calculated from the data (4.4%) was similar to that for *L. buchneri* CUC-3.



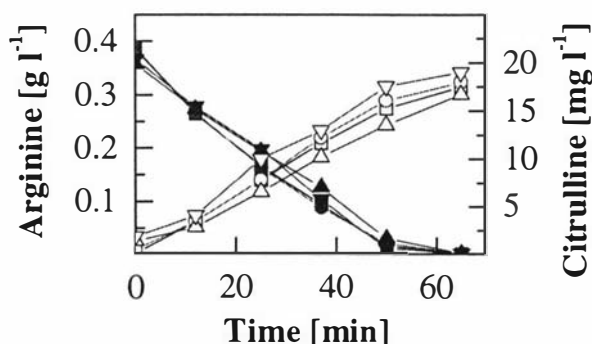
**Figure 5.4** Arginine degradation and citrulline excretion by resting cells of *O. oeni* Lo-111 at initial concentrations of  $0.4 \text{ g l}^{-1}$  arginine and  $0.4 \text{ g l}^{-1}$  glucose (mean values of triplicate rces; error bars represent the standard deviation). Arginine (■) and citrulline (○).



**Figure 5.5** Correlation of arginine degradation rates and citrulline excretion rates for *O. oeni* Lo-111 calculated with data from Figure 5.4. Correlation coefficient  $r = 0.98$  ( $n = 12$ ). [Citrulline excretion rate] =  $-0.27 (\pm 0.06) + 0.044 (\pm 0.003) \times$  [arginine degradation rate]. Function obtained through linear regression (least squares method), standard error in parentheses.

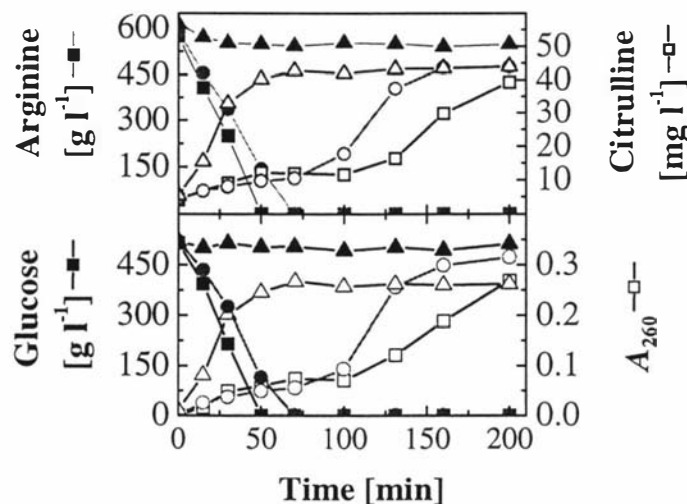
### 5.3.2. Effect of pH and ethanol on arginine metabolism

The course of arginine degradation by strain CUC-3 was similar in resting cell experiments at several pH values within the pH range of wine and no statistically significant difference was found between the conversion ratios at the pH values tested (at  $\alpha = 0.05$ , Figure 5.6). It was not possible to compare the arginine to citrulline conversion ratios from experiments with *O. oeni* at different pH values. Especially at low pH values, cell lysis caused release of intracellular citrulline which interfered with the measurement of citrulline from arginine degradation.



**Figure 5.6** Arginine degradation and citrulline excretion by resting cells of *L. buchneri* CUC-3 at several pH values. Arginine (solid symbols) and citrulline (open symbols): pH 3.3 (■), pH 3.5 (●), pH 3.7 (▲), pH 3.9 (▼). Average values of duplicate measurements shown. The arginine to citrulline conversion ratios (in % $\pm$ SD) calculated from linear regression of the arginine degradation and citrulline excretion rates were:  $4.1 \pm 0.31\%$  (pH 3.3),  $4.2 \pm 0.27\%$  (pH 3.5),  $4.0 \pm 0.26\%$  (pH 3.7),  $4.3 \pm 0.29\%$  (pH 3.9).

Figure 5.7 shows arginine degradation and citrulline excretion by resting cells of *O. oeni* MCW at several ethanol concentrations and pH 3.6. As shown by the course of the absorbance at 260 nm, a measure for cell lysis (cp. Chapter 4), 5% (v/v) ethanol caused faster lysis compared with the control, and 10% ethanol led to immediate lysis and deactivation of cell metabolism. However, in the experiments with 0 and 5% ethanol, the data up to  $t = 75$  min could be evaluated since lysis occurred later in these treatments. At 5% ethanol, both arginine and glucose were degraded slower than without ethanol. Accordingly, citrulline was excreted into the buffer at a lower rate. However, in both treatments (0 and 5% ethanol), the concentrations of citrulline in the medium after arginine depletion and before lysis occurred, were similar. This is also reflected in the arginine to citrulline conversion ratios, which had no statistically significant difference (at  $\alpha = 0.05$ , Figure 5.7).

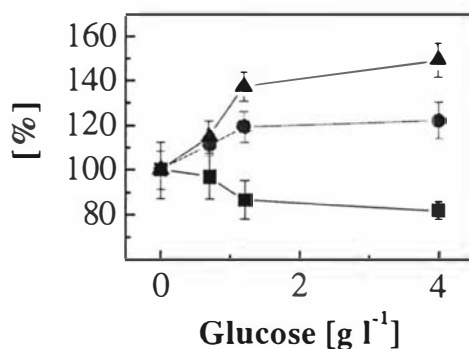


**Figure 5.7** Arginine degradation and citrulline excretion by resting cells of *O. oeni* MCW at several ethanol concentrations and 500 mg l<sup>-1</sup> initial arginine and glucose (pH 3.6). Arginine and glucose (solid symbols); citrulline and  $A_{260}$  (open symbols): 0% (v/v) ethanol (■), 5% (●), 10% (▲). Average values of duplicate measurements shown. The arginine to citrulline conversion ratios (in % $\pm$ SD) calculated from linear regression of the arginine degradation and citrulline excretion rates were: 1.19 $\pm$ 0.25% (0% ethanol) and 1.043 $\pm$ 01% (5% ethanol).

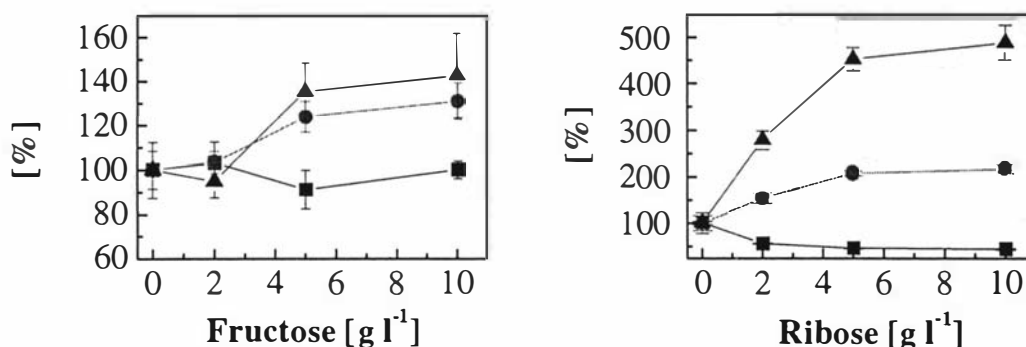
### 5.3.3. Effect of different carbohydrates at several concentrations on arginine metabolism

Resting cell experiments (rces) with *L. buchneri* CUC-3 showed that increasing initial concentrations of glucose, fructose and ribose inhibited arginine degradation (Figure 5.8 and Figure 5.9). Moreover, inhibition of arginine degradation correlated with higher citrulline excretion and thus, higher arginine to citrulline conversion ratios. This increase was most manifest with high ribose concentrations reflecting its greater ability to increase maximum

growth rate ( $\mu_{\max}$ ) and maximum growth yield ( $Y_{\max}$ ) in the growth experiments (Tab. 1, Appendix).



**Figure 5.8** Maximum arginine degradation (■) and citrulline excretion rates (●), and arginine to citrulline conversion ratios (▲) in rces with *L. buchneri* CUC-3 at several glucose concentrations (pH 3.6). Displayed are percent of value without added glucose, error bars display standard error.



**Figure 5.9** Maximum arginine degradation (■) and citrulline excretion rates (●), and arginine to citrulline conversion ratios (▲) in rces with *L. buchneri* CUC-3 at several fructose and ribose concentrations (pH 3.6). Displayed are percent of value without added sugar, error bars show standard error.

The effect of glucose on arginine degradation was also examined for *O. oeni* Lo-111. Glucose at several concentrations (0–3 g l<sup>-1</sup>) did not decrease the rate of arginine degradation by resting cells (Fig. 4, Appendix) although glucose was degraded during the incubation time. The arginine to citrulline ratio decreased slightly with increasing glucose concentrations, but this was attributable to citrulline release from cell lysis, which was highest in the assay with no glucose added (Fig. 4, Appendix). Because of the tendency of *O. oeni* strains to lysis in resting cells experiments, oenococci were not tested further.

## 5.4. Discussion

Granchi et al. (1998) recently published molar ratios of excreted citrulline per arginine degraded for several lactic acid bacteria (LAB) of wine origin. However, for this experiment, LAB were grown at pH 5. Clearly, this is not representative of the wine environment. Also, data for molar ratios was derived from fermentations where only three samples were taken and measured over a period of two weeks. Liu et al. (1995a) reported the importance of the pH on arginine degradation in wine LAB, and it has been shown that some LAB are able to reutilize excreted citrulline in wine and synthetic media (Chapter 4 and Liu et al. (1994)). Therefore, data about arginine to citrulline conversion ratios should be based on meticulous time course studies of the arginine metabolism under conditions similar to wine.

In this chapter, the kinetics of the arginine metabolism of resting cells of *L. buchneri* CUC-3 and *O. oeni* strains Lo-111 and MCW was investigated under different conditions in a tartrate buffer at wine pH. For strain CUC-3, it was found that the arginine degradation pathway was saturated already at the lowest arginine concentration tested. Additionally, citrulline excretion occurred also at minimal arginine degradation rates in both LAB. The rate of citrulline excretion during arginine degradation was correlated linearly with the degradation rate of arginine in both bacteria examined. If these results were confirmed in wine, they would have considerable importance: citrulline excretion would have to be expected in wines even where arginine degradation by LAB is slow. In addition, the linear correlation between arginine degradation rates and citrulline excretion rates would allow the prediction of the total amount of citrulline to be excreted into the wine after degradation of a certain amount of arginine. Arginine to citrulline conversion ratios calculated in this work from experiments with different conditions and strains ranged between 1.0-7.7% (w/w) and were similar to those calculated from data published by Liu et al. (1994) for *L. buchneri* CUC-3 and *O. oeni* OENO in a white wine (3.5 and 7.0%, respectively).

Studying the effect of several pH values and ethanol concentrations on the metabolism of arginine in resting LAB was difficult, since lysis of cells occurred early causing release of intracellular citrulline that interfered with the estimation of citrulline excreted from arginine degradation (cp. Chapter 4). However, where comparisons were possible, it was shown that low pH values and especially high ethanol concentrations, while causing slower arginine (and glucose) degradation, had no direct effect on the arginine to citrulline conversion ratio, itself. Low pH values and high ethanol concentration seemed to have an unspecific effect on general cell stability and metabolism rather than on specific pathways.

Carbon sources which increase the energy status of the cells repress the formation of the inducible ADI pathway enzymes, ADI and OTC (Poolman et al. 1987; Liu and Pilonne 1998) and thus decrease arginine degradation in growing cells. In this study with arginine induced resting cells, arginine degradation was inhibited by ribose, fructose and glucose in a *Lactobacillus* strain. The possibility that production of acids from sugar fermentation and subsequent decrease in medium pH inhibited arginine degradation is unlikely because the medium used was well buffered and pH variances between experiments with different initial sugar concentrations were under 0.1 pH units. Additionally, arginine degradation rates were similar in resting cell experiments (rces) with pH values ranging from 3.3 to 3.9. For *L. buchneri* CUC-3, the arginine to citrulline conversion ratios were augmented at increasing sugar concentrations, suggesting that excess ATP from sugar fermentation inhibited OTC or CK. ATP mediated inhibition of OTC has been in fact reported for the dairy LAB *Streptococcus lactis* (Thompson et al. 1990). In resting cells of an *Oenococcus* strain, however, no inhibition of arginine degradation by glucose could be detected. Therefore, it remains to be studied if inhibition of arginine degradation (either by enzyme inhibition or repression of enzyme formation) occurs in growing cells.

If an inhibition was present in growing cells, a potential way of reducing citrulline excretion in wine would be by conducting MLF in grape must containing high sugar concentrations (180-240 g l<sup>-1</sup>) instead of inoculating the bacteria after the completion of yeast alcoholic fermentation (AF). Indeed, some wine making techniques involve conducting MLF before or simultaneously with AF to avoid inhibition of LAB by alcohol or products of the yeast metabolism (Ribéreau-Gayon 1985). However, the advantage of a reduced citrulline excretion from partially inhibited arginine degradation could be cancelled out by higher arginine to citrulline conversion ratios that were found in experiments here with resting cells of *L. buchneri* for high sugar concentrations. A practical advantage would only exist, if arginine degradation was inhibited completely under the conditions found in grape must. The effect of high sugar concentrations on arginine degradation in wine is further considered in Chapter 8 in connection with a study of simultaneous AF and MLF.

Whereas the use of resting cell experiments described in this chapter was useful in gaining knowledge about kinetic parameters of the ADI pathway, the limitations of this method were clearly shown: the inhibitory effects of any treatments were limited to the effect on enzymes already present in the cells upon incubation. Also, a detailed examination of arginine kinetics by oenococci could not be carried out because of their proneness for lysis in the tartrate buffer. Therefore, studies on arginine degradation were continued with growing cells in wine.

## CHAPTER 6. GROWTH AND ARGININE METABOLISM OF WINE LACTIC ACID BACTERIA AT DIFFERENT PH VALUES, AND ARGININE AND SUGAR CONCENTRATIONS IN WINE

### 6.1. Introduction

After surveying the distribution of the arginine degradation capacity amongst wine lactic acid bacteria (Chapter 4) and further studies on the kinetics of arginine metabolism in resting cells (Chapter 5), it was necessary to evaluate the arginine metabolism of growing wine lactic acid bacteria (LAB) in wine. For this, two strains of *O. oeni* and one strain of *L. buchneri* were investigated in time course studies in wine at several pH values and arginine concentrations. The effect of the strains on arginine and citrulline concentrations and the relationship to malic acid degradation was investigated, as well as the effect of arginine on the growth of the wine LAB used.

Different arginine concentrations were used to examine whether the correlation between arginine degradation and citrulline excretion found in resting cells of *Lactobacillus* and *Oenococcus* also existed in growing cells. Although in resting cells of *L. buchneri* CUC-3 no effect of different pH values in the wine range was found (Chapter 5), several pH values were tested in this experiment to assess possible changes in metabolic activities as suggested by Liu et al. (1995a) that *Oenococcus* degraded arginine at pH 4 but not at pH 3.2. The effect of high residual glucose and fructose concentrations on arginine degradation was tested, as well, since experiments with resting cells (Chapter 5) showed an inhibition of arginine degradation by these sugars.

### 6.2. Experimental conditions

#### 6.2.1. Influence of several arginine concentrations and pH values on arginine metabolism of selected wine lactic acid bacteria

The preparation of the base wine is described in section 2.2.2.10. It had 9.4% (v/v) ethanol and no free SO<sub>2</sub> was detected by the method of Ripper as described by Amerine and Ough (1974). Glucose, fructose and malic acid concentrations were 20 mg l<sup>-1</sup>, 390 mg l<sup>-1</sup> and 1.2 g l<sup>-1</sup>, respectively. Ammonia, urea and arginine were present only in trace amounts. The pH after degassing was 3.2. The base wine was adjusted to 3 g l<sup>-1</sup> malic acid and 1.7 g l<sup>-1</sup> glucose, separated into 1 litre batches and adjusted to several pH values (pH 3.3, 3.6 and 3.9) with NaOH, and to several arginine concentrations (0, 0.5, 1 and 1.5 g l<sup>-1</sup>). After sterile filtration of the wine (2.1.3), 1 litre of wine was filled into glass bottles (1 litre, Schott

Duran, Germany), and MLF was induced as described in section 2.3.2.2. The initial population of the bacteria after inoculation was  $1.8 \times 10^6$ ,  $9.2 \times 10^5$  and  $7.8 \times 10^5$  cfu ml<sup>-1</sup> for strains CUC-3, MCW and Lo-111, respectively. The methods applied for analysis of the experiments are described in section 2.4. Specifically, the measurement of growth was carried out by OD and the concentrations of arginine and citrulline were determined colorimetrically. Some arginine concentrations were verified by the enzymatic method described in Chapter 3.

### 6.2.2. Influence of glucose and fructose degradation on arginine metabolism of *L. buchneri* CUC-3 in wine

The preparation of the base wine is described in section 2.2.2.10. The wine used here had 9.1% (v/v) ethanol and no free SO<sub>2</sub> was detected. Glucose, fructose and malic acid concentrations were 20 mg l<sup>-1</sup>, 900 mg l<sup>-1</sup> and 1.2 g l<sup>-1</sup>, respectively. Ammonia, urea and arginine were present only in trace amounts. The pH after degassing was 3.16. The base wine was adjusted to 3 g l<sup>-1</sup> malic acid and pH 3.5, and separated into 6 × 500 ml batches (3 treatments in duplicate). The control treatment had no further additions, whereas in the two remaining treatments, the glucose or fructose concentrations were adjusted to 20 g l<sup>-1</sup>. The final wine was sterile filtered (2.1.3) into 500 ml glass bottles, and MLF was induced with *L. buchneri* CUC-3 as described in section 2.3.2.2. Samples were taken periodically, centrifuged (10,000 g for 5 min) and the supernatant was frozen (-18°C) until analyzed. The methods applied for analysis of the experiments are described in section 2.4. Specifically, the measurement of growth was carried out by OD and the concentrations of arginine and citrulline were determined by HPLC.

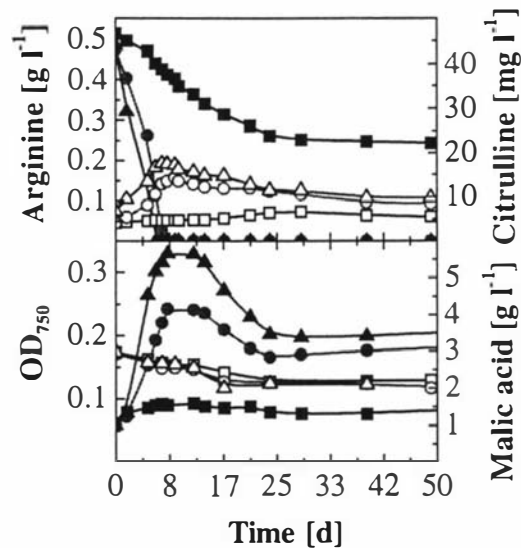
## 6.3. Results

### 6.3.1. Kinetics of arginine degradation at different pH values

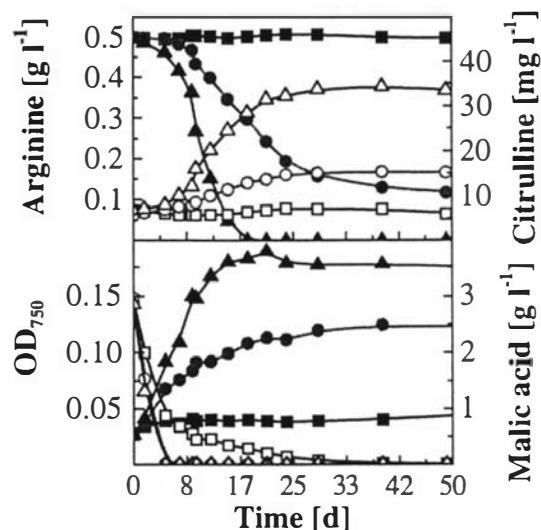
Figure 6.1 shows the time course of arginine and malic acid degradation and citrulline formation by *L. buchneri* CUC-3 at several pH values. Whereas degradation of malic acid was only partially achieved at all pH values, arginine was rapidly depleted at pH 3.9 and 3.6 and degraded to 50% at pH 3.3. Degradation of arginine and excretion of citrulline concurred with the increase in biomass, and citrulline was partially reutilized at the end of arginine degradation.

In contrast, *O. oeni* MCW depleted malic acid at all pH values tested (Figure 6.2). Arginine was fully consumed only at pH 3.9 after 19 days and degraded to 80% at pH 3.6 after 50 days. At all pH values, malic acid degradation was completed before significant degradation of arginine and excretion of citrulline occurred. At pH 3.3, where malic acid degradation was delayed, arginine was not degraded at all. Similar results were obtained with *O. oeni*

strain Lo-111, which depleted arginine at pH 3.9 within 25 days, but degraded only 20% at pH 3.6 after 37 days and none at pH 3.3 (Fig. 6, Appendix). As for strain MCW, malic acid degradation by Lo-111 was finished well ahead of arginine degradation and citrulline excretion. In contrast to *L. buchneri* strain CUC-3, citrulline was not reutilized during the incubation time by either oenococcal strain.



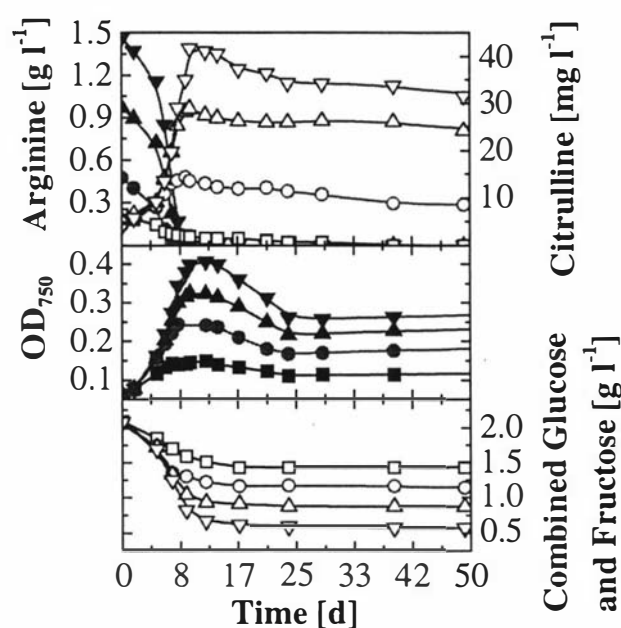
**Figure 6.1** Time course of arginine and malic acid degradation and citrulline formation by *L. buchneri* CUC-3 in wine with  $0.5 \text{ g l}^{-1}$  initial arginine at several initial pH values. Arginine and  $\text{OD}_{750}$ , solid symbols; citrulline and malic acid, open symbols. Initial pH values: 3.3 (■), 3.6 (●) and 3.9 (▲).



**Figure 6.2** Time course of arginine and malic acid degradation and citrulline formation by *O. oeni* MCW in wine with  $0.5 \text{ g l}^{-1}$  initial arginine at several initial pH values. Arginine and  $\text{OD}_{750}$ , solid symbols; citrulline and malic acid, open symbols. Initial pH values: 3.3 (■), 3.6 (●) and 3.9 (▲).

### 6.3.2. Arginine degradation at different arginine concentrations

Figure 6.3 shows arginine and sugar utilization, and citrulline formation by *L. buchneri* CUC-3 at several initial arginine concentrations. Higher initial arginine concentrations led to more rapid growth with the result of faster arginine degradation. This resulted in 1.5 g l<sup>-1</sup> of arginine being degraded in the same time as 0.5 g l<sup>-1</sup>. Arginine degradation rates and corresponding citrulline excretion rates from all experiments carried out with strain CUC-3 correlated well. A linear regression (method of least squares) performed with all the data sets (Fig. 5, Appendix) gave the following function: [Citrulline excretion rate] = -0.003 (±0.008) + 0.023 (±0.002) × [arginine degradation rate] (standard error in parentheses; correlation coefficient  $r = 0.91$ ; number of samples  $n = 41$ ). The slope of this function constitutes an arginine to citrulline conversion ratio (w/w) with a value of 2.3(±0.2)%. Likewise, arginine to citrulline conversion ratios were calculated from pooled data for both oenococci and were 3.8(±0.1)% ( $r = 0.96$ ;  $n = 78$ ) for strain MCW (Figure 6.4) and 3.9(±0.2)% ( $r = 0.96$ ;  $n = 45$ ) for strain Lo-111 (Fig. 7, Appendix).



