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**Carbohydrate Effects on the Inducement of the Arginine
Deiminase Pathway Enzymes in Wine Lactic Acid Bacteria**

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for the Degree of Master of Science in
Microbiology**

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Chapter 1

Introduction to the Thesis

Characterised by a fermentative sugar metabolism resulting in lactic acid as a major end product, the lactic acid bacteria (LAB) may be isolated from a broad range of sources. Dairy products, fermented vegetables, meats and baking products such as sourdough bread involve these organisms in a consistent and intentional manner in present times, no matter how accidental or fortuitous their initial involvement may have been. Alcoholic beverages such as beer, cider and, most pertinently here, wine are also affected by the presence of particular LAB. As conditions differ between nutrient environments so do the LAB found in wine differ to those isolated elsewhere - being both ethanol tolerant to the degree where growth is capable in 10% v/v ethanol and aciduric, able to maintain an active presence at acidic levels as great as pH 4 or less. This ability to remain viable during the primary yeast fermentation of juice into wine leads to these LAB being of no small practical interest in the wine industry. The process of malolactic fermentation (MLF) involves the wine LAB altering the raw materials present in the juice and wine further, increasing the intricacies of the winemaking and final product. Primarily encouraged due to its effect of reducing wine acidity, MLF also alters flavour and aroma in what is generally thought to be an advantageous manner when applied correctly. Another factor thought to be of significance is an increase in biological stability. Found, for example, among the lactobacilli, pediococci and leuconostocs, the wine LAB are classed as either homofermentative or heterofermentative. Homofermenters commonly produce two moles of lactic acid per mole of glucose fermented, while heterofermenters form one mole each of lactic acid and carbon dioxide and varied quantities of ethanol and acetic acid from one mole of glucose.

Natural or chance occurrences of wine LAB, whether as part of the microbiological community on the raw materials or from other sources - such as inoculation from

contaminated equipment - were the original manner in which these organisms were introduced into the vinification equation. With the predilection towards quality control, standardisation and safety in the present day, the use of pure microbial starter cultures to initiate MLF has become increasingly widespread. In order to optimise the manipulation of wine LAB in both the laboratory and industry a thorough insight into their physiology and metabolism is an obvious necessity. Certain areas of interest have undergone more intensive study than others, with, for example, the catabolism of carbohydrates in both wine (Davis *et al.*, 1986) and model wine systems (Liu *et al.*, 1995a) having had a considerable amount of research compared to less primary sources of energy such as nitrogen metabolism.

Utilisation of L-Arginine, a major amino acid found in grapes and wine, occurs in some wine LAB, these being most heterofermentative lactobacilli and leuconostocs (Liu, 1993). The arginine deiminase (ADI) pathway enzymes, namely arginine deiminase, ornithine transcarbamylase and carbamate kinase, are present and active to varying degrees in these heterofermenters, but not inducible in homofermenters. In the process of degradation arginine is converted into ornithine, carbon dioxide, ammonia and adenosine triphosphate (ATP), indicating that arginine is a potential source of energy for wine LAB. Frequently the high ethanol environment in which wine LAB are found tends to be low in sugars; thus an alternate source of ATP could be of particular use in maintaining the continued desired effect of the organisms in wine over time.

With the identification of the arginine deiminase pathway as that responsible for the catabolism of arginine in heterofermentative wine LAB the process of further investigation into factors affecting arginine metabolism begins. Endeavouring to answer in part or in whole questions raised in previous studies (Liu, 1993), should increase understanding of the process and the bacteria in which it is found. It has been noted that arginine utilisation is not initiated in wine LAB in the absence of a fermentable sugar (Liu *et al.*, 1995b) and also that some sugars, such as glucose, are concurrently utilised with arginine, whereas others, such as fructose, are preferentially catabolised, causing arginine to be left until the sugar levels are low. This research project concentrates primarily on the effects of the carbohydrates glucose and fructose on the inducement of the ADI pathway enzymes in heterofermentative wine LAB.