**Figure 6.3** Time course of arginine and sugar utilization and citrulline formation by *L. buchneri* CUC-3 in wine at initial pH 3.6 and several initial arginine concentrations. Arginine and OD<sub>750</sub>, solid symbols; citrulline and combined glucose and fructose, open symbols. Initial arginine concentrations: 0 g l<sup>-1</sup> (■), 0.5 g l<sup>-1</sup> (●), 1 g l<sup>-1</sup> (▲), 1.5 g l<sup>-1</sup> (▼).

### 6.3.3. Effect of arginine concentrations on wine pH and growth

Table 6.1 shows the pH values after malic acid depletion (oenococci only) and at the end of incubations for all wine LAB at several arginine concentrations. Because of its ability to degrade arginine effectively, *L. buchneri* CUC-3 fermentations led to increasing pH values

at higher arginine concentrations. However, with the exception of the fermentations at 1.5 g l<sup>-1</sup> arginine, the final pH values achieved by strain CUC-3 were lower than those attained at the end of the incubation time by strains MCW and Lo-111.

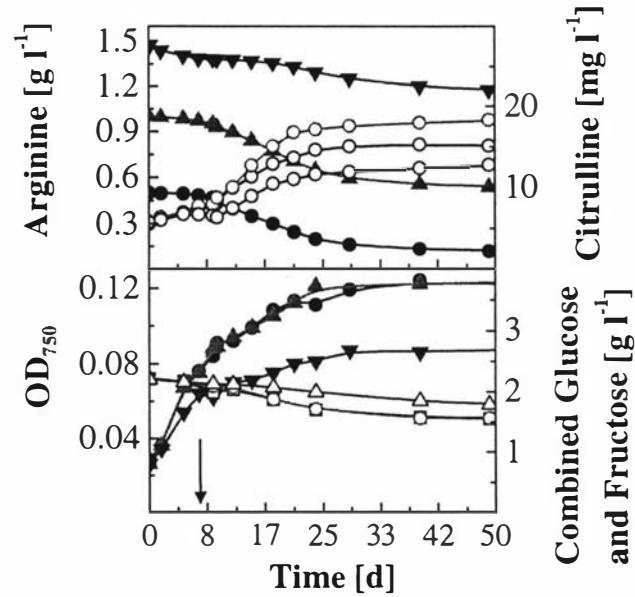
**Table 6.1** Wine pH values at the end of incubation of *L. buchneri* CUC-3 (49 days) and *O. oeni* strains MCW (49 days) and Lo-111 (37 days) at several initial arginine concentrations<sup>a</sup>

Strain	pH values <sup>b</sup> at initial arginine concentration [g l <sup>-1</sup> ]			
	0	0.5	1	1.5
CUC-3	3.61	3.7	3.84	3.93
MCW	(3.8) 3.88	(3.8) 3.90	(3.8) 3.94	(3.82) 3.87
Lo-111	(3.81) 3.84	(3.8) 3.85	(3.8) 3.85	(3.8) 3.85

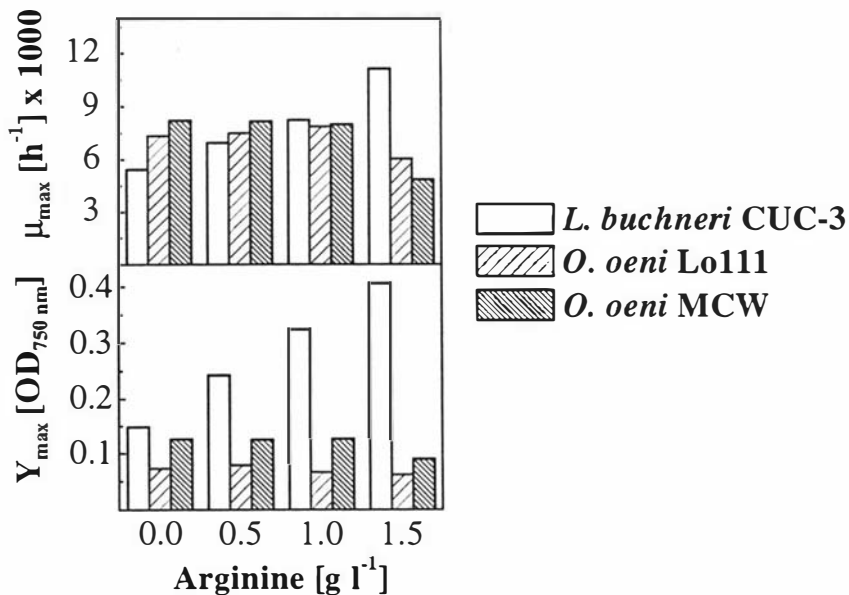
<sup>a</sup> Wine pH prior to MLF was pH 3.6.

<sup>b</sup> Values in brackets show pH values during incubation on completion of malic acid depletion (MCW, after 7.3 days; Lo-111, after 6.9 days).

With increasing initial pH of the wines, all cultures had higher maximum growth rate ( $\mu_{\max}$ ) and higher maximum growth yield ( $Y_{\max}$ ) values (Fig. 8, Appendix). At higher initial arginine concentrations, however, only *L. buchneri* CUC-3 displayed increased growth and faster degradation of fructose and glucose (Figure 6.3). The extent and duration of growth were determined by the arginine available, since growth ceased after arginine depletion even though fermentable hexoses were still present. By way of contrast, high initial arginine concentrations did not increase growth and degradation of glucose and fructose by *O. oeni* MCW. Additionally, growth inhibition was observed at the highest arginine concentration, 1.5 g l<sup>-1</sup> (Figure 6.4). If the sugar concentrations in *O. oeni* cultivations are examined separately, the results show that in all treatments glucose was degraded (decrease of 0.6 - 0.7 g l<sup>-1</sup>) whereas fructose concentrations rose slightly (increase of 0.1 - 0.2 g l<sup>-1</sup>). The same was found for *O. oeni* Lo-111, where sugar degradation was similarly uniform and growth was inhibited at 1 and 1.5 g l<sup>-1</sup> initial arginine (Fig. 7, Appendix). The  $\mu_{\max}$  and  $Y_{\max}$  data of all wine LAB for fermentations at several initial arginine concentrations is summarized in Figure 6.5. A biphasic growth pattern was observed for both *O. oeni* strains MCW (Figure 6.4) and Lo-111 (Fig. 7, Appendix) where growth continued for several days at a lower rate after depletion of malic acid.



**Figure 6.4** Time course of arginine and sugar utilization and citrulline formation by *O. oeni* MCW in wine at initial pH 3.6 and several initial arginine concentrations. Arginine and OD<sub>750</sub>, solid symbols; citrulline and combined glucose and fructose, open symbols. Initial arginine concentrations: 0.5 g l<sup>-1</sup> (●), 1 g l<sup>-1</sup> (▲), 1.5 g l<sup>-1</sup> (▼). Arrow shows time of malic acid depletion for all treatments.



**Figure 6.5** Maximum growth rate ( $\mu_{\max}$ ) and maximum growth yield ( $Y_{\max}$ ) for *L. buchneri* CUC-3 and *O. oeni* strains MCW and Lo-111 from MLFs at several initial arginine concentrations.

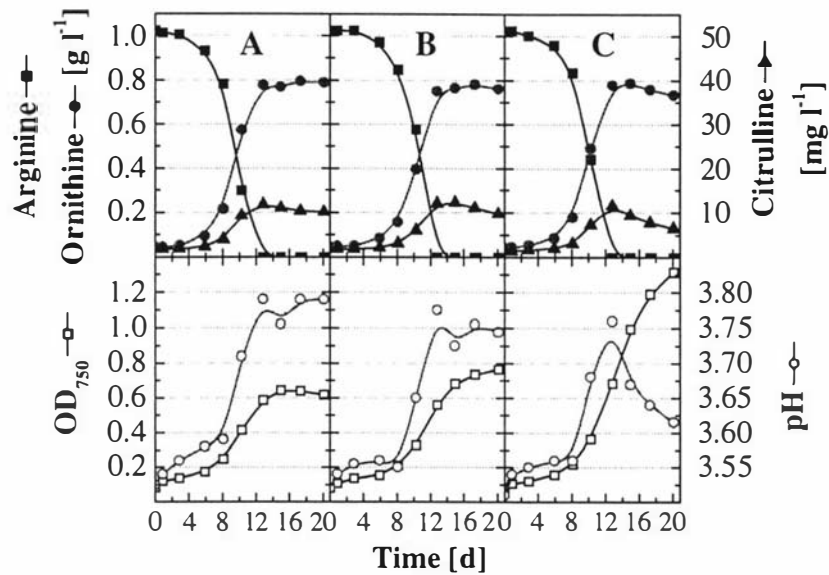
### 6.3.4. Influence of glucose and fructose degradation on arginine metabolism of *L. buchneri* CUC-3 in wine

For this experiment, only a *Lactobacillus* strain was considered, since the general aspect of arginine metabolism inhibition by sugar degradation was to be studied with a strong arginine degrading LAB. Degradation of sugars by oenococci was found to be very weak in wine (Figure 6.4, Fig. 7, Appendix), and degradation of arginine by oenococci could be avoided by other means (this Chapter and Chapter 7). Also, ribose was not considered in this experiment, since its concentration in wines is generally too low as to cause the inhibiting effects observed in section 5.3.3 (Henick-Kling 1993; Ribéreau-Gayon et al. 1998b).

MLF of *L. buchneri* CUC-3 in wines with glucose and fructose added is shown in Figure 6.6. Addition of 20 g l<sup>-1</sup> glucose or fructose to the wine led to increased growth yields compared to the control wine, especially in the case of fructose addition, which doubled the Y<sub>max</sub> of the control experiment during the duration of the experiment. Increased growth in the treatments with added glucose or fructose led to lower pH values, presumably because of the formation of acids from greater sugar degradation. No obvious differences in the kinetics of arginine degradation or citrulline excretion or the amount of excreted citrulline were detectable, but citrulline was reutilized to a greater extent in treatments where sugar degradation permitted ongoing growth after arginine degradation, an observation which was confirmed for the reutilization of ornithine. Therefore, the lowest residual citrulline and ornithine concentrations achieved by the end of the incubation period were found in the treatment with added fructose. The arginine to citrulline conversion ratios shown in Table 6.2 were calculated by linear regression of the respective arginine degradation and citrulline excretion rates (data taken from Figure 6.6; degradation and excretion rates visualized in Fig. 9, Appendix). There was no considerable difference in ratios between the treatments (differences statistically not significant at  $\alpha = 0.05$ ).

**Table 6.2** Arginine to citrulline conversion ratios (w/w) calculated from linear regression of arginine degradation and citrulline excretion rates (Fig. 9, Appendix) of growth data from *L. buchneri* CUC-3 in wine (Figure 6.6) with addition of glucose or fructose at 20 g l<sup>-1</sup>.

Treatment	<i>n</i>	<i>r</i>	% (w/w) Conversion Ratio (±SE)
Control without sugar	6	0.989	1.13 (±0.083)
+Glucose (+20 g l <sup>-1</sup> )	6	0.997	1.07 (±0.035)
+Fructose (+20 g l <sup>-1</sup> )	6	0.990	1.10 (±0.077)
Combined	18	0.991	1.10 (±0.035)



**Figure 6.6** MLF by *L. buchneri* CUC-3 in wine (pH 3.5) with several hexoses. A, control without added sugar; B, with glucose added (20 g l<sup>-1</sup>); C, with fructose added (20 g l<sup>-1</sup>). Arginine (■), ornithine (●), citrulline (▲), OD at 750 nm (□), and pH (○).

## 6.4. Discussion

The arginine metabolism of two oenococcal strains and one *Lactobacillus* strain was investigated under laboratory winemaking conditions. All strains were able to degrade arginine in wine and to excrete considerable amounts of citrulline, underpinning the need to control arginine degradation by wine lactic acid bacteria (LAB). However, differences were found in the minimum pH necessary for degradation of arginine and the kinetics of its degradation.

Liu et al. (1995a) showed that arginine was degraded by oenococci at pH 4 but not pH 3.2 in a synthetic wine. In this study, oenococci were able to degrade arginine at pH 3.9 and partially at pH 3.6, but no degradation occurred at pH 3.3. In addition to the higher minimum pH required, arginine degradation by oenococci was delayed in comparison with malic acid degradation. In contrast, *L. buchneri* CUC-3 degraded arginine at all pH values tested. In practice, this allows the winemaker to avoid arginine degradation by carefully monitoring malic acid degradation and inhibiting bacterial activity after malolactic conversion by pure cultures of *Oenococcus*. This may also be desirable from a sensory point of view, since the concentrations of diacetyl, an important flavour compound produced by wine LAB, has been reported to be highest at the end of malolactic conversion (Nielsen and Richelieu 1999; Bartowsky and Henschke 2000). As well, immediate inactivation of bacterial action after complete malolactic conversion has been recommended to limit the

formation of biogenic amines, degradation products of amino acids with health concerning implications (Soufleros et al. 1998).

As in studies with resting cells (Chapter 5), a linear proportionality was found between arginine degradation and citrulline excretion rates in growing cultures in wine. Arginine to citrulline conversion ratios were calculated that ranged between 2.2 and 3.9% (w/w). These ratios are important, since they allow estimation of the potential addition to the EC precursor pool by citrulline from a given amount of initial arginine. Additionally, ratios could be used for the comparative assessment of the strain-specific risk of citrulline excretion.

It is remarkable that the arginine to citrulline conversion ratios found for both oenococci were over 50% higher than those calculated for *L. buchneri* CUC-3. In a *Mycoplasma* strain, arginine was converted largely to ornithine only during rapid growth. When growth ceased in the presence of excess arginine, on the other hand, arginine degradation continued but citrulline, rather than ornithine, was the predominant product (Barile et al. 1966). These findings are similar to observations made in this experiment since arginine degradation by oenococci occurred in the late log/early stationary phase, at least at pH 3.6 (Figure 6.4 and Fig. 7, Appendix). The growth phase in which arginine degradation takes place could thus play a role in the higher citrulline production by oenococci.

Although *L. buchneri* CUC-3 effectively degraded arginine, it led only to a moderate pH increase because arginine degradation favoured acid formation from sugar degradation. Additionally, strain CUC-3 was able to reutilize some of the excreted citrulline, whereas citrulline reutilization by both oenococci was insignificant or not present, confirming results from studies done by Liu et al. (1994; 1996). Nonetheless, strain CUC-3 would not be the preferred wine LAB to induce malolactic fermentation (MLF), since sugar degradation by heterofermentative LAB leads to the formation of variable amounts of acetic acid which can render a wine unacceptable from a sensory viewpoint or can exceed legal limits for acetic acid. Moreover, malic acid was not degraded efficiently by strain CUC-3. This further supports the preference for the use of oenococci in conducting MLF. Oenococci degraded sugars only marginally and both the degradation of arginine and the resulting pH rise could have been avoided by stopping further microbial activity after complete malolactic conversion.

In experiments with resting cells (cp. Chapter 4), both lactobacilli and oenococci were able to reutilize citrulline previously excreted, but the degree of reutilization depended on the susceptibility of the cells to lysis, and reutilization was weaker in oenococci (cp. Figure 4.1 and Figure 4.2). In wine, only a *Lactobacillus* was able to reutilize citrulline to some extent,

whereas oenococci did not or only marginally (strain MCW at pH 3.9, Figure 6.2), and this confirms results obtained by Liu et al. (1994). In resting cell experiments (cp. Figure 4.1), degradation of citrulline always occurred at a lower rate than arginine degradation by bacteria from the same cell suspension. This may be attributable to the lack of a transport system for citrulline, as suggested by Poolman et al. (1987). As a result, intracellular citrulline concentrations may be too low to drive the unfavourable ornithine transcarbamylase reaction (cp. 1.4.4) and this could lead to growth cessation and loss of viability, leaving residual citrulline in the medium. Accordingly, the degree of citrulline reutilization would depend on the biomass density and the general condition of the cells in wine. In fact, the cell density in fermentations with strain CUC-3, where some citrulline was reutilized, was higher than in fermentations with oenococci. Further, data shown in Figure 6.6 B and C demonstrates that considerable reutilization of citrulline was possible when the medium continued to support growth after depletion of arginine. Reutilization of citrulline by oenococci, therefore, seems to be insignificant, presumably because of low biomass densities and a rapid loss of metabolic activity after malic acid depletion.

Some preliminary results by Liu (1993) on the effect of arginine on growth of wine LAB in a defined medium, indicated that heterofermentative wine LAB (two lactobacilli and one *Oenococcus*) might be able to utilize energy derived from arginine metabolism for growth. Also, Tonon (2000) recently stated that *Oenococcus* was able to couple energy formation from the degradation of arginine to growth. The results shown here differ from the cited works in so far as only *L. buchneri* CUC-3 was able to increase the maximum growth yield ( $Y_{\max}$ ) at higher initial arginine concentrations, suggesting effective energy coupling of arginine degradation to growth in wine. Both oenococci were not able to increase  $Y_{\max}$  under these conditions. Besides, oenococci were inhibited by the higher arginine concentrations used, whereas no inhibition was observed for *L. buchneri* CUC-3. Inhibition of LAB growth by high arginine concentrations has been reported in the past. Manca de Nadra et al. (1981) found growth inhibition of a *L. buchneri* strain of dairy origin by arginine at concentrations that exceeded  $5 \text{ g l}^{-1}$  and Liu (1993) observed prolonged lag phases for two lactobacilli under similar conditions. It has been suggested for *Streptococcus lactis* (now *Lactococcus lactis* subsp. *lactis*) that high arginine concentrations reduce growth by inhibiting the uptake of lysine that is also transported by the arginine-ornithine antiport system (Thompson 1987). A similar mechanism could be responsible for the inhibition found in the present work. Although growth inhibition by high arginine concentrations is not likely to be important because of the rarity of this occurring in wine, the inability of oenococci to use arginine to increase growth in wine could be oenologically significant. That is, non-arginine degrading oenococci could be used for inducing MLF without the risk of being overgrown by undesirable arginine positive strains. However, regarding the contradictory results obtained from experiments described in this chapter and in other

studies, a detailed study of the energetic aspect of arginine degradation in wine clearly remains to be done. The results from this study are presented in Chapter 9 of this thesis.

Growth of *L. buchneri* CUC-3 was clearly driven by the presence of arginine in wine, but growth of both oenococci correlated with malic acid degradation. Significant arginine concentrations remained in oenococcal cultures entering stationary phase and therefore, low arginine degradation rates were more likely a result of growth cessation than a reason for it. However, arginine degradation by oenococci may be beneficial in maintaining some cell growth for a limited time, since a biphasic (diauxic) growth pattern was observed for both strains. Malolactic conversion has been reported to contribute to the acid tolerance of wine LAB at low pH values present in wine (Tourdot-Maréchal et al. 1999). The data presented here suggests that whereas oenococci increase their acid tolerance by the degradation of malic acid, *L. buchneri* CUC-3 achieves this more efficiently by degrading arginine as has been shown for several LAB of non-wine origin (Marquis et al. 1987).

Liu and Pilone (1998) reported that arginine and fructose were degraded sequentially by several strains of wine LAB (*L. buchneri*, *L. brevis* and *O. oeni*) and suggested that fructose may inhibit the uptake or degradation of arginine. Sugar repression of the ADI pathway in many micro-organisms of non-wine origin has also been reviewed (Liu and Pilone 1998). However, the experiments carried out by Liu in a synthetic medium might not be representative of the wine environment. For example *O. oeni* degraded over 5 g l<sup>-1</sup> of glucose in the original experiments described by Liu (1993), whereas normally, oenococci have a very low glucose or fructose consumption, at least during the degradation of malic acid (cp. Figure 6.4, Fig. 7, Appendix and Lafon-Lafourcade (1979) and Bréchet et al. (1974)). Also, the synthetic medium used did not possess the buffer capacity of wine: although initial pHs were representative of wine (pH 3.5), they rose to over 4.5 during fermentations and were probably partly responsible for the high sugar consumption. Thus, the influence of high sugar concentrations on arginine degradation in growing oenococci is probably limited to metabolic activity during late fermentation stages (after malic acid depletion), conditions that should be avoided to reduce the risk of arginine degradation. In the case of lactobacilli, the inhibition by sugars observed was much weaker than for oenococci (Liu 1993) and in this thesis work done in wine, neither glucose nor fructose affected arginine metabolism in growing cells of *L. buchneri* CUC-3 at pH 3.5. It appears that the results obtained from resting cells that indicated an effect of sugars (Chapter 5) are not easily applicable. Whereas sheer metabolic abilities of wine LAB can be rapidly assessed with resting cell experiments, the actual metabolic capacities *in vivo* clearly have to be examined in wine.

Besides citrulline, *L. buchneri* CUC-3 reutilized ornithine after depletion of arginine in wine. While it is possible that ornithine was used in an anabolic pathway by strain CUC-3, it is rather unlikely given that arginine has been found to be essential in many wine LAB (1.4.1). Also, the first enzymatic reaction of the ADI pathway is irreversible (cp. Figure 1, inside back cover) and no reports have been published about the presence in wine LAB of urea-cycle enzymes necessary to synthesize arginine from ornithine (i.e. argininosuccinate synthetase and argininosuccinate lyase). Therefore, it is probable that ornithine was decarboxylated to putrescine as has been shown for strains of *Lactobacillus* isolated from wine and orange juice (Arena and Manca de Nadra 2001). The formation of the biogenic amine putrescine from ornithine would present another negative aspect of the ADI pathway, next to the formation of citrulline and ammonia, and the possible energetic advantage. That is, wine LAB that do not possess the ADI pathway enzymes and are not able to produce putrescine directly from arginine (over agmatine; see Figure 1, inside back cover) could still form putrescine from the decarboxylation of ornithine, which was produced by the ADI pathway of other wine LAB. Therefore, the reutilization of ornithine by *L. buchneri* CUC-3 will require further clarification.

From the results presented in this chapter, it can be concluded that it is possible to reduce the risk of formation of citrulline by wine LAB in wines with high residual arginine concentrations by carrying out MLF with pure oenococcal cultures and precise determination of complete malolactic conversion, followed by inhibition of bacterial activity. To verify the results about the kinetics of arginine degradation by oenococci, further MLFs were carried out with commercial wine LAB under practical conditions. This study is described in Chapter 7.

## **CHAPTER 7. IMPACT OF COMMON WINEMAKING TECHNIQUES ON ARGININE METABOLISM BY DIRECT INOCULATED COMMERCIAL STRAINS OF *OENOCOCCUS OENI* IN CHARDONNAY WINE**

### **7.1. Introduction**

In Chapter 6, the metabolism of arginine by lactic acid bacteria (LAB) was investigated in wine. The oenococcal strains used in Chapter 6 are commercially available, but they were pregrown in a high arginine containing medium to induce ADI pathway enzymes, and the wine used was made from a retail grape juice.

For the experiment presented here, the results obtained in the previous chapter were verified under more practical conditions. The wine used was fermented from Chardonnay grape juice originating from a commercial vineyard and malolactic fermentation (MLF) was induced with recently developed commercial strains for direct inoculation, following the manufacturer's recommendations. Moreover, two treatments were chosen to reflect common winemaking techniques that may have an impact on arginine metabolism.

Natural or spontaneous yeast alcoholic fermentations (AF) are common in 'old-world' winemaking (Europe) and gaining popularity amongst some wine makers in the 'new-world'. e.g. U.S.A., Australia and New Zealand (Eglinton et al. 2000). Some wine makers believe that spontaneous yeast AFs help in creating unique wines with higher complexity, depth and more diverse aromas (Goldfarb 1994; Price 1996; Ross 1997; McCorkindale 1999), although other reports indicate that wines made with spontaneous fermentations had similar or worse sensory properties than those fermented with commercial preparations of freeze-dried yeasts (Gaia and Matta 1984; Bach 1985; Bader 1997; Lorenzini 1999). In any case, differences in the management of AF will lead to wines with a distinct chemical composition, regarding the content of nutrients as well as substances with a potential inhibiting effect on wine LAB growth. The difference in chemical composition of wines may not only affect growth of LAB, but could change the kinetics of arginine degradation, itself.

Storing wines on yeast lees (sedimented yeast cells) after AF is also an important technique in the making of wines, particularly in the production of white and sparkling wines (e.g. Méthode Champenoise sparkling wines), and has a significant effect on the sensory character of the wines that are allowed to rest on lees (Charpentier and Feuillat 1993; Price 1996). The yeast lees have been shown to stimulate wine LAB growth (Lonvaud-Funel et al.