Chapter 2

Literature review

2.1 Arginine catabolism in wine lactic acid bacteria

In wine LAB arginine is catabolised through the actions of the arginine deiminase pathway, an enzymatic sequence also found in a number of other microorganisms. A variety of bacteria including clostridia, bacilli, *Spiroplasma*, *Spirochaeta* and lactic acid bacteria other than wine LAB all have the pathway present. *Lactococcus lactis* (Crow and Thomas, 1982), *Lactobacillus sake* (Montel and Champonier, 1987) and *Lb. buchneri* (Manca de Nadra *et al.*, 1988) have all been shown to have the ADI pathway present with no evidence as to the existence of any alternate methods of arginine metabolism in LAB presented thus far. Through the actions of the three enzymes involved in the pathway (Figure 2.1), arginine deiminase (ADI), ornithine transcarbamylase (OTC) and carbamate kinase (CK), one mole of arginine is converted into one mole of ornithine, one each of carbon dioxide and ATP and two moles of ammonia. (Liu, 1993). These ratios of 1:1 for arginine:ornithine and 2:1 for ammonia:arginine were mostly borne out in experimental results by Liu (1993), adding to the evidence for the active presence of the ADI pathway in wine LAB.

Past reports have been somewhat contradictory as to the ability of wine LAB to degrade arginine, with, for example, *Leuconostoc oenos* indicated as being incapable of arginine catabolism in Bergey's Manual of Systematic Bacteriology (Vol. 2, 1986), but a number of other reports (Kuensch *et al.*, 1974; Weiller and Radler, 1976; Davis *et al.*, 1985; Liu, 1990; Pilone *et al.*, 1991) stating that it is fully capable. As Bergey's statement is in fact a generalised one pertaining to the entire *Leuconostoc* genus it can be thought likely that variation in the ability to initiate the ADI pathway occurs with strains. Indeed, strain variation has been borne out in studies of the pathway enzymes in strains of *Lc. oenos* by Liu *et al.* (1995b). Strains were assigned to one of three groups depending on ADI pathway enzyme presence and activity: group I possessed all three enzymes and degraded all or