1988). Above all, they constitute a major pool of proteins, capable of releasing amino acids into the wine (Stuckey et al. 1991; Ribéreau-Gayon et al. 1998a; Martinez-Rodriguez and Polo 2000). This release is caused first by yeast cell lysis and later by proteolytic activity of residual yeast enzymes (Leroy et al. 1990; Charpentier and Feuillat 1993; Sato et al. 1997). Furthermore, it was shown recently that several wine LAB produce exoproteases, capable of breaking down wine polypeptides into free amino acids, and in particular, arginine (Manca de Nadra et al. 1997; Manca de Nadra et al. 1999). Therefore, the yeast lees represent a potential and significant arginine reservoir after completion of AF and may influence the kinetics of arginine degradation by wine LAB, as well.

In order to study the effect of both techniques presented on the arginine metabolism of commercial wine LAB, the wines used for MLF were produced either by a natural yeast fermentation or by inoculation with commercial freeze dried yeast; and MLF was carried out either in wine containing yeast lees or in wines racked-off the lees.

## 7.2. Experimental conditions

The grape must used for this experiment was obtained from a vineyard in the Fernhill area of Hawke's Bay, New Zealand. The Chardonnay grapes were handpicked at 22 Brix and 30 mg kg<sup>-1</sup> SO<sub>2</sub> was added upon crushing. The must used was free run juice obtained after 45 minutes of skin contact and had a pH of 3.22. The must was separated into two 10 l batches and a temperature controlled AF was carried out at 13°C. One batch was not inoculated with yeast and allowed to ferment by the natural yeast flora, whereas the second was inoculated with 2% (v/v) *S. bayanus* PC, pre-grown in the same but filtered grape juice with 1 g l<sup>-1</sup> yeast extract added.

After completion of AF, the wines were cold-settled for 48 h. The composition of both wines after AF and cold stabilization is shown in Table 7.1. For use in experiments, the only modification was the addition of arginine to increase the concentration in both batches to 750 mg l<sup>-1</sup>, followed by repeated cold-settling of the wines. Then, 5 l of each batch were racked-off and transferred into another container. The remaining 5 l in the original carboys were used as yeast lees treatment. From each of the four batches, 4x1 l aliquots were taken and transferred into 1 l glass bottles for induction of MLF with 2 commercial freeze-dried strains (strains EQ54 and VFO, in duplicate; yeast lees batches were stirred before the division).

The inoculations were carried out according to the manufacturer's recommendation with a modification. The inoculation size was doubled to compensate for the high ethanol content of the wine and potential loss of viability of the starter culture. The growth of bacteria

during MLF was not monitored regularly, but verified at certain times during cultivation by viable cell counts (upon inoculation and  $t = 8, 20$  and  $35$  days). From an initial inoculum of  $9.2 \times 10^5$  and  $2.3 \times 10^6$  cfu ml<sup>-1</sup>, up to  $3.2 - 7.2 \times 10^7$  cfu ml<sup>-1</sup> were formed. Samples were taken periodically, centrifuged ( $10,000 g$  for  $5$  min) and the supernatant was frozen ( $-18^\circ\text{C}$ ) until analyzed. The analysis of arginine, citrulline and malic acid are described in 2.4.4. Specifically, arginine and citrulline were determined by HPLC (2.4.4.5).

**Table 7.1** Characterization of Chardonnay wines after natural AF or AF by *S. bayanus* PC at  $13^\circ\text{C}$ .

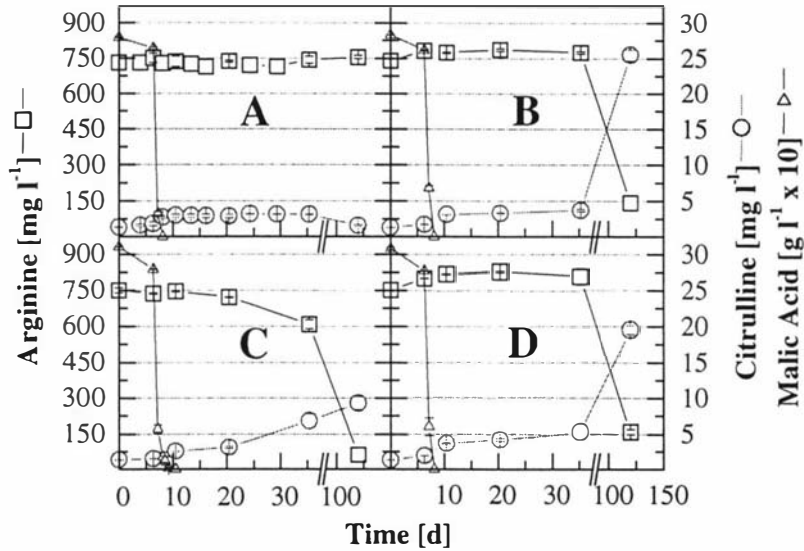
Compound	Type of AF	
	PC	Natural
Ethanol	14.1 % (v/v)	13.9 % (v/v)
pH <sup>a</sup>	3.41	3.42
Malate	3.03 g l <sup>-1</sup>	2.7 g l <sup>-1</sup>
Glucose	80 mg l <sup>-1</sup>	110 mg l <sup>-1</sup>
Fructose	0.882 g l <sup>-1</sup>	2.06 g l <sup>-1</sup>
Arginine	10 mg l <sup>-1</sup>	20 mg l <sup>-1</sup>
Urea	< 0.1 mM	< 0.1 mM
Ammonia	< 0.1 mM	< 0.1 mM
free SO <sub>2</sub>	n.d. <sup>b</sup>	n.d. <sup>b</sup>

<sup>a</sup> after degassing

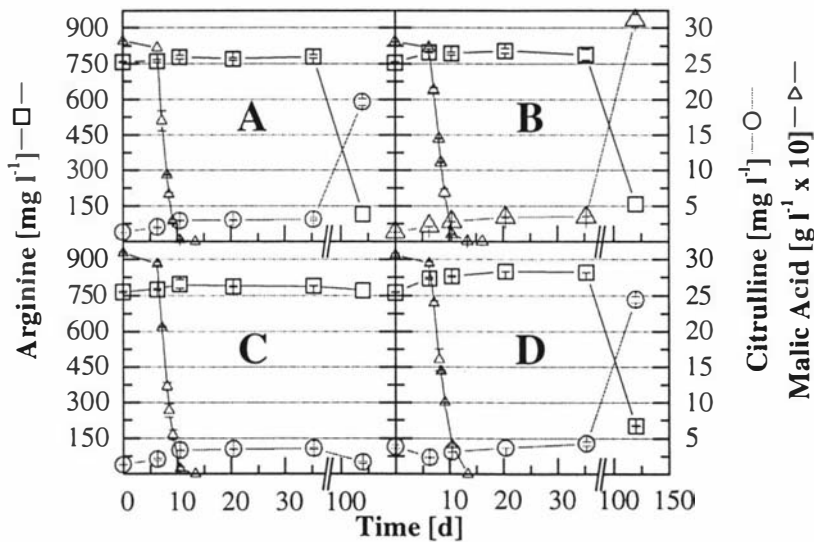
<sup>b</sup> none detected

### 7.3. Results

Figure 7.1 and Figure 7.2 show MLF by *O. oeni* strains EQ54 and VFO under several conditions as described in section 7.2. Arginine degradation by both strains was not noticeable or greatly delayed in comparison with the degradation of malic acid in all treatments. During the entire duration of the experiment, arginine degradation did not occur in only two treatments without yeast lees: after natural AF and MLF by strain EQ54 (Figure 7.1, A), and after AF by *S. cerevisiae* PC and MLF by strain VFO (Figure 7.2, C). In treatments with yeast lees, the arginine concentrations increased after inoculation with both *O. oeni* strains EQ54 and VFO compared to respective treatments without yeast lees. Equally, the citrulline concentrations found at the end of incubation periods were higher in yeast lees treatments than in the respective treatments without yeast lees.



**Figure 7.1** MLF by *O. oeni* EQ54 in Chardonnay wine. A, wine from natural AF; B, wine from natural AF with remaining yeast lees; C, wine from AF by *S. bayanus* PC; D, wine from AF by PC with remaining yeast lees. Arginine ( $\square$ ), citrulline ( $\circ$ ), and malic acid ( $\triangle$ ). Error bars represent standard error.



**Figure 7.2** MLF by *O. oeni* VFO in Chardonnay wine. A, wine from natural AF; B, wine from natural AF with remaining yeast lees; C, wine from AF by *S. bayanus* PC; D, wine from AF by PC with remaining yeast lees. Arginine ( $\square$ ), citrulline ( $\circ$ ), and malic acid ( $\triangle$ ). Error bars represent standard error.

Arginine to citrulline conversion ratios were calculated by using the difference between arginine and citrulline concentrations at the beginning and end of experiments (Table 7.2). In treatments where arginine degradation occurred, the remaining amount of arginine was rather homogenous. Therefore, higher final citrulline concentrations led to higher arginine to citrulline conversion ratios.

**Table 7.2** Arginine to citrulline conversion ratios (% w/w) from MLFs with *O. oeni* EQ54 and VFO in Chardonnay wine (Standard error in parentheses).

Treatment	<i>O. oeni</i> EQ54	<i>O. oeni</i> VFO
Natural AF <sup>a</sup>	n.a. <sup>c</sup>	2.7 (±0.22)%
Natural AF, lees <sup>b</sup>	3.7 (±0.15)%	4.6 (±0.29)%
AF by <i>S. bayanus</i> PC	1.1 (±0.23)%	n.a.
AF by <i>S. bayanus</i> PC, lees	2.7 (±0.18)%	3.4 (±0.18)%

<sup>a</sup> AF<sup>b</sup> MLF carried out in wine containing yeast lees<sup>c</sup> not applicable, no arginine degradation

## 7.4. Discussion

In this Chapter, the potential of common wine making techniques to increase carcinogenic ethyl carbamate precursor citrulline formation during malolactic fermentation (MLF) was investigated. Particularly, the realization of MLF on the yeast lees was considered in view of the possible role of yeast lees in supplying free arginine to wine lactic acid bacteria (LAB). In fact, Rollán et al. (1993) demonstrated extracellular proteolytic activities in four strains of *O. oeni* isolated from wine. The exoproteases degraded polypeptides in white wine (Manca de Nadra et al. 1997) and red wine (Manca de Nadra et al. 1999), releasing predominately arginine into the medium along with other amino acids. According to these studies, exoproteases could be a common feature of *O. oeni* and thus, growth and proteolytic activity of *O. oeni* would have the potential to increase the concentration of free arginine in wines. Sponholz et al. (1991) reported that white wines stored on yeast lees did not have increased ethyl carbamate concentrations compared with wines racked-off the lees. In the same publication, however, Sponholz et al. (1991) reported that wines after MLF always had higher ethyl carbamate concentrations than those without. Therefore, it seems likely that the white wines with yeast lees analyzed by Sponholz et al. did not undergo MLF.

In this experiment, when comparing fermentations where arginine degradation occurred, wines containing yeast lees always had higher citrulline concentrations than wines racked-off the lees. Specifically, the arginine to citrulline ratios were 145% higher when comparing wines fermented with *S. bayanus* PC and MLF by strain EQ54; and 70% higher when comparing naturally fermented wines and MLF by strain VFO. This demonstrates the potential of the yeast lees to increase ethyl carbamate precursor citrulline formation during MLF by increasing the availability of free arginine for degradation by wine LAB.

As well, wines produced by natural AF had higher arginine to citrulline ratios than wines fermented with the commercial yeast strain. This resulted in a 37% higher ratio when

comparing MLF of strain EQ54 in wines with yeast lees; and a 35% higher ratio when comparing MLF of strain VFO in wines with yeast lees. The reason for the increased citrulline formation is not clear, since all wines were adjusted to the same arginine concentration prior to inoculation with malolactic bacteria. Moreover, the increase in arginine concentration during the first days of MLF (presumably due to proteolytic activity) was higher in the wines fermented by the commercial yeast preparation. The difference observed might come from the direct effect of an unknown substrate formed during natural fermentation on the regulation of arginine metabolism, leading to a greater accumulation of citrulline. It is possible, too, that a change in arginine metabolism was caused by growth differences which in turn depend on the medium composition. However, the data collected from viable cell counts carried out at different stages of incubations did not support this suggestion and therefore, this aspect remains to be studied in future work.

Finally, strain specific differences in the ability to form citrulline by the wine LAB used were found. Compared with strain EQ54, the arginine to citrulline ratios of strain VFO were 24% and 26% higher in wines with yeast lees fermented either by *S. bayanus* PC or by natural fermentation.

From the results of this experiment, it can be concluded that the presence of yeast lees increases the citrulline formation potential of MLF and this effect is suggested for wine produced from natural AF, as well. Strain specific differences in the ability to excrete citrulline seem to exist and therefore, studies of the citrulline formation potential may be included in the selection of starter bacteria. The results obtained confirm conclusions made in Chapter 6: arginine degradation by oenococci is avoidable by inactivation of the bacteria after malic acid depletion. It is not possible to assess when degradation of arginine exactly took place because between the sample taken at  $t = 35$  days and  $t = 120$  days no other samples were taken and analyzed. However, it is clear that apart from the treatment with strain EQ54 in wine fermented by *S. bayanus* PC (treatment without yeast lees), where arginine degradation commenced 5 to 10 days after depletion of malic acid, the delay between malic acid depletion and the beginning of arginine degradation was approximately 20 days.

It is not known why arginine was not degraded in two of the treatments. The metabolism of the bacteria may have been inhibited by the prevailing conditions after MLF. It is also possible that arginine degradation was only delayed in these treatments and would have been observed later on. In any case, the results suggest that it is unsafe to allow bacterial metabolism to occur freely in wines after complete malolactic conversion. Compared with the experiments described here, where wines were sterile filtered before inoculation with commercial oenococci, the danger of arginine degradation is greater in practical winemaking all the more, considering that naturally occurring wine LAB may be active, too.

## CHAPTER 8. EFFECT OF INOCULATION TIME ON ARGININE METABOLISM IN COMMERCIAL STRAINS OF *OENOCOCCUS OENI*

### 8.1. Introduction

Experiments presented in Chapter 5 showed that arginine degradation by resting cells of *L. buchneri* CUC-3 was inhibited at concentrations of 2 - 5 g l<sup>-1</sup> of ribose, fructose or glucose in the buffer. This suggested further work to examine if malolactic fermentation (MLF) in high-sugar containing media, like grape must (180 - 300 g l<sup>-1</sup>) or semi-sweet or sweet wine (> ~8 g l<sup>-1</sup>) would lead to decreased arginine degradation. Experiments in Chapter 6, however, indicated that the presence of 20 g l<sup>-1</sup> of glucose or fructose in wine did not influence the degradation of arginine or the arginine to citrulline ratio of *L. buchneri* CUC-3. In addition, it was demonstrated that arginine degradation by oenococci, the microorganisms commonly used to induce MLF, was preventable by inactivating wine lactic acid bacteria (LAB) after completion of malic acid degradation (Chapter 6, Chapter 7). Nevertheless, MLF by oenococci in high sugar containing media (> 2 g l<sup>-1</sup>) has not been examined. Therefore, the current chapter presents results of the arginine metabolism in commercial oenococci during simultaneous alcoholic and MLF in grape must. Grape must was used as the high-sugar containing medium as it is the starting point of any wine, whereas wines with high residual sugar concentrations are not usually allowed to undergo MLF. In addition to the inoculation with commercial strains of *O. oeni*, the grape must was also inoculated with freeze-dried yeasts because spontaneous alcoholic fermentation (AF) by yeasts naturally present in grape must would be expected; thus the inoculation with a commercial yeast strain would give better control of the fermentation course.

Apart from the possible influence of sugars on the metabolism of arginine, concurrent growth of yeasts and wine LAB could lead to microbial interactions which affect the formation of ethyl carbamate precursors. For example, yeasts could break down citrulline produced by oenococci from arginine degradation, a possibility not given when MLF is carried out after AF. In fact, Ough (1991) stated that citrulline, which may be present in some musts, is degraded by yeasts during AF. Also, it was shown in Chapter 7 that the management of yeast fermentation may lead to different arginine to citrulline conversion ratios.

In addition to questions addressed in this thesis, the realization of simultaneous AF and MLF in the production of table wines is of interest regarding a number of microbiological, technical, and financial aspects. This technique may allow efficient malic acid degradation

in difficult wines (e.g. low pH wines) because of the lower alcohol concentrations and higher nutrient content in grape musts compared to wines. Also, wines obtained after successful AF/MLF are immediately ready for further treatments (addition of SO<sub>2</sub>, racking, fining, bottling), increasing processing efficiency, lowering holding costs and leading to higher profitability. However, in spite of the practical interest in carrying out simultaneous alcoholic and MLF, its application is not very common because of fears of wine quality depreciation by LAB metabolism in musts, and the limited scientific data available about this technique. Specifically, growth of some wine LAB in grape must has been reported to cause stuck AF (Huang et al. 1996; Edwards et al. 1998a; Edwards et al. 1998b; Edwards et al. 1999; Edwards et al. 2000) and can lead to increased concentrations of acetic acid, which can render a wine unacceptable (Lafon-Lafourcade 1979; Lafon-Lafourcade et al. 1980). The latter claim, however, is not undisputed (Blackburn 1984; Beelman and Kunkee 1987). Therefore, the outcomes of this experiment were of broader interest for wine microbiology, and time courses of several wine components unrelated to the objectives of this thesis were measured and analyzed. In the present work, however, only the results concerning the arginine metabolism of wine LAB and the production of carcinogenic ethyl carbamate precursors are presented.

For the realization of this study, two commercial strains of *O. oeni* were inoculated concurrently with a commercial yeast strain of *S. cerevisiae* to induce simultaneous AF/MLF in Chardonnay must. Wines produced by this technique were examined for their concentrations in arginine and arginine degradation products, and the results were compared with those from wines produced traditionally by inducing AF and MLF sequentially (using the same yeast and oenococcal strains).

## 8.2. Experimental conditions

Chardonnay grapes were obtained from a commercial Fernhill vineyard in the Hawke's Bay region of New Zealand. The grapes were mechanically harvested (with low portion of stalks) and 620 kg of fruit were pressed at 1.3 bar in a bag press to yield 400 l must that was cold-settled at 4°C for 24h. The must characteristics are shown in Table 8.1. To increase the low ammonia content and avoid yeast fermentation problems, 300 mg l<sup>-1</sup> of diammonium phosphate were added to the wine and mixed by pumping over. The must was then filled into twelve 25 l carboys.

Four treatments were prepared, consisting in combining AF by the yeast *S. cerevisiae* "Lalvin CY-3079" with MLF by the *O. oeni* strains "Lalvin EQ54" or "Enoferm ALPHA", either in simultaneous AF/MLF or sequential AF/MLF. The four treatments were done in triplicate. The micro-organisms used were commercial freeze-dried preparations from

Lallemand Inc. (Montréal, Canada) and were prepared and inoculated as recommended by the manufacturer to give cell counts of  $8 \times 10^7$  cfu ml<sup>-1</sup> for the yeast strain CY-3079 and  $1 \times 10^6$  cfu ml<sup>-1</sup> for the wine LAB. In the sequential AF/MLF treatments, wine LAB were inoculated only after AF finished. Following every inoculation, the wines were stirred for 30 s. The experiment was carried out at the pilot plant of the Eastern Institute of Technology (EIT, Taradale, Hawke's Bay, New Zealand) and manpower was limited. Thus, it was not possible to carry out viable cell counts. However, yeast growth was estimated microscopically by total cell counts (2.4.3.1). The temperature was monitored and maintained between 18-20°C during the fermentations and the sugar degradation was followed with a Brix hydrometer (2.4.1). The end of AF was assessed by a colorimetric semi-quantitative test for determination of reducing sugars (2.4.1) and the completion of malolactic conversion was determined with an enzymatic test for malic acid (2.4.4.4). Any handling of the grape must or wines was accompanied by flushing exposed surfaces with food grade nitrogen gas. Samples were taken periodically during fermentations, centrifuged at 10,000 g for 5 min and the supernatant transferred into 15 ml screw-cap tubes and frozen at -18°C until analyzed. The samples were analyzed for glucose, fructose, ammonia, arginine, citrulline, urea and malic acid as described in section 2.4.

**Table 8.1** Characteristics of Chardonnay must after cold stabilization

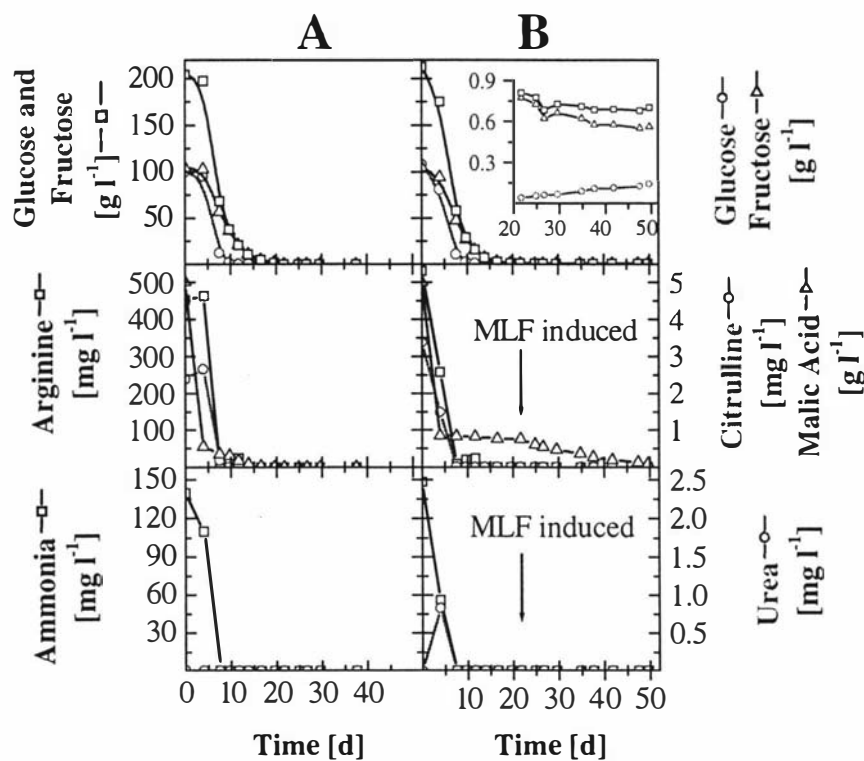
Characteristic	Concentration
pH after degassing	3.28
Soluble solids content (by refractometer)	20.7 Brix
Glucose + Fructose	208 g l <sup>-1</sup>
Total acidity (as tartaric acid)	10 g l <sup>-1</sup>
Malic acid	5.01 g l <sup>-1</sup>
Ammonia (as NH <sub>4</sub> )	8.2 mg l <sup>-1</sup>
Arginine	509 mg l <sup>-1</sup>

### 8.3. Results

Results obtained from the experiments with the *O. oeni* strains EQ54 and ALPHA were similar, thus, only results for strain ALPHA are shown. In both simultaneous (Figure 8.1 A) and sequential (Figure 8.1 B) treatments, arginine was degraded rapidly and no significant increase in citrulline concentrations occurred during arginine degradation. Instead, small amounts of citrulline present in the must at the time of inoculation were depleted quickly. Ornithine concentrations were also very low (<10 mg l<sup>-1</sup>) or undetectable in all treatments, and no residual ornithine remained in any of the finished wines. Equally, ammonia was

rapidly degraded and no ammonia or urea residues were left in the finished wine. In the first 4 days of incubation, over 80% of malic acid was degraded in the simultaneous treatments inoculated with strain ALPHA, but also in the sequential treatments where only yeast was inoculated. After 4 days, however, malic acid degradation stopped in the treatments without inoculated oenococci (Figure 8.1 B) and only continued after inoculation with ALPHA at the end of AF.

The treatments showed considerable differences in the metabolism of fructose and glucose. In the sequential treatments (Figure 8.1 B), traces of glucose and approximately 750 mg l<sup>-1</sup> of fructose remained. After inoculation with strain ALPHA (at t=21.6 d), the glucose concentration increased and fructose concentrations decreased leaving a combined fructose and glucose residue of ~750 mg l<sup>-1</sup> (insert in Figure 8.1 B). This was observed for both strains ALPHA and EQ54. By contrast, neither glucose nor fructose was detectable in the simultaneous treatments after completion of AF/MLF (both strains).



**Figure 8.1** Time course of several substrates and metabolites during the production of wine from Chardonnay must with yeast *S. cerevisiae* Lalvin CY-3079 and wine LAB *O. oeni* Enoferm ALPHA. AF and MLF were induced either simultaneously at t=0 d (A, left column), or sequentially: AF induced at t=0 and MLF at t=21.6 d (B, right column). The insert shows details of the concentrations of glucose and fructose during MLF in the sequential treatments.

## 8.4. Discussion

In this experiment, the arginine metabolism of oenococcal wine lactic acid bacteria (LAB) was examined in musts where alcoholic fermentation (AF) and malolactic fermentation (MLF) were induced either simultaneously or sequentially. Significant malic acid degradation occurred in the simultaneous treatment with added yeast and commercial LAB, but also in the sequential treatment where only yeast was inoculated. It is suggested that this degradation was due to the activity of LAB naturally present in the must, since the yeast strain used degrades malic acid only marginally, according to the manufacturer's information. In fact, the presence of wine LAB in the yeast-only inoculated must was confirmed microscopically. However, malic acid degradation by the indigenous bacteria was inhibited after 4 days, probably because of the rising alcohol levels from sugar fermentation by yeasts, and only continued after inoculation with malolactic starter after completion of AF.

In any case, it is unlikely that the growth and metabolism of natural or inoculated wine LAB influenced the degradation of arginine. In both treatments, the course of arginine degradation was very similar and no increase in citrulline or ornithine could be detected. In previous chapters it was shown that arginine degradation by wine LAB was weak at low pH values, or (in the case of oenococci) clearly delayed in comparison with malic acid degradation (Chapter 6, Chapter 7). Since the initial pH value was below pH 3.3 and arginine and malic acid were depleted nearly simultaneously in both treatments, the results suggest that arginine was predominantly degraded by the yeast. In any event, the effect of high sugar concentrations on arginine degradation by oenococci could not be assessed in this experiment. However, it is questionable if a potential effect of sugars would have been oenologically relevant since the results suggest that if wine LAB and yeasts have access to arginine at the same time, yeasts will play a primary role in its degradation. This is at least so if *O. oeni*, the micro-organism commonly used for inducing MLF, is the predominant wine LAB. Consequently, the time of inoculation with commercial *O. oeni* starters is not expected to lead to an increased risk of citrulline formation. On the contrary, the observed depletion of citrulline, which is most probably attributable to yeast metabolism, demonstrates that during simultaneous AF/MLF, potential citrulline excretion by wine LAB can be neutralized by yeast citrulline degradation. However, if the arginine concentration during simultaneous AF/MLF is so high that yeasts do not deplete arginine, the remaining arginine may be degraded by wine LAB before the end of AF. If this is the case and a dry wine is desired (i.e. complete degradation of sugars), the possibility of stopping bacterial metabolism after malic acid depletion will be jeopardized since addition of preservatives or filtration would also affect AF. To reduce this risk, it is recommended that MLF be carried out after AF when arginine levels in musts are high ( $>1 \text{ g l}^{-1}$ ). The effect of high sugar

concentrations on the arginine degradation by oenococci remains to be studied. This could be done, for example, by inoculating oenococci in arginine containing must with residual sugars ( $> 10 \text{ g l}^{-1}$ ) produced by stopping AF by removal of yeasts.

In the present experiments, no urea, another carcinogenic ethyl carbamate precursor in wine (1.5.3), remained in any of the finished wines. It is suggested that not only arginine degradation by wine LAB, but more generally the formation of ethyl carbamate precursors urea and citrulline can be avoided if arginine levels in musts are adequate (Ough et al. 1990); that is, high enough to allow growth of yeasts but low enough to prevent free arginine or urea residues in wine. Arginine concentrations in grape musts should not exceed  $1 \text{ g l}^{-1}$ , but the exact concentration needed for sufficient growth will depend on the yeast strain used and the availability of other nitrogen sources in the must. The basis for the concentration of arginine in musts has to be seen in the nitrogen management of vineyards (cp. 1.5.3.3 and Ough et al. (1989)).

An additional risk, however, exists even where arginine is present in moderate concentrations and would normally be degraded by yeasts during AF. It has been shown that ammonia exerts a negative effect on transport and metabolism of many yeast nitrogen sources, including amino acids. This phenomenon is known as nitrogen catabolite repression (Cooper and Sumrada 1983). Specifically, the gene for the enzyme arginase (cp. Figure 1, inside back cover) is repressed when readily transported and catabolized nitrogen sources like ammonia are available (Sumrada and Cooper 1987; Cooper et al. 1992; Smart et al. 1996). A gene required for the active transport of urea is also sensitive to nitrogen catabolite repression (El-Berry et al. 1993). In fact, it has been demonstrated that nitrogen supplementation of musts with diammonium phosphate (DAP) can lead to increased ethyl carbamate concentrations in wines. Specifically, Albert (2000) showed a positive linear correlation between the time of DAP addition and the final ethyl carbamate concentration in the wine. Addition of DAP to musts with 75% of the sugars consumed led to a two-fold increase of ethyl carbamate levels in wine compared to the treatments with DAP added at the onset of AF. In the cited work, it is not mentioned if MLF was carried out. Therefore, the increased formation of ethyl carbamate was probably attributable to higher urea concentrations in the wine. The effect of DAP additions on arginine residues in wines still has to be studied. In order to avoid potential urea or arginine residues in wine, the nitrogen requirements of the yeast strain used and the existing nutritional status of the must should be considered carefully, prior to addition of ammonia.

## CHAPTER 9. ENERGETICS OF ARGININE AND CITRULLINE DEGRADATION BY SEVERAL STRAINS OF WINE LACTIC ACID BACTERIA

### 9.1. Introduction

Results from experiments with growing lactic acid bacteria (LAB) in wine (Chapter 6) showed that *L. buchneri* CUC-3 increased maximum growth rate ( $\mu_{\max}$ ) and maximum growth yield ( $Y_{\max}$ ) by degradation of arginine. However, increasing arginine concentrations did not lead to higher  $\mu_{\max}$  or  $Y_{\max}$  of two oenococci, although arginine was degraded. This suggests that oenococci are either unable to produce ATP by the ADI pathway or are inefficient or lacking in coupling ATP formed by the ADI pathway to anabolic metabolism. These results, though, are not in accordance with results of other studies. Amoroso et al. (1993) reported that arginine had a stimulatory effect on growth of three *O. oeni* strains grown in synthetic medium, and Tonon and Lonvaud-Funel (2000) suggested that arginine degradation was efficiently coupled to growth of an oenococcus. *O. oeni* is the preferred bacterium to induce malolactic fermentation (MLF) and, with one exception, the only species offered commercially (cp. 1.1). Therefore, the question whether arginine stimulates growth of *O. oeni* or not is of particular oenological interest. If there was no growth stimulation by arginine, then non-arginine degrading oenococci could be selected and used for inducing MLF without the risk of being overgrown by arginine positive strains. This would eliminate the negative consequences of arginine degradation by the ADI pathway (cp. 1.5).

In order to examine the discrepancies cited above, the growth behaviour of several selected LAB was studied in wine adjusted to different pH values and arginine concentrations. Different pH values were used since this parameter has been shown to influence the ability of wine LAB to degrade arginine (Chapter 6, and Liu et al. (1995a)) and the pH might affect the ability of wine LAB to couple energy to cell growth, too. The energetic impact of the degradation of citrulline by wine LAB also was investigated at several pH values, since citrulline may be excreted by wine LAB and subsequently serve as a substrate for the same (citrulline-excreting) micro-organism or other bacteria.

Some other aspects concerning the energetic role of arginine/citrulline degradation are also presented in this chapter. In Chapter 6 it was shown that *L. buchneri* CUC-3 increased growth rate at higher arginine concentrations (Figure 6.5). This could be attributable to efficient coupling of ATP formation to anabolic pathways. However, it could also be due to the increase in medium pH from the formation of ammonia, since the same micro-organism

increased growth yields at higher pH values (Fig. 8, Appendix). In fact, higher arginine concentrations in wine led to a greater degradation of sugars and this could be the direct cause for increased growth (Figure 6.3). Because of this possibility, it was of interest to conduct cultivations that allow the assessment of the role of increased pH from ammonia formation in the growth enhancing effect of arginine degradation. This could be done by growing cultures at a fixed pH value, where acid or base is added to compensate for decrease or increase in pH. However, fixed pH cultivations do not represent the pH behaviour of wine when MLF occurs. Thus, an experiment was carried out where the pH value of cultures in wine with added citrulline or ornithine, or without any additions, was continually adjusted with concentrated ammonia or sodium hydroxide to match the pH value of a cultivation in wine with added arginine and freely developing pH. This was done to simulate ammonia formation from arginine degradation by the ADI pathway and to assess its contribution to cell growth.

It was also important to assess if the growth enhancing role of arginine in *L. buchneri* CUC-3 was based only on the stimulation of the assimilation of carbon sources, or if arginine constitutes a major carbon source itself. To study this, the recovery of arginine metabolites, citrulline and ornithine, was analyzed in cultures of *L. buchneri* CUC-3 in wine.

Finally, on the basis that *L. buchneri* CUC-3 preferentially degraded arginine over malic acid, whereas two oenococci degraded malic acid well before arginine (Chapter 6), growth studies were made where lactobacilli were inoculated into wine which had previously undergone MLF by oenococci, and *vice versa*. This was done to investigate the microbial stability of wines after the degradation of arginine by a *Lactobacillus* or after malic acid degradation by an *Oenococcus*. Also, in order to consider the biological removal of citrulline in wines, the ability of a *Lactobacillus* and an *Oenococcus* to degrade citrulline excreted during arginine degradation was evaluated.

## 9.2. Experimental conditions

The preparation of the base wine is described in section 2.2.2.10. The wine used here (cp. 6.2) had 8.9% (v/v) ethanol and no free SO<sub>2</sub> could be detected. Glucose, fructose and malic acid concentrations were 20 mg l<sup>-1</sup>, 900 mg l<sup>-1</sup> and 1.2 g l<sup>-1</sup>, respectively. Ammonia, urea and arginine were present only in traces. The pH after degassing was 3.16. The base wine was adjusted to 3 g l<sup>-1</sup> malic acid and 1 g l<sup>-1</sup> glucose. Pimaricin was added to inhibit yeast growth (2.1.7). Other additions and/or adjustments were made depending on the experiment, as specified below.

### 9.2.1. Effect of arginine and citrulline concentrations

For the investigation of the influence of arginine on growth of wine LAB, 450 ml batches of the base wine were adjusted to give 0, 300, 600 and 900 mg l<sup>-1</sup> of arginine or citrulline. The batches were subsequently subdivided into 150 ml batches, which were adjusted to pH 3.1, 3.3 and 3.5 for each arginine concentration with 5M HCl or 5M NaOH. The twelve batches were sterile filtered and 10 ml aliquots were pipetted into sterile screw-cap spectrophotometer glass tubes (25 ml capacity, Pyrex 9826, U.S.A.). To inoculate the wines, either cultures pre-grown in AMRS at pH 4.5 (2.2.2.5) or commercial strains for direct inoculation were used. In the first case, the cells were separated from the medium by centrifugation (5,000 g, 10 min) to avoid carryover of media constituents and metabolic products, and were then washed twice with water before inoculation occurred to give an initial OD of 0.05. In the latter case, the commercial starters were reactivated in water at room temperature for 15 min, and then treated as the pre-grown cultures, including the washing steps. The growth of bacteria was calculated by measuring the OD at 600 nm and subtracting the values of an inoculated control. All cultivations were done in duplicate. Samples were taken at the time of inoculation and during the death phase of the cultures. Arginine degradation was determined qualitatively in these samples by the enzymatic method (2.4.4.1) to verify whether arginine was degraded.

For the investigation of the influence of citrulline on growth of wine LAB, wines were prepared as described above, but arginine was replaced by citrulline and the wines were adjusted to pH 3.3, 3.5 and 3.7, because growth of all micro-organisms in the previous experiment (with arginine) had been relatively low at pH 3.1. Samples were taken at the time of inoculation and during the death phase of the cultures, and citrulline was quantified by HPLC (2.4.4.5).

### 9.2.2. Contribution of increasing pH to growth stimulation of *L. buchneri* CUC-3 by arginine degradation

For this experiment, the base wine was used as described in 9.2, above. The wine was divided into 7 duplicate treatments of 200 ml each, and different substrates were added. The treatments are described in Table 9.1. After addition of substrates, the pH of all treatments was adjusted to 3.9 and the wines were sterile filtered into sterile 200 ml glass bottles. During cultivations, the OD and the pH of the cultures were closely monitored and concentrated ammonia or sodium hydroxide solution were added to some cultures as required to match the pH of the arginine treatment (Table 9.1). Samples were taken at the

time of inoculation and during the death phase of the cultures. Arginine, citrulline and ornithine were determined in these samples by HPLC (2.4.4.5).

**Table 9.1** Specification of treatments for the study of the effect of pH increase from arginine degradation on growth of *L. buchneri* CUC-3 (cp. 9.3.2).

Treatment Name	Additions to base wine (to give final concentration)	pH adjustment to match pH of Arg treatment during cultivation
Arg	Arginine (6.75 mM)	-
Citr + NH <sub>3</sub>	Citrulline (6.75 mM)	3.5 % NH <sub>3</sub> solution (12.33 mol l <sup>-1</sup> )
Citr	Citrulline (6.75 mM)	-
Ctrl + NH <sub>3</sub>	-	3.5 % NH <sub>3</sub> solution (12.33 mol l <sup>-1</sup> )
Orn + NH <sub>3</sub>	Ornithine (6.75 mM)	3.5 % NH <sub>3</sub> solution (12.33 mol l <sup>-1</sup> )
Ctrl + NaOH	-	NaOH solution (2 mol l <sup>-1</sup> )
Ctrl	-	-
Orn	Ornithine (6.75 mM)	-

### 9.2.3. Recovery of arginine metabolites

For this experiment, the base wine used was as described in 9.2, above. The base wine was adjusted to 1 g l<sup>-1</sup> arginine and pH 3.9 and divided into seven 200 ml batches. Inoculation was carried out with pre-grown and washed cells of *L. buchneri* CUC-3 as described in 9.2.1, above. Degradation of arginine was followed by pH development, and the depletion of arginine was confirmed by enzymatic arginine analysis. A sample taken at the time of inoculation and one after complete arginine degradation were analyzed by HPLC (2.4.4.5) for arginine, citrulline and ornithine concentrations. The amounts of arginine degraded and citrulline and ornithine formed were corrected for substrate already present or remaining upon inoculation or end of incubation, respectively.

### 9.2.4. Microbiological stability of wines after degradation of malic acid or arginine

For this experiment, the base wine used was as described in 9.2, above. The base wine was adjusted to 1 g l<sup>-1</sup> arginine and the pH adjusted to 3.5 with 5 M sodium hydroxide. 500 ml aliquots were sterile filtered into eight sterile 500 ml bottles (four treatments in duplicate). For three of the treatments, *O. oeni* EQ54 was inoculated according to the manufacturer's recommendations and for the other, *L. buchneri* CUC-3 was inoculated as described

previously (2.3.2.2). In the EQ54 treatments, the pH course was monitored in order to stop metabolic activity by sterile filtering the wines after depletion of malic acid in one case, and after depletion of malic acid and arginine in the other two cases. The wines were then re-inoculated with strain CUC-3, or again *O. oeni* EQ54. Accordingly, the pH was also monitored in the CUC-3 cultures and the wines were sterile filtered after arginine depletion, followed by re-inoculation with strain EQ54. Samples were taken periodically throughout the experiment, centrifuged (10,000 g for 5 min) and the supernatant was frozen (-18°C) until analyzed. The methods applied for analysis of the experiments are described in section 2.4. Specifically, the measurement of growth was carried out by OD and the concentrations of arginine and citrulline were determined by HPLC.

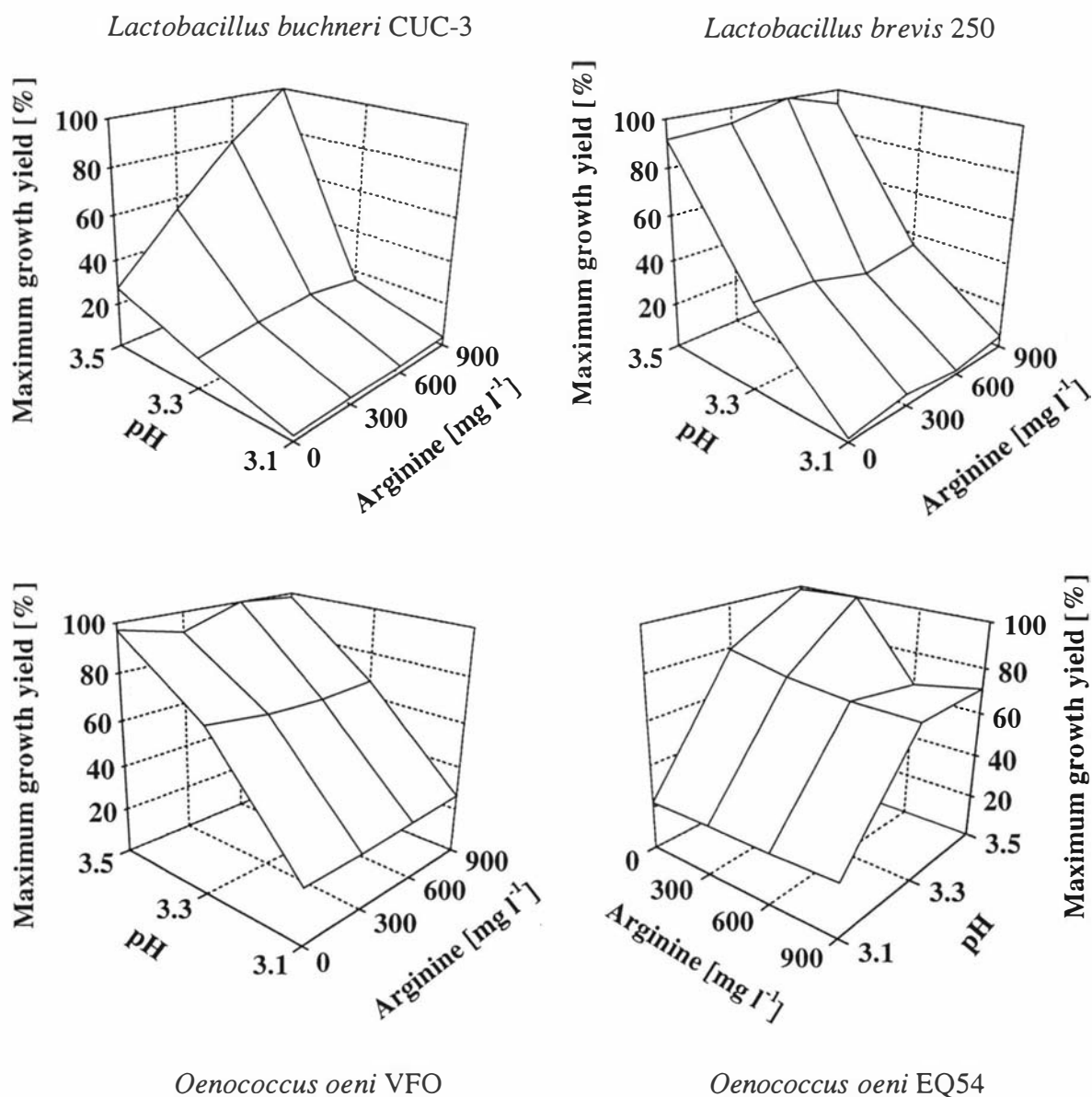
## 9.3. Results

### 9.3.1. Influence of several arginine and citrulline concentrations at different pHs on growth of selected wine lactic acid bacteria

Figure 9.1 shows results of growth experiments with selected wine LAB at different initial pH values and arginine concentrations. Maximum growth yields ( $Y_{\max}$ ) and respective maximum growth rates ( $\mu_{\max}$ ) obtained from all experiments corresponded well (as found with data in Chapter 6). Thus, only  $Y_{\max}$  values were retained for graphical representation here. At pH 3.1 and 3.3, growth of *L. buchneri* CUC-3 was weak and increasing concentrations of arginine had no effect on  $Y_{\max}$  values. However, at pH 3.5,  $Y_{\max}$  of strain CUC-3 increased considerably at higher initial arginine concentrations. In contrast,  $Y_{\max}$  of *L. brevis* 250, another heterofermentative, arginine-degrading *Lactobacillus* (cp. Chapter 4), did not increase with increasing arginine concentrations, at any pH.

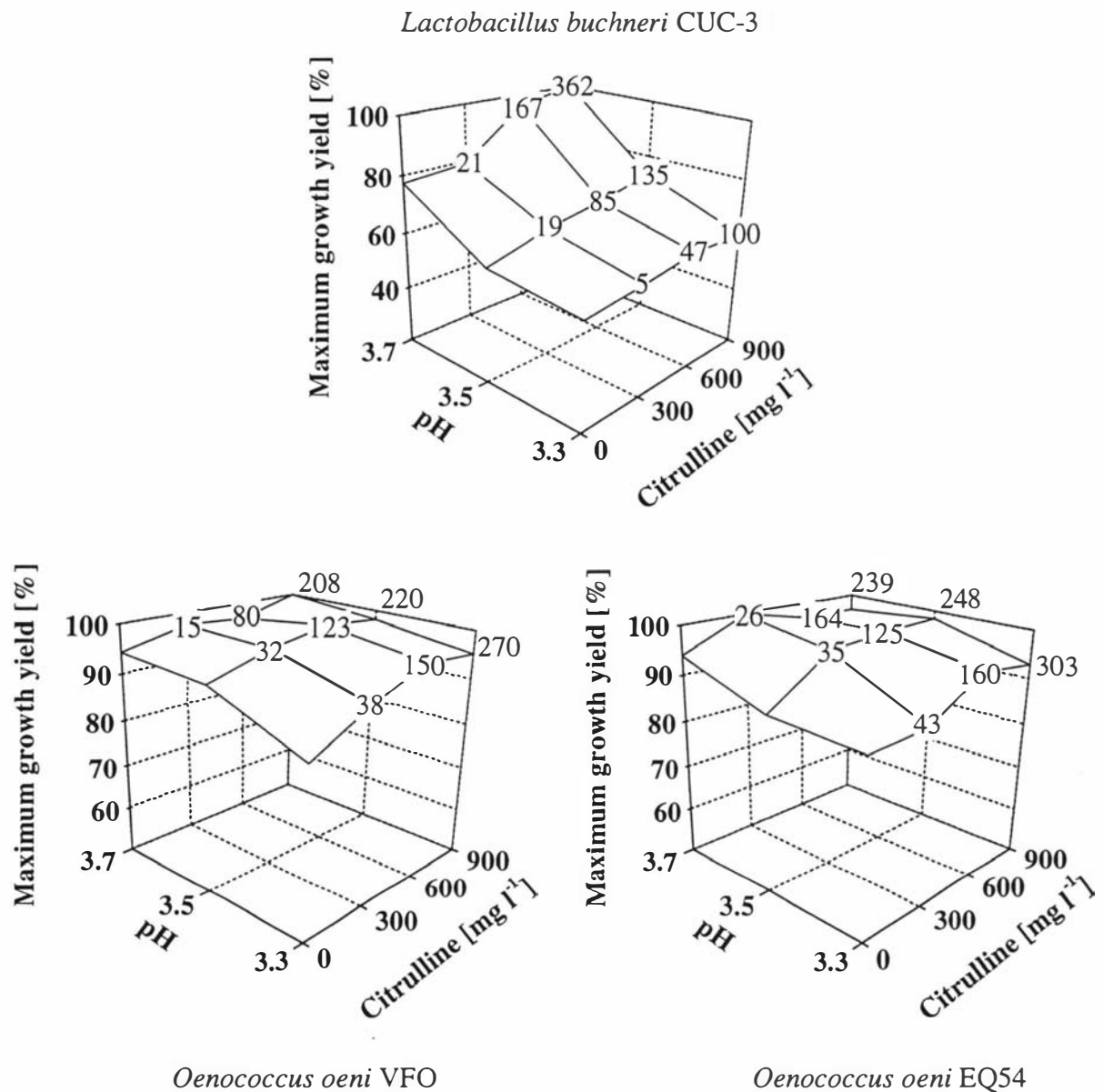
$Y_{\max}$  values of oenococci were not influenced as markedly below pH 3.5, as with the lactobacilli. However, as for *L. brevis* 250, none of the oenococci showed an increase in  $Y_{\max}$  at higher arginine concentrations at any pH value. Nevertheless, arginine had been degraded at least partly during all incubations at pH 3.5 and pH 3.3. At pH 3.1, no arginine degradation was observed for any strain.

Whereas no stimulation was found for *O. oeni* strain EQ54 and VFO, growth of *O. oeni* EQ54 was inhibited (statistically significant at  $\alpha = 0.05$ ) by arginine concentrations of 600 and 900 mg l<sup>-1</sup> at pH 3.5. This inhibition was not seen with *O. oeni* VFO.