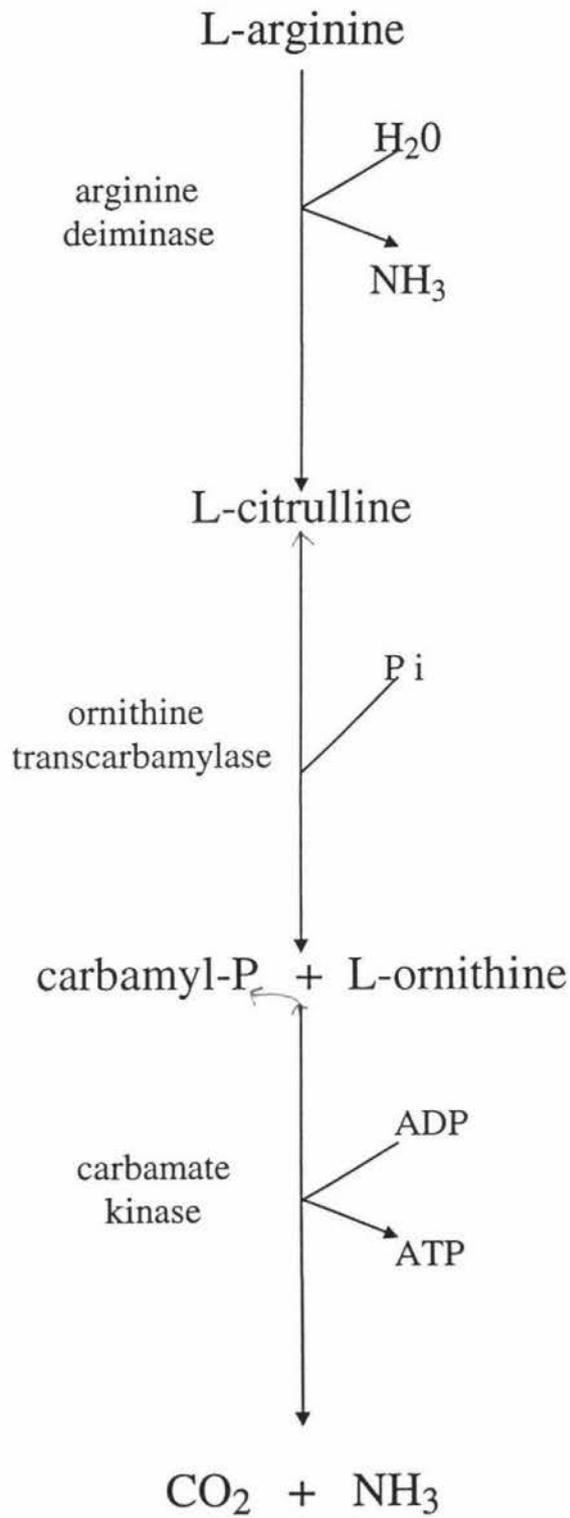


Fig 2.1 The arginine deiminase pathway (after Liu, 1993)

nearly all of the arginine in solution; group II leuconostocs were unable to degrade arginine particularly well despite their enzyme activities appearing, for the most part, to be

similar to group I; lastly, group III members contained either very low levels of OTC and CK activity or entirely lacked detectable ADI pathway enzymes, thus being unable to degrade arginine (Table 2.1). All the homofermenters tested for ADI pathway enzymes showed negative for any of the enzymes and were incapable of arginine consumption.

2.2 Carbon source effects on enzyme induction

The presence or absence of fermentable sugars appears to have a limiting effect on the ability of some wine LAB to degrade arginine. In the absence of sugars two strains of *Lc. oenos* and one of *Lb. brevis* have been indicated as incapable of arginine catabolism, yet utilised arginine when in a medium supplemented with the carbohydrates (Liu, 1990). This data suggests that the presence of a fermentable substrate is necessary in order for arginine catabolism to be initiated in these organisms, and quite possibly others, at least to significant levels.

Repressive effects on the ADI pathway by the carbon source glucose have been reported in some literature (Konings *et al.*, 1991) but this has not been indicated more recently in lengthy time course studies (Liu, 1993). The effectiveness of arginine utilisation was not noticeably reduced in the study, with glucose and arginine metabolised concurrently; consequently the sugar was thought unlikely to affect the synthesis of the ADI pathway enzymes adversely. A sequential metabolism of glucose and arginine, on the other hand, as has been observed in some homofermentative LAB (Crow and Thomas, 1982; Jonsson *et al.*, 1983), would suggest that glucose did repress the synthesis of the particular enzymes in question. Another sugar, fructose, was preferentially utilised in just such a sequential manner with arginine, suggesting that it is indeed a repressor.

The ADI pathway is known to be regulated, at least in part, through the availability of ATP in some bacteria, for example *Pseudomonas aeruginosa* PA01 and *Str. faecalis* ATCC 11700 (Mercenier *et al.*, 1980; Simon *et al.*, 1982; Cunin *et al.*, 1986). When a

Group I*Leuconostoc oenos* (heterofermentative)

OENO

2008

2043

2035

DSIR-C

Ey2d

Lactobacillus spp.(heterofermentative)

CUC-3 32.7

EQUILAIT

Microenos HP

250

Group II*Leuconostoc oenos*

1222

252

MCW

Er1a

Microenos B1

Microenos B2

2001

DSIR-A

DSIR-B

INOACTER

Lactobacillus spp.(heterofermentative)

ML30

Group III*Leuconostoc oenos*

ML34

PSU-1

L181

Microenos B

Microenos B3

Lactobacillus spp. (homofermentative)

CUC-1

Viniflora LP

49

Pediococcus spp. (homofermentative)

44.40

2672

93

C5

CUC-4

Table 2.1. Groupings of wine LAB by presence or absence of ADI pathway enzymes (from Liu *et al.*,1995b)

mixture of glucose, fructose and arginine was present in media the arginine was not catabolised until the fructose was degraded to levels less than 1 g/L (Liu, 1993). Further studies (Liu, 1994) observed that it was the *Leuconostoc* strains of group II (Table 2.1) that exhibited a marked decrease in the formation of ammonia from arginine. It seems likely that fructose metabolism in group I strains may be markedly faster than that of group II strains, reducing the effective time period of inhibition drastically in comparison. It is possible that additional ATP from fructose metabolism is the cause of reduced ADI pathway activity in the sugar's presence. During heterofermentation fructose can be reduced to mannitol and thus act as a hydrogen acceptor for the reoxidation of NADH and NADPH (Kandler, 1983). Additional ATP can then be produced from the acetyl phosphate formed from the phosphoketolase reaction, along with acetate, instead of being reduced to ethanol. This extra ATP per mole of substrate could be the reason for the repressive effect of fructose on the ADI pathway. Differences in mechanism or rate of transport of glucose and fructose are other possible reasons for the repressive effect.