**Figure 9.1** Maximum growth yields of selected wine LAB from cultivations in wine at different pH values and arginine concentrations. Mean results of duplicate cultivations are shown. The values are displayed in percent of the highest  $Y_{\max}$  value found for each strain with 100% growth corresponding to 144 mg l<sup>-1</sup> dry weight for strain CUC-3, 39 mg l<sup>-1</sup> for strain 250, 91 mg l<sup>-1</sup> for strain EQ54, and 56 mg l<sup>-1</sup> for strain VFO.

The effect of different citrulline concentrations at pH 3.3 - 3.7 on growth of several wine LAB is shown in Figure 9.2. Growth at pH 3.1 was not considered for this experiment, since growth of all strains had been weak at this pH in the previous experiment, and no arginine was degraded. Instead, the effect of citrulline on growth was studied at pH 3.3, 3.5 and 3.7. *L. brevis* 250, being unable to increase its growth with arginine at any pH, was not considered in the current study.



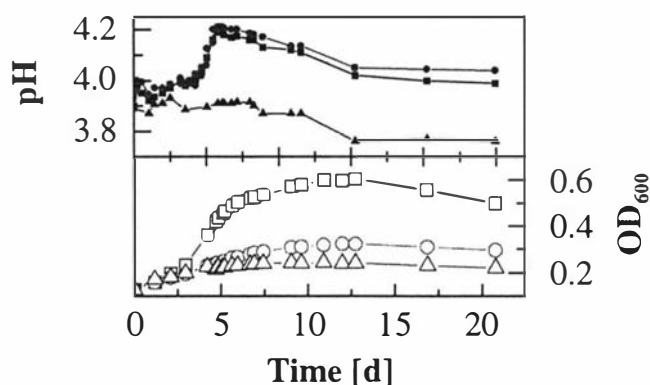
**Figure 9.2** Maximum growth yields of selected wine LAB from cultivations in wine at different pH values and citrulline concentrations. Mean results of duplicate cultivations are shown. The values are displayed in percent of the highest  $Y_{\max}$  value found for each strain with 100% growth corresponding to 67 mg l<sup>-1</sup> dry weight for strain CUC-3, 165 mg l<sup>-1</sup> for strain EQ54, and 171 mg l<sup>-1</sup> for strain VFO. The numbers displayed in the graphs represent the reduction in citrulline concentration (mg l<sup>-1</sup>) observed after incubations.

For strain CUC-3, a statistically significant increase (at  $\alpha = 0.05$ ) of the maximum growth yield ( $Y_{\max}$ ) was observed at pH 3.5 and pH 3.7 in treatments having 600 and 900 mg l<sup>-1</sup> of citrulline compared with those having 0 and 300 mg l<sup>-1</sup> of citrulline. In contrast, no statistically significant increase or decrease could be found for any combination of treatments with both *O. oeni* strains.

In all treatments, citrulline was degraded (Figure 9.2). Whereas CUC-3 degraded more citrulline at higher pH values and the amount degraded corresponded with the increase in  $Y_{\max}$ , (cp. arginine degradation by CUC-3 in Chapter 6), this was not the case for the oenococci examined. Here, the amount of citrulline degraded decreased with increasing pHs (with the only exception of the treatment at pH 3.7 and 600 mg l<sup>-1</sup> citrulline of strain EQ54), and there was no correlation between the amount of citrulline degraded and  $Y_{\max}$ .

### 9.3.2. Contribution of increasing pH to growth stimulation of *L. buchneri* CUC-3 by arginine degradation

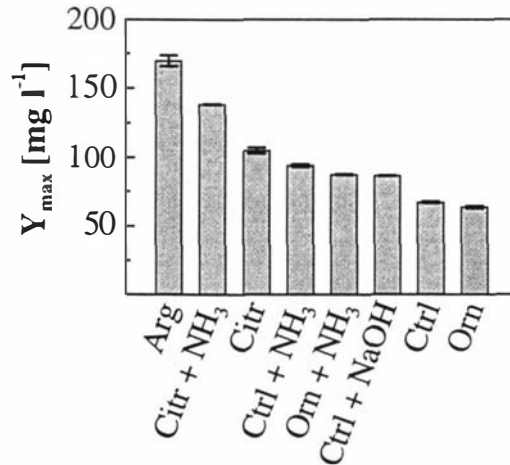
Figure 9.3 shows an example of the cultivations carried out to assess the contribution of pH increase from ammonia excretion to the growth stimulating effect of arginine degradation in *L. buchneri* CUC-3. In the treatment with added arginine, the pH was measured regularly and the value obtained was used to adjust the pH value of the regulated treatments with added ornithine (shown in Figure 9.3), citrulline or those without added substrate (cp. Table 9.1). All treatments with addition of ammonia or sodium hydroxide were also compared with a similar but unadjusted treatment.



**Figure 9.3** Growth of *L. buchneri* CUC-3 and time course of pH in several cultivations, adjusted to 6.75 mM of arginine ( $\square, \blacksquare$ ) or 6.75 mM of ornithine (adjusted pH,  $\circ, \bullet$ ; free pH,  $\triangle, \blacktriangle$ ). In the arginine treatment ( $\square, \blacksquare$ ) and one ornithine treatment ( $\triangle, \blacktriangle$ ), the pH during fermentations was left unadjusted. In the second ornithine treatment ( $\circ, \bullet$ ) the pH was adjusted periodically by adding ammonia to equal or exceed the corresponding pH of the arginine ( $\square, \blacksquare$ ) treatment.

The average maximum growth yield values ( $Y_{\max}$ ) obtained from duplicate cultivations as described above and in section 9.2.2 are shown in Figure 9.4. Treatments which had the pH adjusted by addition of ammonia or sodium hydroxide to equal the pH of the treatment with arginine always had higher  $Y_{\max}$  values than the respective treatments without pH adjustment. Nevertheless, the higher  $Y_{\max}$  in the treatment with added arginine was not attained by any other treatment. Similarly, but less marked, the cultivations with added

citrulline gave higher  $Y_{\max}$  values than those with added ornithine or the controls without any additions. Addition of ornithine to wine led to growth inhibition of strain CUC-3 compared with the control without any additions, an observation also made when ammonia was replaced by sodium hydroxide for adjusting pH values.



**Figure 9.4**  $Y_{\max}$  data from cultivations of *L. buchneri* CUC-3 in wine (pH 3.9) containing several substrates (treatments described in 9.2.2; mean values of measurements from duplicate cultivations shown (error bars indicate standard deviation). Arginine (Arg), citrulline (Citr), ornithine (Orn), control (Ctrl). Analysis of variance revealed that at the 0.05 confidence level only the treatments [Orn + NH<sub>3</sub>] and [Ctrl + NaOH] were not significantly different; all other combinations of treatments had significant differences at  $\alpha = 0.05$ .

Table 9.2 compares data obtained from the treatment with added arginine and the treatment with added citrulline and adjusted pH. Whereas arginine was depleted in the treatment with this substrate added, citrulline was only partially degraded (58% of initial 6.75 mM citrulline, Table 9.2). In the treatment with citrulline added, the pH course was rigorously adjusted to the pH course of the arginine treatment. Nevertheless, to maintain the same pH values, only 60% of the ammonia formed from arginine degradation in the arginine treatment were either produced from citrulline degradation or originated from addition of ammonia solution in the citrulline treatment.

**Table 9.2** Degradation of arginine and citrulline and formation of ammonia during growth experiments with *L. buchneri* CUC-3 in wine with added arginine or citrulline.

	Treatment	
	Arg	Citr + NH <sub>3</sub>
Arginine or citrulline degraded ( $\pm$ SD)	6.78( $\pm$ 0) mM	3.91( $\pm$ 0.16) mM
Ammonia formed + ammonia added <sup>a</sup> ( $\pm$ SD)	13.57( $\pm$ 0) mM	8.22( $\pm$ 0.001) mM

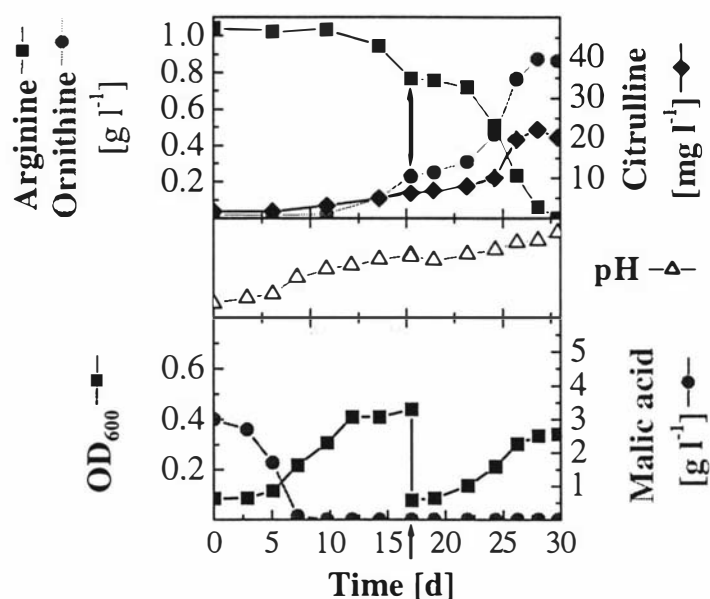
<sup>a</sup> only Citr + NH<sub>3</sub> treatment

### 9.3.3. Recovery of arginine metabolites

After complete arginine degradation by *L. buchneri* CUC-3 in wine, the amounts of citrulline and ornithine formed were combined and compared to the amounts of arginine degraded. Expressed as a molar ratio, 98.14 ( $\pm 1.63$ )% of arginine degraded during incubation was recovered as citrulline or ornithine in the medium ( $n = 7$ , SE in parentheses).

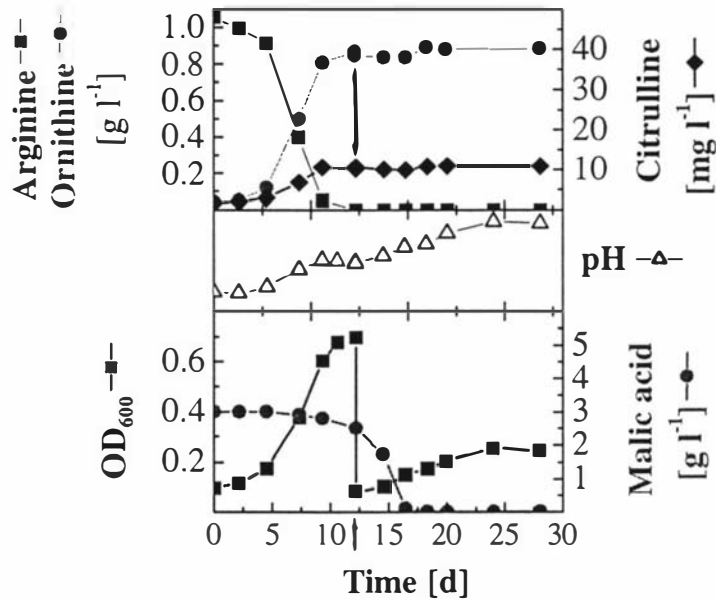
### 9.3.4. Microbiological stability of wines after degradation of malic acid or arginine

A wine that had supported successful growth, malolactic conversion and partial arginine degradation by a commercial *O. oeni* strain was still able to support growth and arginine degradation after sterile filtration and inoculation with *L. buchneri* CUC-3 (Figure 9.5). During the complete degradation of arginine by *L. buchneri* CUC-3, the arginine to citrulline conversion ratio was 2.1( $\pm 0.21$ )% (SE in parentheses).



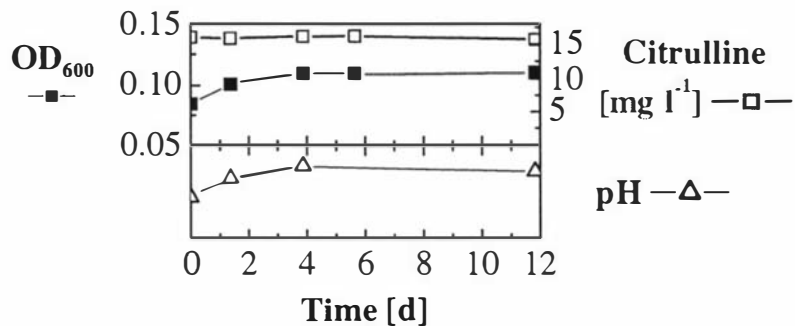
**Figure 9.5** Sequential growth of two wine LAB in wine (initial pH, 3.5; final pH, 4.01). Growth of *O. oeni* EQ54 followed by growth of *L. buchneri* CUC-3. Wine was sterile filtered and inoculated with the second strain at the time indicated by the arrow. Data points are averages from duplicate incubations.

Correspondingly, a wine was able to support growth and complete degradation of malic acid by *O. oeni* EQ54 following growth, arginine depletion, and partial malic acid degradation by *L. buchneri* CUC-3 (Figure 9.6). During the first incubation with strain CUC-3, arginine was converted to citrulline at a ratio of 0.84( $\pm 0.16$ )%.



**Figure 9.6** Sequential growth of two wine LAB in wine (initial pH, 3.5; final pH, 4.01). Growth of *L. buchneri* CUC-3 followed by growth of *O. oeni* EQ54. Wine was sterile filtered and inoculated with the second strain at the time indicated by the arrow. Data points are averages from duplicate incubations.

A wine which had supported complete malic acid and arginine degradation by *O. oeni* EQ54 and had a citrulline concentration of 16 mg l<sup>-1</sup>, was not able to support growth of the same micro-organism after sterile filtration and re-inoculation (data not shown). The same wine supported weak growth of *L. buchneri* CUC-3, but citrulline was not degraded during the incubation (Figure 9.7).



**Figure 9.7** Growth of *L. buchneri* CUC-3 in a wine depleted of malic acid and arginine by previous incubation with *O. oeni* EQ54 (initial pH, 4; final pH, 4.025). Data points are averages from duplicate incubations.

## 9.4. Discussion

### 9.4.1. Energetic aspects of arginine and citrulline degradation by wine lactic acid bacteria

The effect of arginine and citrulline on growth of selected wine lactic acid bacteria (LAB) was studied at different pH values. When investigating the role of potential energy sources on growth of micro-organisms, cultivations can be carried out in a chemically defined medium with known composition. The advantage of a defined medium is that the energy and carbon sources available can be restricted and considered. In this way, the contribution of a putative energy source, like arginine, to growth can be quantified by subtracting the contribution of other energy sources utilized (e.g. glucose or fructose). However, chemically defined media also have disadvantages. The development of a chemically defined medium supporting satisfactory growth of bacteria, especially LAB, can be a challenge in itself. Further, even if the stimulation of growth by a putative energy source has been demonstrated and quantified by the calculation of substrate specific molar growth yields, this stimulation may not be relevant in the natural environment of the micro-organism examined. This is especially true in the case of wine LAB, whose natural environment is relatively harsh in terms of acidity, a condition that is often relieved in cultivations to avoid growth difficulties. Given the ambiguous results about stimulation of oenococcal growth by arginine degradation (cp. 9.1) and the oenological importance of this characteristic, it was necessary to study the growth of wine LAB in wine and at wine pH. Whereas this method gave results that are significant for the wine environment, the quantification of the contribution of arginine or citrulline to growth of wine LAB (e.g. in substrate specific molar growth yields) was not possible, since the degradation of the main energy sources by LAB in wine cannot be assessed with precision. For example, glucose or fructose concentrations have been shown to increase during growth of wine LAB in this thesis (6.3 and 8.3) and elsewhere (Costello et al. 1985; Fleet 1985; Davis et al. 1986a; Davis et al. 1986b). Since the pool of hexoses and other sugars available to LAB in wine is difficult to measure, it is not possible to discriminate the contribution of these sugars to the growth of micro-organisms from the possible contribution of arginine in the calculation of molar growth yields.

Results obtained from this investigation in wine confirmed results presented in Chapter 6 (Figure 6.5). That is, *L. buchneri* CUC-3 was able to derive a growth advantage from the degradation of arginine, but two arginine-degrading oenococci did not. These results are reflected in the data from experiments with citrulline in this chapter, too. Although to a lesser degree, strain CUC-3 was able to increase growth from citrulline degradation, but not oenococci. The results of this detailed growth study and similar data presented in Chapter 6

demonstrate clearly that oenococci, while capable of degrading arginine or citrulline by the ADI pathway, are not able to couple ATP formation from this metabolism to cell growth in wine and at wine pH values. Contrary results obtained by Tonon and Lonvaud-Funel (2000) may originate in the experimental conditions applied: growth of oenococci was studied in a complex broth (a modified MRS medium) and at a non-physiological pH (pH 4.5). Moreover, molar growth yields were calculated for arginine, but the cultivations carried out in MRS broth were not corrected for other energy sources found in the components of this complex medium other than glucose. Specifically, MRS broth contains, amongst others, yeast extract (Anonymous 1998), which is rich in trehalose, a disaccharide that is likely to support growth of oenococci (cp. Tab. 1, Appendix, and Liu et al. (1995a)). However, only the concentration of free glucose was considered for the calculation of molar growth yields for arginine (Tonon and Lonvaud-Funel 2000). So too, studies carried out by Amoroso et al. (1993) suggesting a stimulatory effect of arginine on growth of three *O. oeni* strains were carried out at non-physiological pH (4.8) in a semi-synthetic medium.

As mentioned in section 6.4, the inability of oenococci to increase growth by way of the ADI pathway has oenological significance because this allows the winemaker to induce malolactic fermentation (MLF) with arginine negative strains of *O. oeni* that would not have an ecological disadvantage, compared with arginine degrading oenococci. Arginine negative oenococci could be selected from wines undergoing spontaneous MLF or culture collections, or be constructed by genetic engineering. In fact, one arginine negative strain of *O. oeni* for the induction of MLF has already been selected and promoted commercially (*O. oeni* CH35, Chr. Hansen, Denmark). The results also show that not all heterofermentative lactobacilli are able to increase growth by way of the ADI pathway, since *L. brevis* 250 could not increase growth from arginine degradation at any pH. It is therefore desirable to assess this characteristic for more lactobacilli.

Apart from the differences in their ability to couple arginine or citrulline degradation to growth, oenococci and *L. buchneri* CUC-3 also differed in the pattern of citrulline degradation. That is, citrulline degradation by *L. buchneri* CUC-3 was stronger at higher pH values where greater growth occurred, whereas oenococci degraded more citrulline at lower pH values. This difference may be caused by the growth phase in which the ADI pathway is active in oenococci and its physiological role in the respective bacteria. *L. buchneri* CUC-3 uses the ADI pathway in order to gain an ecological advantage in wine. Therefore, arginine or citrulline degradation takes place during rapid growth. In contrast, oenococci mainly gain an ecological advantage from the degradation of malic acid. The degradation of arginine or citrulline occurs during late-log or stationary phase. The role of the ADI pathway in oenococci might therefore lie in increasing acid resistance of stationary phase cells, rather than gaining an ecological advantage during growth. This would explain why the ADI

pathway in oenococci was more active at lower pH values in wine. In fact, it was shown that viable but non-culturable cells of *O. oeni*, obtained from energy starved cultures, were able to recover the ability of cell division upon addition of arginine (Tonon and Lonvaud-Funel 2000).

Although oenococci could not increase growth by arginine degradation in experiments shown here, Tonon et al. (2001) demonstrated recently that arginine degradation in *O. oeni* actually leads to formation of ATP. The lack of growth stimulation in oenococci may be due to missing or inefficient coupling of ATP formation to anabolic functions, as discussed above. In *L. buchneri* CUC-3, the formation of ATP by the ADI pathway is probably a reason for the growth stimulating effect of arginine degradation. In addition to this effect, it was demonstrated here that the increase of pH from ammonia formation also contributes to the growth stimulating effect of the ADI pathway in *L. buchneri* CUC-3. However, the contribution of increasing pH to growth stimulation was much smaller than the growth enhancing effect ascribed to ATP formation from arginine degradation. Even in the treatment with added citrulline, which could still be degraded to yield ATP, addition of ammonia was not able to simulate the growth enhancing effect of added arginine. This is partly due to the fact that citrulline was degraded incompletely in the citrulline treatment with added ammonia (58%), whereas arginine was depleted in the arginine treatment. Moreover, although pH values in the treatments were maintained similar, the total amount of ammonia formed from citrulline degradation and added for pH adjustment in the citrulline treatment accounted only for 60% of the ammonia formed from arginine degradation in the arginine treatment. This implies that in the arginine treatment more acids were formed from sugar degradation to compensate for the pH increase that ammonia formation alone would have caused. A reason for this observation could be that intracellular formation of ammonia from the ADI pathway was more effective in promoting sugar fermentation than the extracellular addition of ammonia. Because a complex medium with unknown composition (wine) was used as a medium in this experiment, it is impossible to make detailed energetic considerations to explain the observations made. However, if wine is replaced by a synthetic wine with known composition, the experimental design applied here could be used to study the dependence of the molar growth yield of arginine under dynamic pH conditions. This would be useful to quantify the exact contribution of pH increase by ammonia formation and ATP formation to the growth stimulating effect of arginine degradation in *L. buchneri* CUC-3.

It has been reported that ornithine has an inhibitory effect on growth of wild yeasts such as *Hansenula minuta* (Mayer et al. 1973; Kuensch et al. 1974). The results presented here show that ornithine also inhibited the growth of *L. buchneri* CUC-3 in wine. Even though the growth inhibition by ornithine was relatively low (~7%), it may be more significant in

other wine LAB. Also, ornithine is also a metabolic product of yeast arginine degradation (Figure 1, inside back cover) and concentrations high enough to cause oenologically significant LAB growth inhibition may be encountered in wines already after alcoholic fermentation. High extracellular ornithine concentrations may lead to growth inhibition by reducing the import of arginine into the cell by the arginine-ornithine exchange system, which has been reported for dairy LAB (Poolman et al. 1987; Thompson 1987; Driessen et al. 1987; Konings et al. 1989). However, the exact mechanism of ornithine inhibition is not known at present and is subject to further studies.

#### 9.4.2. Recovery of arginine metabolites

In this chapter it was shown that *L. buchneri* CUC-3 was able to increase its growth by the degradation of arginine. In order to examine if this stimulation was attributable to the utilization of arginine as a carbon source, the recovery of citrulline and ornithine from arginine degradation was measured in growing cultures in wine. The analysis showed that arginine was nearly completely retrievable as ornithine and citrulline. Data taken from other cultivations presented in this thesis had similar values to the 98% recovery obtained in this experiment. Specifically, sequential growth experiments presented in this chapter (including arginine degradation of both an *Oenococcus* and a *Lactobacillus*; Figure 9.5, Figure 9.6) resulted in an average recovery of  $104.3(\pm 1.2)\%$  (SE in parentheses) and a value calculated from experiments on the effect of sugar on arginine degradation by *L. buchneri* (Figure 6.6) yielded an average recovery of  $96.2(\pm 0.56)\%$  (6.3). Even without having studied the fate of arginine more rigorously (e.g. by carrying out tracing experiments with radioactively labelled arginine in a chemically defined medium), the data presented suggests that arginine does not serve as a carbon source for wine LAB; its incorporation is likely to be limited to proteinacious structures and possibly to the formation of pyrimidines (Bringel et al. 1997; Bringel 1998), a pathway which has yet to be confirmed in wine LAB.

#### 9.4.3. Microbiological stability

The microbiological stability of wines exhausted of malic acid or arginine after growth of wine LAB was analyzed. This was done by assessing the ability of an *Oenococcus* and a *Lactobacillus* to grow in wine pre-fermented by the opposite micro-organism. It was demonstrated that wines with depleted malic acid after growth of an *Oenococcus* or wines with depleted arginine after growth of a *Lactobacillus* had the potential to support growth and metabolism of arginine or malic acid by the opposite micro-organism. Thus, degradation of arginine and formation of citrulline is possible in wines even after successful malolactic conversion. Furthermore, more citrulline was excreted from arginine degradation when growth and arginine degradation by *L. buchneri* CUC-3 took place after depletion of malic acid from growth of *O. oeni* EQ54 (Figure 9.5, conversion ratio was 2.5 times higher than in Figure 9.6). In fact, in Chapter 6 it was shown that oenococci had significantly

higher arginine to citrulline conversion ratios than a *Lactobacillus*, and it was suggested that this was caused by the growth phase in which arginine degradation took place. This suggestion was based on work with *Mycoplasma* where more citrulline was excreted from arginine degradation during weak growth (Barile et al. 1966). Interestingly, in the experiment described here, the higher arginine to citrulline conversion ratio of *L. buchneri* CUC-3 in wine pre-fermented by *O. oeni* EQ54 corresponded with lower growth of strain CUC-3 in this incubation (Figure 9.5). Specifically, the maximum growth yield ( $Y_{\max}$ ) of strain CUC-3 in the incubation with the higher arginine to citrulline conversion ratio (2.1%, Figure 9.5) was only 48% of the  $Y_{\max}$  in the incubation with the lower conversion ratio (0.84%, Figure 9.6). This observation raises the question, if apart from the reasons discussed in the introduction (unfavourable equilibrium of the citrulline degrading reaction, section 1.4.4; enzymatic feedback inhibition, 5.1), the excretion of citrulline may be regarded as an overflow metabolism (Russell and Cook 1995). The excretion of this partly degraded arginine metabolite could serve in dissociating the hydrolysis of arginine from ATP production and biomass formation during weak growth when arginine is still available in excess. Citrulline excretion could thus be an adaptation to a medium with lower nutrient availability, and allow wine LAB to control the formation of biologically useable energy (ATP) relative to the anabolic requirements (Tempest and Neijssel 1992; Teixeira de Mattos and Neijssel 1997).

Regarding the risk of arginine degradation and formation of citrulline in wines, these results suggest that the separation of bacteria from the wine (e.g. by sterile filtration) will only stop arginine degradation and citrulline excretion efficiently when bacterial contamination in the further handling of the wine can be prevented. If degradation of arginine by spoilage bacteria happens in wine after MLF, the amount of citrulline excreted could even be higher than if arginine is degraded during MLF. Where wines after completion of malolactic conversion are held under conditions that do not allow filtration (e.g. wine stored in barrels), any bacterial activity should be eliminated by using appropriate preservatives, such as SO<sub>2</sub>.

The reason for the ability of the tested bacteria to grow in pre-fermented wine probably lies in the different energetic significance of the substrates malic acid and arginine for strain EQ54 and strain CUC-3. The degradation of malic acid has a stimulating effect on growth of oenococci (Henick-Kling 1993; Cox and Henick-Kling 1995) and in fact, malic acid was degraded before arginine by *O. oeni* EQ54 confirming previous results (cp. Figure 6.2, Figure 6.4, Figure 7.1 and Figure 7.2). On the other hand, a potential spoilage micro-organism like *L. buchneri* CUC-3 could increase growth efficiently by the degradation of arginine (cp. results above) but degrades malic acid only weakly (cp. Figure 6.1, Figure 6.3).

In wines with high citrulline concentrations after MLF, it would be desirable to apply methods that eliminate the citrulline residues. Wines with urea residues from arginine degradation by yeasts can be treated with acid urease (Ough and Trioli 1988; Trioli and Ough 1989; Famuyiwa and Ough 1991). However, no such method has been presented for citrulline removal in wines. In this work, *L. buchneri* CUC-3 grew in a wine with depleted malic acid and arginine from growth of *O. oeni* EQ54. However, the growth was too weak to enable degradation of citrulline present in this wine. Biological citrulline removal by selected wine LAB that do not negatively affect sensory characteristics of wines may still be possible if the bacteria are prepared appropriately for inoculation (e.g. by pre-growth in a citrulline containing medium).

The pH course during the incubation of *O. oeni* EQ54 did not allow a clear identification of complete malic acid degradation because there was only a minor delay between malic acid depletion and the beginning of arginine degradation. It becomes clear that indicators of malolactic conversion that are used by some winemakers to assess the course of malolactic conversion, like the pH increase or the "sound of a tank undergoing MLF" (bubbling due to CO<sub>2</sub> formation from malolactic conversion), may not allow the accurate determination of malic acid depletion, since both qualities are a characteristic of arginine degradation by wine LAB, as well. Hence, in order to avoid arginine degradation by oenococci after complete malolactic conversion, the concentration of malic acid, itself, has to be followed.

## CHAPTER 10. KINETICS OF ARGININE DEGRADATION IN RESTING CELLS OF *L. BUCHNERI* CUC-3 BETWEEN PH 3 AND 10

### 10.1. Introduction

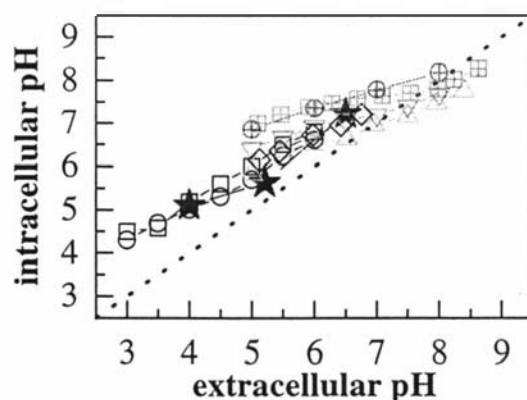
Several experiments presented in this thesis manifested the impact of the medium pH on the arginine metabolism in wine lactic acid bacteria (LAB). For example, growing cells of *L. buchneri* CUC-3 degraded arginine at different rates depending on the pH (Figure 6.1). Also, differences were found in the ability of oenococci to degrade arginine at pH 3.6 or 3.3 (Figure 6.2 and Fig. 6, Appendix). A certain minimum pH seemed to be necessary above which oenococci could degrade arginine. The effect of pH on the metabolism of other substrates by wine LAB is well established. For example, Ramos et al. (1995) showed that the products formed from citrate and pyruvate metabolism of *O. oeni* depended on the pH. The pH was also found to be an important factor in the production of biogenic amines by LAB in wine (Lonvaud-Funel and Joyeux 1994). Wibowo et al. (1985) reviewed the influence of the pH on several other metabolic activities in wine LAB, such as the production of acetic acid and diacetyl, and the degradation of tartaric and malic acid.

The effect of the medium pH on metabolic activities is understandable given the narrow pH optima of many enzymes and the relationship between extracellular or medium pH and intracellular pH in LAB. In fact, many workers have investigated intracellular pHs of LAB using radioactively labelled weak organic acids (Nannen and Hutkins 1991a; Russell 1992; O'Sullivan and Condon 1999) or fluorescent probes (Molenaar et al. 1991; Siegumfeldt et al. 2000). Figure 10.1 summarizes results from some of the publications and shows the tight relationship between extracellular and intracellular pH values in several LAB.

In fact, an important mechanism in pH homeostasis of fermenting bacteria is the pH optimum of the proton-translocating membrane-bound ATPase (Kashket 1987; Nannen and Hutkins 1991b; Hutkins and Nannen 1993; Siegumfeldt et al. 2000). This fact raises the question if the pH optimum of ADI pathway enzymes would have an effect on the kinetics of arginine degradation in wine LAB, too.

According to the data shown in Figure 10.1, the intracellular pH of wine LAB would be approximately between 4 and 5.5 at the pH values usually found in wine. In a *L. buchneri* strain, the pH optimum for arginine deiminase (ADI, cp. Figure 1, inside back cover) was reported to be 6 (Manca de Nadra et al. 1988), whereas the pH optimum of ornithine

transcarbamylase (OTC) was 8.5. Similar results were obtained by Liu (1993) who determined the pH optimum of ADI in 3 wine LAB to be 5.8 and the pH optima of OTC from 2 lactobacilli and 2 oenococci were at pH 8.5 and pH 8, respectively. For *Mycoplasma*, lower pH optima for ADI as opposed to OTC (pH 6.6 and 8.4, respectively) were reported, as well (Barile et al. 1966). Thus, it becomes clear that the optimum pH of OTC is far from the intracellular pH that prevails in LAB growing in wine.



**Figure 10.1** Intracellular and respective extracellular (medium) pH values in LAB. Data taken from several publications: *Leuconostoc mesenteroides* sp. (□) and *Leuconostoc plantarum* sp. (○) (McDonald et al. 1990); *O. oeni* sp. (★) (Ramos et al. 1995); *Leuconostoc lactis* sp. (△) (Fitzgerald et al. 1992); *Lactococcus lactis* sp. (▽) and (◇), (Kashket et al. 1980); *Lactococcus lactis* sp. (⊕) and (⊞), (Maloney 1983).

Further, it was suggested in Chapter 9 that the pH value of the media used to study the effect of arginine degradation on *O. oeni* growth may have led to the differences found between results in this thesis and those from other workers. Considering these differences, the tight relationship of extracellular and intracellular pH values in LAB, and the position of the pH optimum of OTC in several wine LAB, the question was raised whether the medium pH also had an immediate contribution to the intracellular accumulation and excretion of citrulline by wine LAB.

To test this hypothesis, resting cells of an arginine degrading strain, *L. buchneri* CUC-3, were investigated for citrulline excretion from arginine degradation over a wide pH range in a synthetic buffering system. It was not the aim of this experiment to extend knowledge of the properties of individual ADI pathway enzymes; therefore, whole cells were used over cell extracts, because this could give insights into the metabolic flow of the integral and functional ADI pathway in viable bacteria.

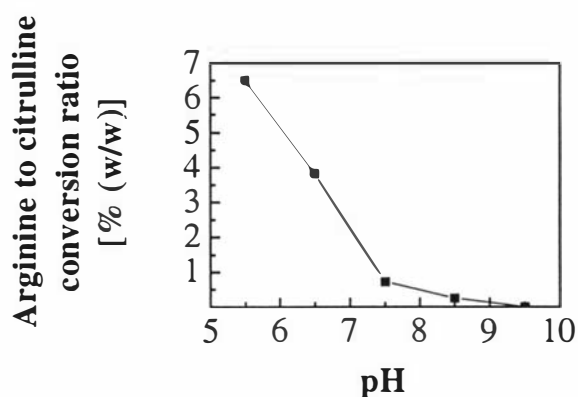
In order to ensure similar conditions for the cells at a widest possible pH range, a buffer system had to be developed. A study of several biological buffers and their  $pK_a$  values, solubility, toxicity, availability, and price led to the establishment of a quaternary buffering system ( $P_2T_2$ -buffer). Several titrations carried out with the  $P_2T_2$ -buffer showed a well balanced buffering behaviour over the desired range (pH 3.5 - 9.5) and a sufficient buffer capacity was achieved with the composition described in section 2.2.2.9.

## 10.2. Experimental conditions

For the study of the arginine metabolism over a wide pH range, resting cell experiments (2.3.2.1) were used with modifications: The sodium hydrogentartrate rce-buffer (2.2.2.8) was replaced by  $P_2T_2$ -buffer (2.2.2.9). As substrates, arginine and glucose were present in the buffer at  $1\text{ g l}^{-1}$  each. The experiments were started by adding 1 ml of *L. buchneri* CUC-3 suspension (prepared by resuspending the pre-grown and washed cells in sterile water) to 19 ml of  $P_2T_2$ , adjusted to several pH values. During a preliminary experiment, samples were taken at  $t = 0$  and a second after 30 minutes. For this, 1 ml of a cell suspension was placed into a 1.7 ml micro-centrifugation tube and cooled immediately in chilled ice ( $-20^\circ\text{C}$ , prepared by adding 1 kg of NaCl and 500 ml of water to 3 kg of ice and mixing) before centrifugation (10,000  $g$  for 5 min) and storage of the supernatant ( $-18^\circ\text{C}$ ) for subsequent analysis. The analysis of substrates is described in section 2.4. The lysis of cells during incubations was assessed as described in section 2.4.3.5. The main experiment was carried out similarly, but with one modification. Samples were taken at the time of inoculation, after 2 minutes and then every 5 minutes for a total time of 30 minutes.

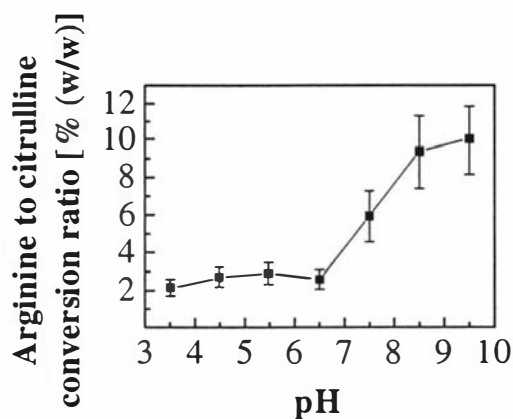
## 10.3. Results

Figure 10.2 shows the arginine to citrulline conversion ratios from the incubation of *L. buchneri* CUC-3 in  $P_2T_2$ -buffer at several pH values. This preliminary experiment was carried out to test the suitability of the method. Conversion ratios were calculated by dividing the amount of citrulline excreted by the amount of arginine degraded in the medium after 30 min. The resulting conversion ratios decreased with increasing buffer pH.



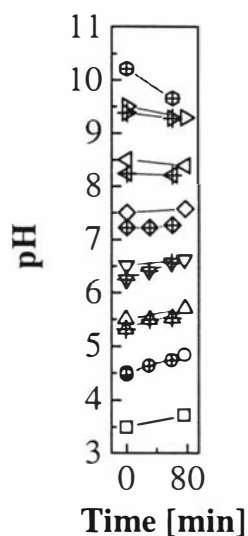
**Figure 10.2** Arginine to citrulline conversion ratios from incubations of resting cells of *L. buchneri* CUC-3 in P<sub>2</sub>T<sub>2</sub>-buffer at pH values ranging from 5.5 to 10.5. Ratios calculated from linear regression of arginine degradation and citrulline excretion rates (see text).

Figure 10.3 shows the results from a similar but more detailed study from that above, carried out in duplicate. The conversion ratios displayed were calculated by dividing the actual citrulline excretion rates by the corresponding arginine degradation rates for every pH value used. In turn, the excretion and degradation rates were obtained from the time courses of arginine and citrulline concentrations (Fig. 10, Appendix) during the incubation. The conversion ratios from incubations between pH 3.5 and 6.5 had no statistically significant difference (at  $\alpha = 0.05$ ). Above pH 6.5, however, conversion ratios increased considerably.



**Figure 10.3** Arginine to citrulline conversion ratios from incubation of resting cells of *L. buchneri* CUC-3 in P<sub>2</sub>T<sub>2</sub>-buffer at pH values ranging from 3.5 to 9.5. Ratios were calculated from linear regression of arginine degradation and citrulline excretion rates (see text). Averaged results of duplicate measurements shown. Error bars display the standard deviation.

Data taken from both the preliminary experiment and the main experiment shows that during incubations, the pH increased when the initial pH had been adjusted to values of 7.5, or below (Figure 10.4). However, when the initial pH was adjusted to 8, or higher, the medium pH decreased during incubations.



**Figure 10.4** Time course of pH values during several incubations of resting cells of *L. buchneri* CUC-3 in P<sub>2</sub>T<sub>2</sub>-buffer at pH values ranging from 3.5 to 10.5. Data taken from the preliminary experiment is represented by hollow symbols with a cross. Data from the main experiment (hollow symbols) represents averaged results from duplicate measurements.

## 10.4. Discussion

The effect of the external medium pH on the kinetics of arginine degradation by resting cells of *L. buchneri* CUC-3 was studied. Results from a preliminary study (Figure 10.2) seemed to confirm the hypothesis that citrulline excretion from arginine degradation could be lowered if the medium pH, and consequently the intracellular pH, was closer to pH 8.5, the pH optimum of the citrulline degrading enzyme, ornithine transcarbamylase (OTC, cp. Figure 1, inside back cover). This, however, could not be confirmed in a more comprehensive study of the pH effect on arginine degradation (Figure 10.3). For the calculation of conversion ratios from the preliminary experiment, the substrate concentrations in only one sample taken after 30 minutes had been compared with the initial concentrations at every pH. Thus, the experimental error of these measurements could not be considered. Additionally, it was not possible to assess if citrulline was reutilized in some of the treatments. Therefore, the results from the detailed study carried out in duplicate are certainly more meaningful. In fact, in the detailed study it was revealed that the arginine to citrulline ratios were similar between pH 3.5 and 6.5. Similarly, in studies with resting cells of the same strain shown in Chapter 5 (5.3.2), no difference in the arginine to citrulline conversion ratios could be found in the wine pH range of 3.3-3.9.

Higher pH values than 6.5 caused a significant increase in the arginine to citrulline conversion ratio (Figure 10.3). It was not possible to ascribe this characteristic clearly to a pH mediated effect on the arginine degradation kinetics since evaluation of cell lysis, which can lead to release of intracellular citrulline into the medium independent of arginine degradation (cp. Chapter 4), gave ambivalent results. If the increase in citrulline excretion at pH values over 6.5 truly originated from arginine degradation, several explanations are possible. For example, the pH dependence of the carbamate kinase (CK) activity could result in an accumulation of metabolites like carbamoyl phosphate, which in turn could inhibit OTC causing higher citrulline excretion. In fact, the pH optimum of CK from a *L. buchneri* strain has been reported to be 5.4 (Manca de Nadra et al. 1988). However, Liu (1993) reported the pH optimum of CK from several wine lactic acid bacteria (LAB) including a *L. buchneri* strain to be at pH 7.6 - 7.9. An indirect inhibition of OTC causing citrulline accumulation also might have been caused by a pH effect on transport systems. Clearly, more data is needed about the transport of arginine, ornithine and citrulline in wine LAB to evaluate the physiology of the ADI pathway in more depth. In any case, the results clearly demonstrate that the relatively high pH optimum of the citrulline degrading enzyme, OTC, is very unlikely to be the direct reason for the intracellular accumulation and excretion of citrulline from arginine degradation by heterofermentative wine LAB.

An interesting observation was made during the incubation of resting cells of *L. buchneri* CUC-3 at different pH values. This is, the buffer pH increased or decreased during incubations, depending on the initial pH value adjusted. The graphical representation of the pH courses shows that there was a trend towards pH 8, approximately (Figure 10.4). In fact, the buffer contained both glucose and arginine. Thus, by favouring one or the other degradation pathway, the bacteria were able to decrease the pH (increased formation of acids from heterofermentation of glucose) or increase the pH (increased formation of ammonia from arginine degradation). It is remarkable, though, that the pH trend towards pH 8 causes conditions that reduce the proton gradient across the bacterial membrane; i.e. similar intracellular and extracellular pHs, as shown in Figure 10.1. It remains to be verified if the metabolism of *L. buchneri* CUC-3 in actively growing cultures would also lead to similar pattern of pH courses if the substrates available in the medium allowed for rapid pH increase or decrease. This could be tested, for example, by addition of generous amounts of glucose and arginine to a weakly buffered growth medium. If growing LAB displayed the same behaviour, the acidification of media by growing cultures of LAB could be regarded as the result of either the exclusive availability of metabolizable acid generating substrates or the preference of micro-organisms to utilize acid generating substrates rather than base generating substances. LAB able to utilize substrates to increase or decrease acidity would thus have a greater metabolic flexibility.

## CHAPTER 11. GENERAL DISCUSSION

This thesis studied the degradation of the amino acid arginine via the arginine deiminase (ADI) pathway in heterofermentative wine lactic acid bacteria (LAB). Whereas the results presented in previous chapters are summarized in the abstract of this thesis, in this chapter findings will be put into context within the wider oenology and microbiology.

It is remarkable that the degradation of arginine via the ADI pathway in wine LAB shares some similarities with the formation of biogenic amines from arginine and other amino acids by the same micro-organisms.

From a health point of view, both the metabolism of arginine via the ADI pathway and the formation of biogenic amines lead to the formation of substances with toxicological significance. Biogenic amines, especially histamine and tyramine, have been connected with several medical conditions, including nausea, headaches, digestive disturbances and hyper- or hypotension (Bardocz 1995). Other biogenic amines (e.g. putrescine) are not considered to be toxic themselves, but can act as potentiators for toxic amines (Pfannhauser and Pechanek 1984; Snelten and Schaafsma 1993; Rollán et al. 1995). Additionally, highly carcinogenic nitrosamines may form from the reaction of amines and nitrite (Pfannhauser and Pechanek 1984; Bardocz 1995) and are found in some alcoholic beverages (Kune and Vitetta 1992). For example, increased cancer incidences have been correlated with the nitrosamine content in some African palm wines (Sen and Dalpe 1972; Maduagwu and Bassir 1979; Maduagwu et al. 1979; Maduagwu 1981), beer and spirits (Walker et al. 1979; Riboli et al. 1991). By analogy, the degradation of arginine by the ADI pathway leads to the production of varying amounts of citrulline, a precursor in the formation of carcinogenic ethyl carbamate (cp. 1.5.3).

From a physiological point of view, the ADI pathway may contribute to pH increase by formation of ammonia (cp. 1.5.1). Similarly, the decarboxylation reactions taking place during the formation of different biogenic amines lead to consumption of protons with a pH increasing effect. Therefore, both pathways can contribute to protecting cells from acid stress (Marquis et al. 1987; Curran et al. 1998; Nomura et al. 1999). Finally, an increase in pH leads to decreased microbiological stability in wines by increasing the risk of growth of spoilage micro-organisms that would be inhibited at lower pH values.

From a metabolic regulation point of view, the kinetics of biogenic amine formation and the degradation of arginine via the ADI pathway have further similarities in oenococci. Both metabolic activities seem to be inhibited at lower pH values (Chapter 6 for the ADI

pathway, and Lonvaud-Funel and Joyeux (1994) for biogenic amines) and occur during later stages of oenococcal growth, i.e. mainly after depletion of malic acid (Chapter 6, 7 and 9 for ADI pathway, and Coton et al. (1999) for biogenic amines). The timing of both metabolic activities may be in relation to the acid protecting role. Both metabolic processes seem to help maintain viability in stationary phase cells following the phase of rapid growth with malic acid degradation.

Regarding the similar consequences of biogenic amine formation and arginine degradation and the kinetics and potential biological role of both metabolic processes, it is not surprising that conclusions drawn from this work to avoid arginine degradation (and citrulline formation) and recommendations given in the literature to avoid formation of biogenic amines are congruent.

In order to avoid the degradation of arginine or formation of biogenic amines during malolactic fermentation (MLF), pure strains of *O. oeni* or other wine LAB with similar physiological properties should be used. Such as many lactobacilli, oenococci are able to degrade arginine and to form biogenic amines in wine (cp. Chapter 6, 7 and 8 for arginine degradation, and Coton et al. (1998; 1999) and Lonvaud-Funel and Joyeux (1994) for biogenic amine formation). However, oenococci are still preferred as starter bacteria for MLF, since these transformations can be circumvented more easily than in lactobacilli: namely, after completion of malolactic conversion by oenococci, any further bacterial activity can be inhibited in order to avoid arginine degradation and biogenic amine formation (Chapter 6, 7 and 9 for ADI pathway, and Soufleros et al. (1998) and Theodore and Gerland (1998) for biogenic amine formation). This is especially important at pH values above 3.6, where both the degradation of arginine by oenococci (Figure 6.2 and Fig. 6, Appendix) and the formation of biogenic amines (Lonvaud-Funel and Joyeux 1994; Coton et al. 1999) will be stimulated and occur earlier compared with the degradation of malic acid.

Other factors will lead to a higher risk of formation of citrulline from arginine degradation or formation of biogenic amines. These factors are generally those which will increase the concentration of amino acids in the wine, or more specifically, the concentration of arginine, one of the most important amino acids in grape musts and wines (cp. 1.2). The basis of the amino acid concentrations found in musts and wine has to be seen in the nitrogen management of the vineyards. Over-fertilization will lead to high amino acid (arginine) concentrations in musts which may still be high in the wine after alcoholic fermentation (cp. 1.5.3.3). Addition of ammonia as a yeast nutrient during alcoholic fermentation may also cause increased arginine residues in wine (cp. 8.4). There are also reports that a high content of amino acids may enrich the body and enhance the mouth feel of dry white wines, and it

has been suggested to use yeasts deficient in amino acid uptake to obtain wines with these characteristics (Kishimoto et al. 2000). Further, storing the wines on yeast lees has been shown to increase the formation of citrulline in this work (Chapter 7) and to increase the formation of biogenic amines (Lonvaud-Funel and Joyeux 1994; Nouadje et al. 1997) since the yeast lees will enlarge the amino acid pool through the activity of yeast and bacterial proteases. It also has been reported that under poor nutrient conditions (e.g. absence of glucose and malic acid) the formation of biogenic amines by oenococci is favoured (Lonvaud-Funel and Joyeux 1994). Similarly, data shown here (cp. 9.3.4 and 9.4.3) suggests that the arginine to citrulline conversion ratio may be higher in wines that support weak growth.