2.3 Other products of arginine catabolism

As arginine is degraded and ATP produced the concentrations of ornithine and ammonia increase consistently, as is to be expected from the projected output of the ADI pathway (Figure 2.1). Most strains of *Lc. oenos* and all heterofermentative wine lactobacilli have been shown to produce and release ammonia during the process of arginine degradation (Pilone *et al.*, 1991). Homofermentative wine LAB, conversely, do not show these products and are unable to degrade arginine, lacking the necessary enzymes for the process (Liu, 1993).

2.4 Arginine catabolism in non wine lactic acid bacteria

2.4.1 Occurrence of the arginine deiminase pathway enzymes

Other LAB possess a varying occurrence of the ADI pathway enzymes - from the full complement of ADI, OTC and CK to two, one or none of them. Species and strains affect the occurrence of the enzymes, with lactobacilli covering a range of the possibilities (Manca de Nadra *et al.*, 1982). Several strains of *Lb. plantarum* have been

shown to lack all three enzymes; strains of *Lb. helveticus* and *Lb. jensenii* to possess only ADI.

2.4.2 Regulation of the arginine deiminase pathway enzymes

As an alternate source of energy production, the ADI pathway is typically induced when energy depletion affects cells in order for it to begin generation of ATP, as with the pseudomonads (Cunin *et al.*, 1986). Pathway employment requires the substrate, arginine, and thus its presence being an enhancer of induction of enzyme synthesis is not entirely a surprise. Both of these conditions can be necessary for the induction of pathway enzymes (Simon *et al.*, 1982). Conversely, repression of the pathway occurs when energy is readily available to the cell - such as in the presence of a fermentable carbohydrate.

Evidence of a trend of carbohydrate effects on the specific activities of the ADI pathway enzymes in certain bacteria has been gathered, but tends to be inconsistent as a general rule. Galactose was found to increase the specific activities of ADI and OTC in *Lactococcus lactis* subsp. *lactis* when compared to glucose or lactose as the growth substrate (Crow and Thomas, 1982). All three enzymes indicated induction by galactose in *Lb. buchneri* NCDO 110 (Manca de Nadra *et al.*, 1986a), but in *Lb. leichmanii* ATCC 4797 the specific activities of the enzymes were reduced by two or threefold (Manca de Nadra *et al.*, 1986b). A broad rule as to carbohydrate effects in the induction of the ADI pathway enzymes seems unlikely from this disparate data, although groupings of LAB from specific environments would be more likely to produce similar effects from specific sugars.

2.4.3 Genetics of the arginine deiminase pathway system

There have been plasmids found in strains of *Lc. oenos* (Janse *et al.*, 1987) but no evidence as to whether these are linked to arginine catabolism has been found. It is unknown if the ADI pathway is plasmid borne or chromosomally encoded and so the reasons for the lack of the enzymes ADI, OTC and CK in some strains presently remains a point of speculation. In *Pseudomonas aeruginosa* the ADI pathway is known to be encoded by a chromosomal four gene cluster: *arcI* (ADI), *arcB* (catabolic OTC), *arcC* (CK), and *arcD* (arginine uptake) in which mutation of any gene will affect arginine

utilisation (Mercenier *et al.*, 1982; vander Wauven *et al.*; 1984; Luthi *et al.*, 1986). Similar gene clusters seem likely to be present in other bacteria containing the ADI pathway due to the continuously observed coinducibility and simultaneous repression of the enzymes.