Reports about the occurrence of biogenic amine-forming micro-organisms of wine origin suggest that the ability of oenococci and lactobacilli to form biogenic amines is strain specific (Straub et al. 1995; Moreno-Arribas et al. 2000; Leitão et al. 2000). In this work, it was shown that all heterofermentative wine LAB strains of the genus *Lactobacillus* and *Oenococcus* analyzed in resting cell experiments at wine pH had the ability to degrade arginine and to excrete citrulline (cp. Chapter 4). However, the occurrence of arginine negative oenococci has been reported (Liu et al. 1995b) and an arginine negative oenococcal strain is being commercialized (CH35, Chr. Hansen, Denmark). In preliminary studies carried out by the author, resting cells of this strain, in fact, failed to degrade arginine (results not shown). Therefore, it is suggested that the occurrence of arginine positive strains is also strain specific, but with a higher frequency than the occurrence of biogenic amine-forming wine LAB strains.

Since wine LAB strains do exist that do not have the ability to degrade arginine or to form biogenic amines, it is desirable to test potential MLF starter bacteria for these metabolic characteristics in order to exclude strains that possess these unwanted physiological abilities. In the case of the ADI pathway, the selection of arginine negative strains can be recommended, since in this work it was shown that commercial oenococcal starter bacteria did not have a growth advantage in degrading arginine in wine. Thus, arginine negative starter strains would not have an ecological (growth) disadvantage compared with naturally present and likely arginine positive oenococci.

Potential MLF starter bacteria can be assessed for arginine degradation or biogenic amine formation by their phenotype. That is, after growth of bacteria in media containing the necessary substrates, the chemical transformations are determined. In the case of biogenic amines, this method would be tedious if used to screen many bacteria, since quantitative analysis of biogenic amines is usually carried out by time-consuming and costly HPLC or gas chromatography methods (Soufleros et al. 1998; Vazquez-Lasa et al. 1998; Soufleros

and Bertrand 1998; Glória et al. 1998; Mafra et al. 1999; Sass-Kiss et al. 2000). To test for arginine degradation in wine LAB, Pilone et al. (1991) proposed a method where cells were grown in an arginine containing medium followed by analysis of ammonia formation by the Nessler's reaction after a certain time of incubation. This method, however, is not sensitive enough to identify weak arginine degraders (Liu et al. 1995b) and may also give false positive results. For example, Granchi et al. (1998) suggested that amino acids other than arginine can be degraded leading to ammonia formation. They reported, as well, that some oenococci degraded arginine but that ornithine was not found in the medium and explained this observation with the formation of putrescine, a biogenic amine (cp. Figure 1, inside back cover). Therefore, in order to screen for the occurrence of an operative ADI pathway in micro-organisms, the direct measurement of arginine degraded and one of its metabolites formed (citrulline or ornithine) is necessary. Since colorimetric methods available for the metabolites of the ADI can present interferences when used with samples from complex media (cp. Chapter 3), the application of HPLC would be required, as well.

Generally, methods including the culturing of bacteria may conceal the ability to degrade arginine or form biogenic amines. That is, the conditions chosen may not lead to these metabolic activities, whereas under different conditions the transformations may occur. Other methods can be more effective in order to screen bacteria for the ability to form biogenic amines or degrade arginine via the ADI pathway. For example, Le Jeune et al. (1995) presented genetic tests using specific PCR primers or DNA/DNA hybridisation to assess the ability of potential MLF starter bacteria to form histamine. The tests are based on the fact that HDC (histidine decarboxylase) is present in all bacteria producing histamine, and that therefore the protein coding *hdc* gene is also present (Coton et al. 1999). So far, no similar test is available to detect the ability of bacteria to degrade arginine via the ADI pathway. Using specific PCR primers for genes of ADI pathway enzymes (cp. 1.4.3), Tonon et al. (2001) detected all three genes for the ADI pathway enzymes ADI, OTC and CK in several oenococcal strains that were arginine negative by phenotype. As well, Ferrer (S. Ferrer, Personal Communication) tested arginine negative wine LAB positive for the gene encoding for OTC using PCR with a specific primer. The inability to degrade arginine seems not to be linked to the absence of ADI pathway enzymes and consequently, it is impossible to discriminate strains which by phenotype are able or unable to catabolize arginine by detecting the genes of ADI pathway enzymes. Accordingly, the ability of bacteria to degrade arginine and to excrete citrulline, so far, has to be assessed by the phenotype. Since growth of wine LAB at (physiological) low pH values is slow and degradation of arginine may only be revealed after weeks of incubation, it is suggested that resting cell experiments be used in order to assess the potential ability of strains to degrade arginine in wine via the ADI pathway. This is preferred over using growing cultures at non-physiological pH values. In this thesis, results obtained using this method were similar to

results from growth in wine. Resting cell experiments were also used with good results in order to assess the metabolism of free and SO<sub>2</sub>-bound acetaldehyde in wine LAB (Osborne et al. 2000). Moreover, since the buffer used in resting cell experiments is a simple tartaric acid solution, cheap and fast colorimetric methods can be used to measure arginine and citrulline concentrations. Conclusions from resting cell experiments can later be complemented with results from detailed time course studies of growing cultures.

Recently, Krieger (2000) suggested that the course of MLF by oenococci can be subdivided into three phases. During the first phase, growth occurs accompanied by sugar catabolism and weak acetate production. During the second phase, no further growth or sugar degradation occurs. The predominant transformation during this second phase is the malolactic conversion. The third phase is characterized by the degradation of citric acid, and the production of significant amounts of acetate. Indeed, the formation of acetate during late stages of MLF was observed before by Lafon-Lafourcade (1979; 1980) and by Bréchet et al. (1974), who suggested that the "pique lactique" (increased concentrations of lactic and acetic acid from malolactic fermentation) is caused by MLF in total or nearly complete absence of malic acid. Results obtained from several MLFs with oenococci in this work principally agree with the model proposed by Krieger et al. (2000), at least at low pH values (i.e. below pH 3.5: see data for pH value 3.3 in Figure 6.2 and Fig. 6, Appendix). A modification is suggested for pH values over 3.5, where malic acid degradation always occurred simultaneously with growth; malic acid degradation was even completed before growth ceased (pH values 3.6 and 3.9 in Figure 6.2 and Fig. 6, Appendix; see also at pH 3.5 in Figure 9.5). Therefore, the first two phases overlapped significantly at pH values of 3.5 and higher. In any case, both the results from this thesis, and those presented by Krieger et al. (2000) and by Bréchet et al. (1974) agree in that the metabolic transformations taking place in the third phase were inhibited by the presence of malic acid. According to the present work on the ADI pathway, the degradation of arginine by oenococci happens in the third phase, as well as the formation of biogenic amines. From an oenological point of view, the first two phases are desired and required for successful malolactic conversion. The third phase, however, is unwanted regarding the metabolic transformations taking place (formation of acetic acid, biogenic amines and citrulline) and should be avoided. The biological role of arginine degradation for oenococcal growth seems to be relatively small. It was shown that neither arginine nor citrulline stimulated growth of selected oenococci at different wine pH values (Chapter 9). Additionally, complete malolactic conversion by *O. oeni* EQ54 was possible in a wine depleted of free arginine from previous growth of *L. buchneri* CUC-3 (cp. 9.3.4 and 9.4.3). Degradation of arginine by stationary phase oenococci, therefore, might mainly provide maintenance energy. In comparison, arginine degradation strongly stimulated growth of *L. buchneri* CUC-3, and if the model of Krieger et al. (2000) is applied to this micro-organism, arginine degradation takes place in the

second phase (at pH 3.3 in Figure 6.1) or a combined first and second phase (at pH 3.6 and 3.9 in Figure 6.1). Hence, the degradation of arginine and malic acid has analogous functions in *L. buchneri* CUC-3 and oenococci, respectively. Both transformations lead to an increased pH. Additionally, they contribute to the conservation of energy and are coupled to growth, at least at pH values of 3.5 and above.

In the future, more work needs to be done to identify the degradation kinetics of other substrates with respect to malic acid degradation, and to assess the possible stimulating effect of such substrates on wine LAB growth. Apart from sugars, malic acid or arginine, the metabolism of acetaldehyde, for example, can increase growth in some oenococci (R. Mira de Orduña, unpublished work). Knowledge of potential energy sources and the conditions under which they can be utilized would be useful for several reasons. It would allow for the selection of bacteria with appropriate metabolic capacities that would give good growth and successful malolactic conversion in wines. Also, it would allow a better assessment of the microbiological stability of wines. It has been demonstrated that wines after malolactic fermentation are able to support growth of different wine LAB on re-inoculation (Costello et al. 1983; Davis et al. 1986a; Wibowo et al. 1988). In this thesis, it was shown that the depletion of either arginine or malic acid could not prevent further microbial growth upon re-inoculation. Thus, the risk of spoilage could be better assessed with more information about energy sources in wines and their stimulating effect on wine LAB growth. Finally, further knowledge of the energy yielding substrates in wines and the reactions involved in energy conservation could open the possibility of using wine LAB with other objectives than the degradation of malic acid. For example, white wines from hot countries that are low in natural acidity where malic acid reduction from MLF is not recommended, could still benefit from some of the metabolic conversions that wine LAB can realize, such as the degradation of acetaldehyde (see Liu and Pilone (2000) for recent review on acetaldehyde) or the formation of flavour compounds (Bartowsky and Henschke 1995; Laaboudi et al. 1995; Henick-Kling and Acree 1998; Nielsen and Richelieu 1999). This could be achieved with wine LAB that do not or only weakly degrade malic acid but can utilize other energy yielding substrates.

From an energetic point of view, the activity of the ADI pathway in late exponential or stationary phase of oenococci is of particular interest. Tonon et al. (2001) reported that arginine degradation via the ADI pathway leads to ATP formation. However, the gain in energy from arginine degradation was not coupled to growth of oenococci in wine (cp. Chapter 6 and 9). This suggests that oenococci possess the ability to turn over ATP by processes unassociated with growth. It is true in the case of *L. buchneri* CUC-3 that arginine degradation stimulated growth. However, strain CUC-3 must also possess the ability to turn over ATP by other means, since all wine LAB tested in resting cell experiments for their

ability to degrade arginine (including CUC-3) did so in the absence of cellular growth. Considering the large amounts of arginine degraded by wine LAB in resting cell experiments (up to  $5 \text{ g l}^{-1}$ , cp. Chapter 5), it is unlikely that energy was uniquely used for cell maintenance, e.g. protein turnover, keeping a constant membrane potential or acid stress response (Guzzo et al. 2000). Therefore, the question arises if arginine degrading wine LAB possess the ability to spill energy deliberately in order to dissociate the energy conservation from arginine degradation from energy consuming (anabolic) reactions. The excretion of citrulline during arginine degradation, itself, could be regarded as a mechanism to prevent ATP formation, since the latter requires the degradation of citrulline and carbamoyl phosphate (cp. Figure 1, inside back cover). However, citrulline excretion, up to now, has been exclusively described as a result of the unfavourable thermodynamic balance of the citrulline degrading enzymatic reaction (cp. 1.4.4), and to some degree, citrulline excretion is regulated by mechanisms of enzymatic feedback inhibition (cp. Chapter 5). Nevertheless, it is remarkable that the arginine to citrulline conversion ratios of oenococci were mostly higher than those of *L. buchneri* CUC-3 and that there was a correlation to the growth phase where arginine degradation occurred. Also, the arginine to citrulline conversion ratio of strain CUC-3, itself, was flexible. In some experiments shown in this thesis, the ratio was higher when arginine degradation occurred in a medium supporting only reduced growth (cp. 9.3.4 and 9.4.3). In fact, these results suggest that in *Oenococcus* and *L. buchneri* CUC-3, higher arginine to citrulline conversion ratios may also be linked to arginine degradation during weak or non-existing growth and this behaviour has been reported for citrulline excretion in cultures of *Mycoplasma hominis* (Barile et al. 1966). Therefore, the excretion of citrulline could also have aspects of an overflow metabolism (Russell and Cook 1995; Teixeira de Mattos and Neijssel 1997) and may well be an adaptation to media with lower nutrient availability allowing wine LAB the control of biologically useable energy formation (ATP) relative to their anabolic requirements (Tempest and Neijssel 1992; Teixeira de Mattos and Neijssel 1997). Nevertheless, the percentage of arginine excreted as citrulline was relatively low in all experiments presented in this work ( $< 5\%$  (w/w) of citrulline excreted per arginine degraded). Therefore, arginine degrading wine LAB may possess other regulatory mechanisms in order to spill energy produced from complete arginine degradation. Mechanisms causing a net loss of ATP may include energy spilling by the membrane bound ATPase, resulting from changes in the membrane resistance and thus the proton conductance (Russell and Stroebel 1990; Cook and Russell 1994). Poolman et al. (1987) reported that uncouplers of the membrane bound ATPase increased the activity of the ADI pathway in a *Streptococcus*, but they did not assess the arginine to citrulline ratio. Hence, it remains to be studied if the arginine to citrulline conversion ratio could be decreased upon addition of ionophores to arginine degrading cultures. In stationary oenococci, it could also be possible that the membrane resistance is not actively regulated but just modified as a result of membrane stresses (e.g. acid stress). Then, membrane-

stabilizing substances like magnesium ions that delay lysis of resting cells could also have an effect on the kinetics of arginine degradation. On the basis that conditions that led to slow growth in some cases led to increased citrulline excretion, high ethanol concentrations (>10% [v/v]) could also have this effect, since ethanol can cause slow or sluggish growth (Wibowo et al. 1985). However, it has to be considered that not all data presented in this thesis supports the hypothesis of an influence of growth on arginine to citrulline excretion ratios (cp. results in Chapter 6), and this area needs further study.

The results presented in this thesis on the energetics of arginine degradation are preliminary and clearly more work has to be done to clarify the microbiological significance of arginine degradation by heterofermentative wine LAB. Apart from detailed growth studies in defined media, future work should also include the assessment of intracellular fluxes of ADI pathway metabolites. For example, the possibility exists that carbamoyl phosphate accumulates intracellularly, or is diverted towards the biosynthesis of pyrimidines in growing cells (Bringel 1998). Consequently, carbamoyl phosphate would not be available for the ATP forming carbamoyl kinase reaction in these organisms. Knowledge of the intracellular metabolite flows during arginine degradation might also help to understand the regulation of the ADI pathway.

It was a principal concern of this thesis to study arginine degradation by wine LAB in order to control the formation of carcinogenic ethyl carbamate precursor citrulline. The arginine to citrulline conversion ratios obtained from all experiments done in wine (Chapter 6, 7 and 9) ranged from 0.8 to 4.6% (w/w) and had an average of  $3.8(\pm 1.2)\%$  (SD in parentheses). Using this average and the ratio of 0.15% for the conversion of citrulline to ethyl carbamate (after 3 years of storage at 15°C, cp. 1.5.3.1), it can be concluded that the complete degradation of an arginine residue of approximately  $500 \text{ mg l}^{-1}$  by wine LAB has the potential to form  $30 \mu\text{g l}^{-1}$  of ethyl carbamate under normal storage conditions. Regular consumption of wines containing this concentration of ethyl carbamate would constitute a significant increase in the lifetime cancer risk (Schlatter and Lutz 1990), which is reflected in the legal and voluntary limits adopted in the U.S.A. and Canada (cp. 1.5.3.2). However, several suggestions have been given in this work for controlling the formation of citrulline from arginine degradation by wine LAB, and these recommendations are summarized in the abstract of this thesis.

In any case, the potential presence of ethyl carbamate in table wines should not obscure the fact that more and more evidence (epidemiological and experimental) is being presented on the health promoting effect of moderate table wine consumption, especially in the reduction of coronary heart diseases and other cardiovascular diseases (Ellison 1998; Broustet 1999; Bujanda et al. 1999; de Lorimier 2000; German and Walzem 2000). Specifically, different

wine phenolics have been shown to possess antioxidant, vasorelaxing and antithrombic effects (Teissedre et al. 1996; Halpern et al. 1998; Wamhoff et al. 1998) and inhibition of nitrosamine formation has been shown for anthocyanins (Tomera 1999). Additionally, wine plays an important role for social interactions, cultural activities and gastronomy.

Compared with many other food products, the concentration of potentially toxic compounds in wine is low (Cerutti 1980). Nevertheless, it is desirable to further decrease the concentration of potentially harmful substances in wine; not only in view of possible legal maxima, but also to limit the contribution of wine to the pool of health affecting substances already present in our daily food. This work intends to contribute to the general effort in keeping grape wine a healthy beverage. Regarding sensationalistic messages that regularly invade the press with mostly unobjective messages of toxic foodstuffs, I would like to conclude this thesis with a statement of Paracelsus, who recognized early: "All things are poison, and nothing is without toxicity. Only the dose permits anything not to be poisonous" (Theophrastus von Hohenheim *alias* Paracelsus, 1493-1541).

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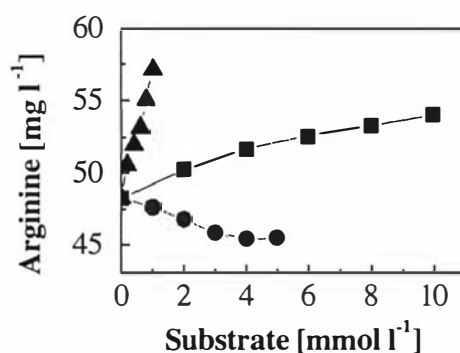
## APPENDIX

**Tab. 1** Growth of selected wine LAB in VJ medium (pH 3.8) with several carbohydrates added at 2 g l<sup>-1</sup>. Qualitative evaluation of  $\mu_{\max}$  and  $Y_{\max}$  values (as measured by OD at 700 nm) in comparison to a control without added carbohydrate.

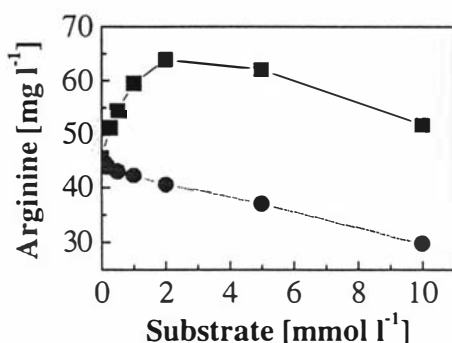
	Monosaccharides										Disacch.		Polyol	
	Pentose		Hexoses						Deoxyhexose					
	Ribose		Fructose	Glucose	Galactose	Rhamnose		Trehalose		Mannitol				
	$\mu_{\max}$	$Y_{\max}$	$\mu_{\max}$	$Y_{\max}$	$\mu_{\max}$	$Y_{\max}$	$\mu_{\max}$	$Y_{\max}$	$\mu_{\max}$	$Y_{\max}$	$\mu_{\max}$	$Y_{\max}$	$\mu_{\max}$	$Y_{\max}$
CUC-3	++	++	+	+	+	+	-	-	-	-	-	-	-	-
Lo-111	++	++	+	+	+	+	-	-	-	-	+	-	-	-
MCW	++	++	+	+	+	+	-	-	-	-	-	-	-	-

"-", no increase of  $\mu_{\max}$  and  $Y_{\max}$  compared with control without added carbohydrate.

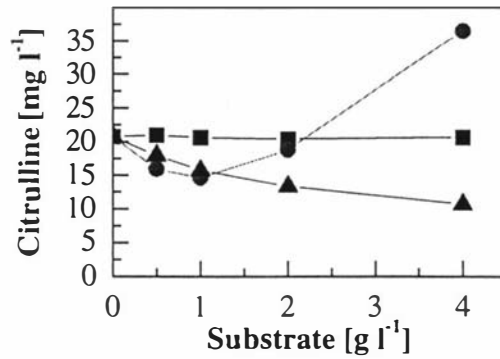
"+" or "++", increase or strong increase of  $\mu_{\max}$  and  $Y_{\max}$  compared with control



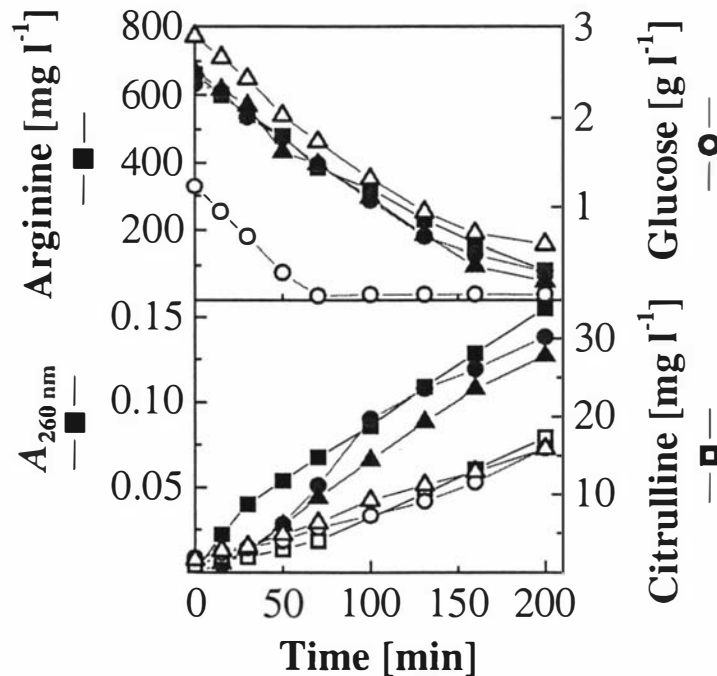
**Fig. 1** Interference of ornithine, ammonia and citrulline with arginine determination by the Voges-Proskauer method (cp. 3.1): An arginine standard (48 mg l<sup>-1</sup>) was adjusted to several concentrations of ammonia, citrulline and ornithine, and the arginine determined. Ammonia (■) and citrulline (▲) gave false positive results, whereas lower apparent arginine concentrations were obtained with ornithine (●).



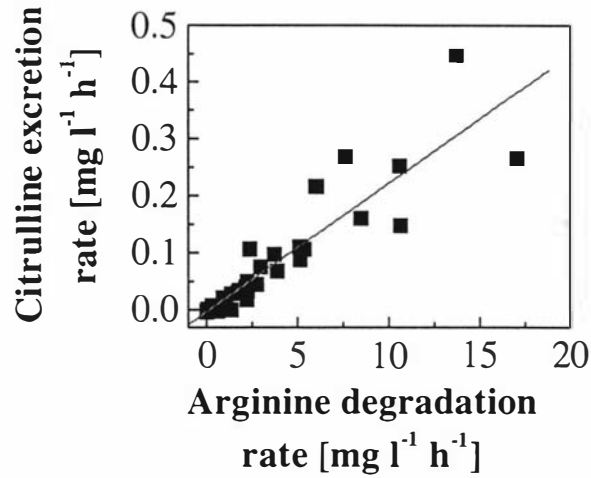
**Fig. 2** Interference of ammonia and ornithine with arginine determination by the Staron-Allard method (cp. 3.1): An arginine standard (48 mg l<sup>-1</sup>) was adjusted to several concentrations of ammonia and ornithine, and the arginine determined. Ammonia (■) gave false positive results at concentrations of 0 - 10 mmol l<sup>-1</sup> of ammonia. Presence of ornithine (●) led to false negative results.



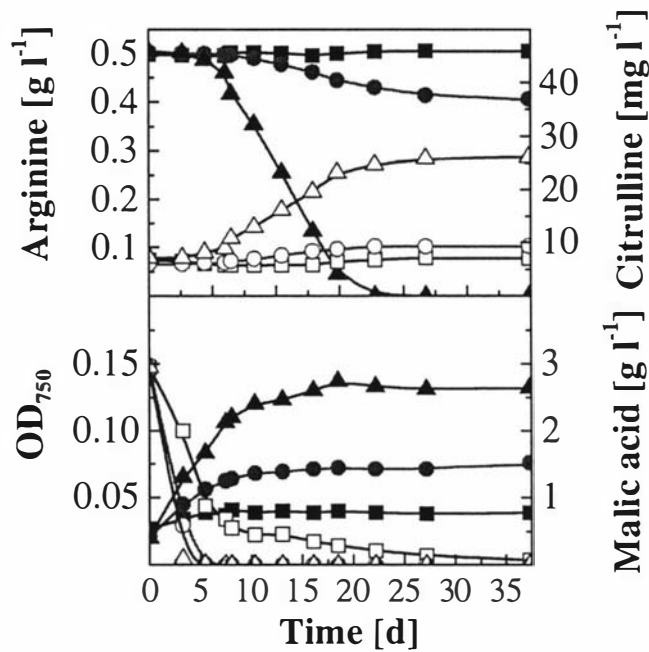
**Fig. 3** Interference of glucose, fructose and ribose with citrulline determination by the method of Archibald as modified by Spector and Jones (cp. 3.1). A citrulline standard ( $22 \text{ mg l}^{-1}$ ) was adjusted to several concentrations of D-glucose, D-fructose and D-ribose, and the arginine determined. Glucose (■), fructose (●) and ribose (▲). Fructose and ribose gave variable false results, whereas glucose did not interfere with the citrulline determination at the concentration range studied.



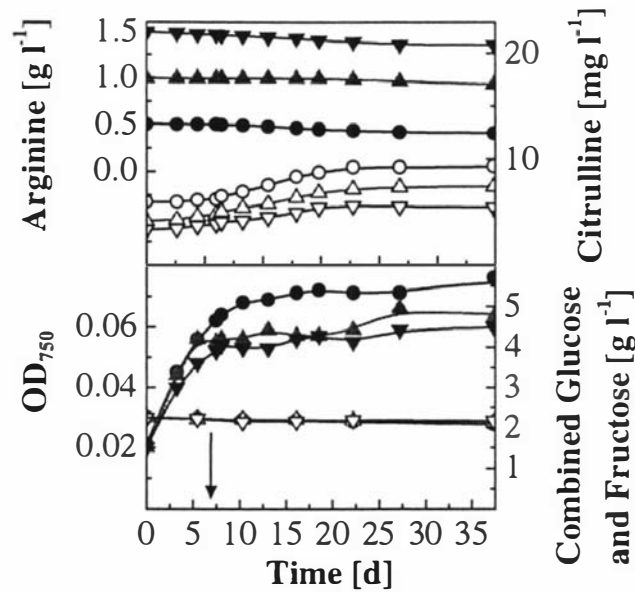
**Fig. 4** Degradation of arginine and excretion of citrulline by resting cells of *O. oeni* Lo-111 at several glucose concentrations (pH 3.6). Arginine and  $A_{260 \text{ nm}}$  (solid symbols), glucose and citrulline (open symbols):  $0 \text{ g l}^{-1}$  glucose (■, □),  $1.2 \text{ g l}^{-1}$  (●, ○),  $3 \text{ g l}^{-1}$  (▲, △). Average values from duplicate incubations shown. The arginine to citrulline conversion ratios calculated from linear regression of the arginine degradation and citrulline excretion rates were:  $2.8 \pm 0.3\%$  ( $0 \text{ g l}^{-1}$ ),  $2.5 \pm 0.26\%$  ( $1.2 \text{ g l}^{-1}$ ),  $2.3 \pm 0.27\%$  ( $3 \text{ g l}^{-1}$ ).



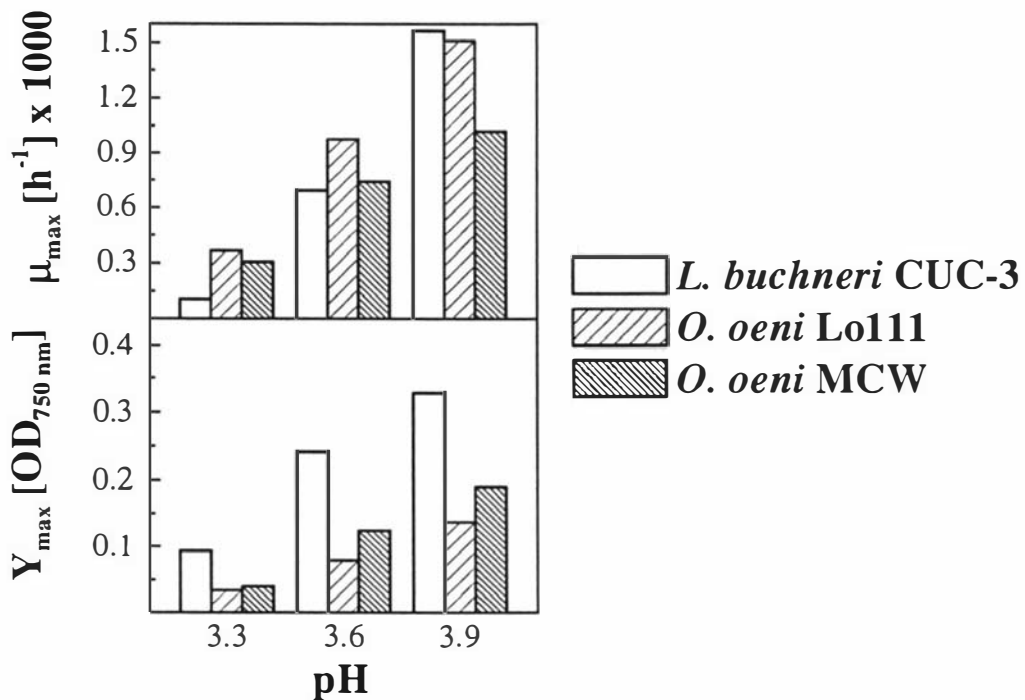
**Fig. 5** Correlation of arginine degradation and citrulline excretion rates from experiments with *L. buchneri* CUC-3 in wine. The data was taken from MLFs at several pH values and arginine concentrations. Correlation coefficient  $r = 0.91$  ( $n = 41$ ).



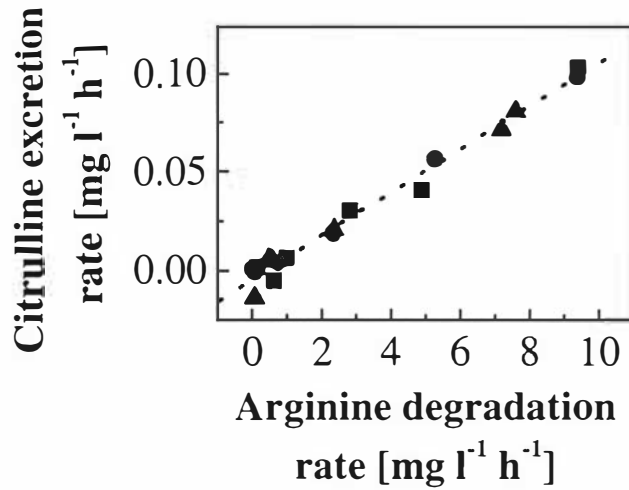
**Fig. 6** Time course of arginine and malic acid degradation and citrulline formation by *O. oeni* Lo-111 in wine at  $0.5 \text{ g l}^{-1}$  initial arginine and several initial pH values. Arginine and  $\text{OD}_{750}$ , solid symbols; citrulline and malic acid, open symbols. Initial pH values: 3.3 (■), 3.6 (●) and 3.9 (▲).



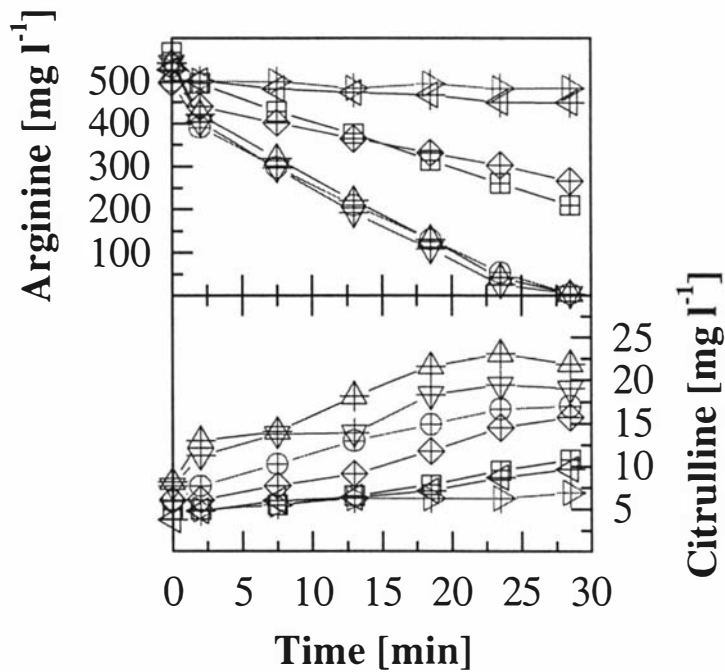
**Fig. 7** Time course of arginine and sugar utilization and citrulline formation by *O. oeni* Lo-111 in wine at initial pH 3.6 and several arginine concentrations. Arginine and OD<sub>750</sub>, solid symbols; citrulline and combined glucose and fructose, open symbols. Initial arginine concentrations: 0 g l<sup>-1</sup> (■), 0.5 g l<sup>-1</sup> (●), 1 g l<sup>-1</sup> (▲), 1.5 g l<sup>-1</sup> (▼). Arrow shows time of malic acid depletion for all treatments.



**Fig. 8** Maximum growth rate ( $\mu_{\max}$ ) and maximum growth yield ( $Y_{\max}$ ) for *L. buchneri* CUC-3 and *O. oeni* strains MCW and Lo-111 from MLFs at several initial pH values.



**Fig. 9** Arginine degradation and citrulline excretion rates of *L. buchneri* CUC-3 in fermentations with added hexoses. Data taken from Figure 6.6. Control (■), with 20 g l<sup>-1</sup> added glucose (●), with 20 g l<sup>-1</sup> added fructose (▲). Straight line obtained from linear regression of data taken from all treatments.



**Fig. 10** Degradation of arginine and excretion of citrulline by resting cells of *L. buchneri* CUC-3 in P<sub>2</sub>T<sub>2</sub>-buffer at several pH values. Initial pH 3.5 (⊞), 4.5 (⊕), 5.5 (⊞), 6.5 (⊞), 7.5 (⊕), 8.5 (⊞), 9.5 (⊞). Averages of duplicate measurements shown.

## PUBLICATIONS FROM THIS THESIS

This list only contains refereed publications and only those that are connected with the subject of this thesis. Other refereed publications and oral presentations can be found in the Curriculum Vitae, below. Further manuscripts are being prepared for submission.

1. **Mira de Orduña, R.** 2001. Quantitative determination of L-arginine by enzymatic end-point analysis. *J. Agric. Food Chem.* **49**:549-552.
2. **Mira de Orduña, R., M. L. Patchett, S.-Q. Liu, and G. J. Pilone.** 2001. Growth and arginine metabolism of wine LAB *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. *Appl. Environ. Microbiol.* **67**.
3. **Mira de Orduña, R., S.-Q. Liu, M. L. Patchett, and G. J. Pilone.** 2000. Kinetics of the arginine metabolism of malolactic wine LAB *Lactobacillus buchneri* CUC-3 and *Oenococcus oeni* Lo-111. *J. Appl. Microbiol.* **89**:547-552.
4. **Mira de Orduña, R., S.-Q. Liu, M. L. Patchett, and G. J. Pilone.** 2000. Ethyl carbamate precursor citrulline formation from arginine degradation by malolactic wine LAB. *FEMS Microbiol. Lett.* **183**:31-35.

## CURRICULUM VITAE

### Education and Practical Training

- 1998 - 2001 Ph.D. thesis on arginine metabolism of malolactic bacteria at the Institute of Molecular BioSciences, Massey University under supervision of Dr. G. J. Pilone (until June 2001).
- 1997 Practical training at Boehringer Mannheim, Mannheim (Germany) in the division „Biochemica - Marketing“ (now Roche Molecular Biochemicals).
- 1996 - 1997 M.Sc. thesis on growth and physiology of the antibiotic producing *Streptomyces coelicolor* at the Dept. of Microbiology (Biotechnology-Antibiotics) of the Universität Tübingen under Prof. H.-P. Fiedler.
- 1993 - 1994 Study of biochemistry and molecular biology at the Universidad Autónoma de Madrid, Spain with a scholarship of the German DAAD and the Spanish Foreign Ministry.
- 1990 - 1996 Study of biology at the Universität Tübingen (Germany) with majors in microbiology, biochemistry and plant physiology.

### Professional Experience, Memberships

#### Teaching Experience

Demonstrating and laboratory supervision of undergraduate students; supervision of Dip.Tech. and M.Sc. graduate students.

#### Financial Responsibility

Research budget management (totalling over 130,000 NZD).

#### Languages

German (native), Spanish (native), French, English

#### Computing

Text editing software: MS Word, MS Excel, MS Powerpoint, Adobe Acrobat

Bibliographic software: RIS ProCite, Reference Manager

Graphical and statistical software: Microcal Origin, MS Excel, Corel Draw

Web design: Macromedia Dreamweaver, Fireworks (Wine Cellar Homepage)

Relational database design: MS Access

#### Professional Memberships

New Zealand Microbiological Society, American Society for Enology & Viticulture, American Society for Microbiology

## Scholarships, Awards and Funding

- 2001 5,000 USD financial support from Lallemand, Inc. for a project entitled "Effect of malolactic strain and timing of malolactic fermentation on the physiochemical parameters and sensory characteristics of Chardonnay wine".
- 2000 Best Oenology Student Paper at the Annual Meeting of the American Society for Enology and Viticulture, Seattle, 2000, sponsored by Lallemand, Inc. (1,000 USD).
- 2000 Travel grant of the Institute of Molecular BioSciences, Massey University (1,000 NZD).
- 1999 NZMS Student Prize for oral presentation at the International Annual Conference of the New Zealand Microbiological Society (300 NZD).
- 1999 Travel grant of the New Zealand Microbiological Society (500 NZD).
- 1998 Commendation for oral presentation at the International Annual Meeting of the New Zealand Microbiological Society and the New Zealand Society for Biochemistry and Molecular Biology, Masterton, 1998.
- 1998 – Ph.D. research project funded by American Vineyard Foundation
- 2001 (54,000 USD over 3 years).

## Scientific Presentations

### Refereed Publications (corresponding author in bold type)

- **Mira de Orduña, R.**, Patchett, M. L., Liu, S.-Q. and Pilone, G. J. (2001) Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. *Appl. Environ. Microbiol.* 67, 1657-1662.
- **Mira de Orduña, R.** (2001) Quantitative determination of L-arginine by enzymatic end-point analysis. *J. Agric. Food Chem.* 49, 549-552. (incl. Supplementary Information)
- Osborne, J. P., **Mira de Orduña, R.**, Liu, S.-Q. and Pilone, G. J. (2000) Acetaldehyde metabolism by wine lactic acid bacteria. *FEMS Microbiol. Lett.* 191, 51-55.
- **Mira de Orduña, R.**, Liu, S.-Q., Patchett, M. L. and Pilone, G. J. (2000) Kinetics of the arginine metabolism of malolactic wine lactic acid bacteria *Lactobacillus buchneri* CUC-3 and *Oenococcus oeni* Lo-111. *J. Appl. Microbiol.* 89, 547-552.
- Mira de Orduña, R., Liu, S.-Q., Patchett, M. L. and **Pilone, G. J.** (2000) Ethyl carbamate precursor citrulline formation from arginine degradation by malolactic wine lactic acid bacteria. *FEMS Microbiol. Lett.* 183, 31-35.
- Mira de Orduña, R. and **Theobald, U.** (2000) Intracellular glucose 6-phosphate content in *Streptomyces coelicolor* upon environmental changes in a defined medium. *J. Biotechnol.* 77, 209-218.

- **Mira de Orduña, R.** (1997) Characterization of growth and physiology of *Streptomyces coelicolor* A3(2). M.Sc. Thesis. Universität Tübingen, Germany [German with English abstract].

### Oral Conference Presentations (presenting author in bold type)

- **Mira de Orduña, R.**, Pilone, G. J., and Pickering G.J. (July 2001, invited) Simultaneous Alcoholic and Malolactic Fermentations in the Production of a New Zealand Chardonnay - Chemical and Sensory Evaluation. Eastern Section of the American Society for Enology and Viticulture, Niagara-on-the-Lake, Ontario, Canada.
- **Mira de Orduña, R.**, Liu, S.-Q. and Pilone, G. J. (June, 2001) Effect of Malolactic Strain and Timing of Inoculation on Chemical and Sensory Characteristics of Chardonnay. International Annual Meeting of the American Society for Enology and Viticulture, Convention Center, San Diego, USA.
- **Mira de Orduña, R.**, Osborne, J.P, Pilone, G. J. and Liu, S.-Q. (April 2001, invited) Acetaldehyde Metabolism in Malolactic Bacteria and its Effect on Bacterial Growth. Lallemand International Technical Meeting, Perugia, Italy.
- **Mira de Orduña, R.** (18. Jun. 2000) Arginine degradation by lactic acid bacteria in wine: toxicological and enological implications. International Annual Meeting of the American Society for Enology and Viticulture, Convention Center, Seattle, USA. (*Presentation awarded with Best Enology Student Paper Prize, sponsored by Lallemand, Inc.*)
- **Mira de Orduña, R.** (4. May 2000) Arginine metabolism in malolactic bacteria and its oenological implications. Eastern Institute of Technology Viticulture and Oenology Seminar Series 2000, Taradale, Hawke's Bay, New Zealand.
- **Mira de Orduña, R.** (12. Feb. 2000) Lactic acid bacteria in wine: arginine degradation and its consequences. Meeting of the Microbial Ecology Special Interest Group of the New Zealand Microbiological Society, AgResearch, Palmerston North, New Zealand.
- **Mira de Orduña, R.**, Liu, S.-Q. and Pilone, G. J. (23. Nov. 1999) Arginine degradation by lactic acid bacteria in wine: energetic, toxicological and oenological implications. International Annual Conference of the New Zealand Microbiological Society, Dunedin, New Zealand. (*Presentation awarded with NZMS Student Prize*)
- **Mira de Orduña, R.** (30. Nov. 1998) Arginine Metabolism in Wine Lactic Acid Bacteria. International Annual Conference of the New Zealand Microbiological Society and the New Zealand Society for Biochemistry and Molecular Biology, Masterton, New Zealand. (*Presentation received commendation within NZMS Student Competition*)

### Poster Conference Presentations (presenting author in bold type)

- Osborne, J. P., **Mira de Orduña, R.**, Liu, S.-Q. and Pilone, G. J. (18. Jun. 2000) Acetaldehyde metabolism in malolactic bacteria. Int. Annual Meeting of the American Society for Enology and Viticulture, Convention Center, Seattle, USA.

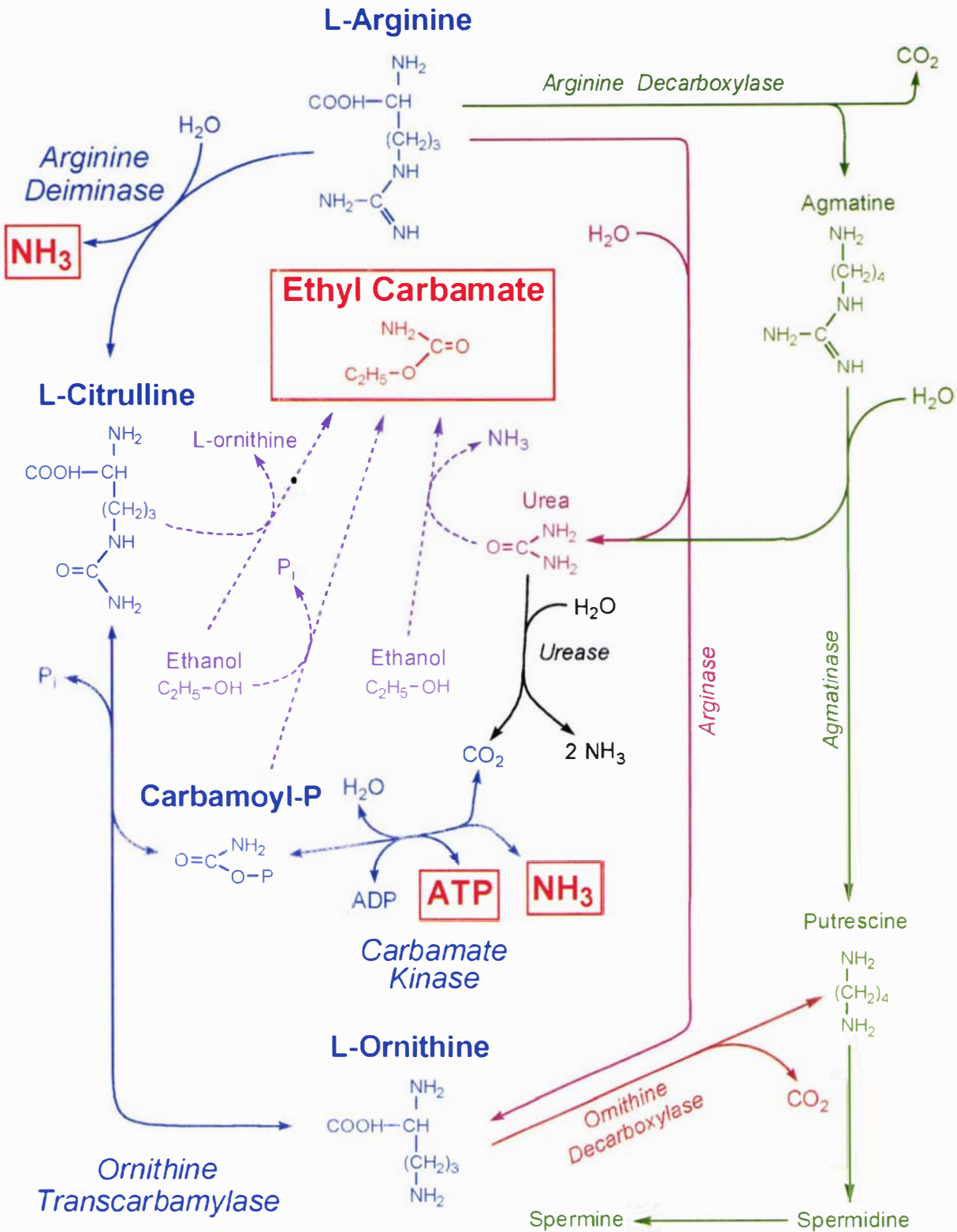
- **Pilone, G. J.**, Nuttall, E., Henry, R., Mira de Orduña, R., Patchett, M. L. and Liu, S.-Q. (23. Nov. 1999) Detecting the arginine deiminase pathway in wine lactic acid bacteria. International Annual Conference of the New Zealand Microbiological Society and the New Zealand Society for Biochemistry and Molecular Biology, Masterton, New Zealand.

## Conference Abstracts

- Mira de Orduña, R., Liu, S.-Q., Patchett, M. L. and Pilone, G. J. (18. Jun. 2000) Arginine degradation by lactic acid bacteria in wine: toxicological and enological implications. Abstracts of the International Annual Meeting of the American Society for Enology and Viticulture, Seattle, USA. Page 59.
- Osborne, J. P., Mira de Orduña, R., Liu, S.-Q. and Pilone, G. J. (18. Jun. 2000) Acetaldehyde metabolism in malolactic bacteria. Abstracts of the International Annual Meeting of the American Society for Enology and Viticulture, Seattle, USA. Page 146.
- Mira de Orduña, R., Liu, S.-Q. and Pilone, G. J. (23. Nov. 1999) Arginine degradation by lactic acid bacteria in wine: energetic, toxicological and oenological implications. Abstracts of the International Annual Conference of the New Zealand Microbiological Society, Dunedin, New Zealand. Page 35.
- Osborne, J. P., Liu, S.-Q., Mira de Orduña, R. and Pilone, G. J. (23. Nov. 1999) The impact of malolactic bacteria on acetaldehyde in wine. Abstracts of the Int. Annual Conference of the New Zealand Microbiological Society, Dunedin, New Zealand. P36.
- Mira de Orduña, R. and Pilone, G. J. (30. Nov. 1998) Arginine metabolism in wine lactic acid bacteria. Abstracts of the International Annual Conference of the New Zealand Microbiological Society and the New Zealand Society for Biochemistry and Molecular Biology, Masterton, New Zealand. Page 75.
- Pilone, G. J., Nuttall, E., Henry, R., Mira de Orduña, R., Patchett, M. L. and Liu, S.-Q. (30. Nov. 1998) Detecting the arginine deiminase pathway in wine lactic acid bacteria. Abstracts of the International Annual Conference of the New Zealand Microbiological Society and the New Zealand Society for Biochemistry and Molecular Biology, Masterton, New Zealand. Page P48.

## Reports

Regular reports about progress within the Ph.D. project have been submitted to the main funding agency, the American Vineyard Foundation (AVF). Summaries of the reports for the funding periods 1997-1998, 1998-1999 and 1999-2000 are accesible on the respective AVF web pages. Full reports can be requested on the AVF homepage, as well



**Figure 1** Pathways of arginine degradation by micro-organisms in wine and their **main consequences**. Shown is the arginine deiminase (ADI) pathway of heterofermentative wine LAB. The pathway consists of three enzymes: arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK). Also shown are the **degradation of arginine by yeast** (by the enzyme **arginase**), the formation of biogenic amines from arginine and its metabolite **ornithine**, and the formation of ethyl carbamate via citrulline, urea and carbamoyl phosphate (carbamoyl-P).