2.5 Fluoride inhibition of bacteria

Fluoride is known to be an effective antibacterial agent, acting optimally as an inhibitor at low pH values. Inhibition of glycolysis in *S. mutans* GS-5, a LAB, was shown to be complete at concentrations well below 0.1 mM at a pH value of 4.0, while at a pH value of 6.0 a substantially greater concentration of nearly 10 mM fluoride was necessary to produce the same effect (Belli *et al.*, 1995). The effect on the bacterial glycolytic pathway is through strong inhibition of the enzyme enolase. This inhibition appears to be a complex mixture of competitive and non-competitive mechanisms and is quasi-irreversible in some situations, although not in intact glycolyzing cells. Cytoplasmic acidification is thought to occur, thus inhibiting acid sensitive glycolytic enzymes such as enolase. Due to its small size and similar molecular dimensions to water, Fluoride is thought to pass readily through the cell membrane. LAB maintain a pH gradient across the cell membrane through proton locating F-ATPases, but do not maintain a constant intracellular pH value, leading to the opportunity to alter it. Although environmental pH has effects on intracellular pH levels even LAB in extremely low environmental pH's have been seen to be affected by fluoride, thus indicating its efficacy as an inhibitor of glycolysis.

Chapter 3

General Materials and Methods

3.1 Lactic acid bacterial strains

Two strains of wine LAB were used in this study, both being heterofermentative and ammonia positive, as tested previously (Liu, 1993). MCW is a *Leuconostoc oenos* strain while CUC-3 is a strain of *Lactobacillus buchneri*; each obtained from the cell collection of the Microbiology and Genetics Department of Massey University, Palmerston North, New Zealand. MCW was originally taken from a commercially freeze-dried culture isolated by M. Bannister, Vinquiry, Healdsburg, California, USA. The initial source for CUC-3 was a laboratory culture obtained by R. Kunkee, Department of Viticulture and Enology, University of California, Davis, USA.

3.2 Medium preparation, culturing and harvesting of wine LAB

Wine LAB were grown in a vegetable juice broth supplemented with glucose and arginine. Arginine was added in order to prime ADI pathway enzyme expression (Liu, 1993), initiating it during the growth of the organisms. This medium consisted of the following components per litre of deionised (DI) water: tryptone, 5 g; peptone, 5 g; yeast extract, 5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.05 g; Tween 80 (5% aqueous solution), 1 mL; D-Glucose, 5 g; L-Arginine, 5 g and clarified vegetable juice, 200 mL. Campbell's V8 Vegetable Juice™ (Campbell's Soups, Australia) with no added sugar was used as the source of the clarified juice and filtered through Whatman No. 1 filter paper and diatomaceous earth filter aid (Witco Corp., Kenite Diatomite 3000). This Tomato Juice Broth-Arginine-Glucose medium (TJBAG) was adjusted to pH 5.5 before making up to volume and autoclaving at 121°C for 15 minutes.

Cells were initially inoculated into 5 mL aliquots of TJBAG from separate stock maintained in either TJBAG or Apple Rogosa (AR) broth. AR broth was prepared from 500 mL of 2X Basal Broth (BB) medium, 200 mL of apple juice, adjusted to pH 5.5 and made up to 1000 mL. BB contains Tryptone, 5 g; Peptone, 5g; Yeast Extract 5 g;

MgSO₄·7H₂O, 0.2 g, MnCl₂·4H₂O, 0.05 g; Tween 80 (5% aqueous solution) adjusted to pH 5.5 and made up to 1000 mL. Apple juice was prepared from a concentrate (Fresh-up™, the New Zealand Apple and Pear marketing Board) by diluting to normal strength with DI water. These actively growing cells were used to inoculate (10% v/v) sequentially larger TJBAG media quantities, which were each incubated at 30°C. Growth was continued in the final 500 mL aliquots until the period between late log and early stationary phase, which was determined through observation of the initial settling of the cells. The harvesting of cells was carried out by centrifugation at 6,500 x g for 10 minutes at 4°C in a Sorvall RC-5B refrigerated centrifuge. After harvesting, cells were washed twice with 100 mL of 10 mM phosphate buffer (pH 7) and spun at the same specifications, retaining cell pellets each time. Phosphate buffer was formed by adding 0.697 g K₂HPO₄ and 0.817 g KH₂PO₄ to DI water, altering the pH value to 7 and increasing the volume to 1000 mL to produce a 10 mM solution.

3.3 Preparation of non-growing cells

Non-growing or resting cells were formed when growth was halted by removing the cells from their source of nutrients and then were suspended in a neutral, buffered solution with no access to carbon or nitrogen sources. In this relatively inactive state the enzymatic processes of the cells were still viable and areas of interest could be investigated by specific alterations in the limited environment.

Once cell pellets were obtained they were then resuspended in an amount and molarity of pH 3.6 KHT buffer dependent on the particular micro-organism (either MCW or CUC-3) and its degree of activity. Buffer levels were configured to keep a minimal alteration in environmental pH throughout the experimental period and thus limit its effects on the results observed. Cell concentration was likewise maintained at levels where activity could be measured in a meaningful manner, utilising substrate at a rate slow enough to allow differentiation through sampling. The leuconostoc MCW was resuspended in 45 mL of 10 mM potassium bitartrate (KHT) buffer as standard, while the lactobacillus CUC-3, which was considerably swifter in utilising both glucose and arginine, was typically resuspended in 225 mL of a saturated solution of KHT buffer. Chosen as being simple but somewhat similar to a wine environment, 10 mM KHT

buffer was made up of KHT, 1.88g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 g; adjusted to pH 3.60 and made up to 1000 mL. Saturated solution was typically made by increasing the quantity of KHT by a factor of 10. Buffer capacity was decided upon after varied concentrations were tested with cells, as sensitivity could be limited if it were too high.

A calibrated pH electrode was inserted into the cell suspension and equilibrated in a 30°C water bath where the cells were maintained in suspension by the use of a submersible stirring plate. This stirring also contributed to avoiding disruption of the experiment during sampling. Continuous measurement of pH was used as an indicator to recognise a steady state in the cells. At this time, with background activity predictable, additions to the solution mixture best showed their effects.

3.4 Obtaining starved cells

Starved cells were obtained by resuspending the cells in 50 mL 10 mM phosphate buffer (pH 7) as used for washing. The metabolism of internal glucose was observed by measuring the pH changes over time, following the decline in pH until it leveled off and became stable. A steady pH indicated exhaustion of cellular glucose reserves. At this point the cells were centrifuged again and the pellet resuspended in KHT buffer in order to conduct resting cell fermentations.

3.5 Analytical methods

This section describes the common analytical methods employed in the experimental work carried out.

3.5.1 Determination of citrulline concentration

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

Reagents

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

- 2) Diacetyl monoxime (3%): 3 g diacetyl monoxime (2,3-butanedione monoxime) in 100 mL DI water.
- 3) Citrulline stock standard (100 mg/L): 10.0 mg citrulline (Sigma) in 100 mL DI water. Stored frozen.
- 4) Citrulline working standard (10 g/mL): 1 mL stock standard in 10 mL DI water.

Procedure

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

3.5.2 Determination of arginine concentration

Two procedures were utilised over the course of the study.

3.5.2.1 The Sakaguchi reaction method

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

Reagents

- 1) Urea (40%): 200 g urea dissolved in a small amount of DI water first and made up to 500 mL with DI water.
- 2) 8-Hydroxyquinoline (0.02%): 0.2 g 8-Hydroxyquinoline dissolved in 100 mL ethanol (95%) first and made up to 1000mL with DI water.
- 3) Sodium hydroxide (10%): 50 g NaOH in 500 mL DI water.
- 4) Sodium hydroxide (5%): 25g NaOH in 500 mL DI water.
- 5) Sodium hypobromite (1%): 0.68 mL liquid bromine in 200 mL 5% NaOH.
- 6) Arginine standard stock solution (500 µg/mL): 60.50 mg arginine.HCl (Sigma) in 100 mL DI water.

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

Procedure

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

3.5.2.2 Staron-Allard (S-A) Method

Again the method is also centred around the formation of a coloured reaction product, in this case yellow. This method was based on the modified version of Micklus and Stein (1973).

Reagents

- 1) Sodium hydroxide solution (2N): 40 g NaOH in 500 mL DI water.
- 2) Chloromethylphenol (2.5%): 2.5 g Chloromethylphenol dissolved in 50 mL ethanol (100%) first and made up to 10 mL with DI water.
- 3) Sodium hypochlorite (1%): 14 mL 13.5% sodium hypochlorite solution added to 175 mL DI water.
- 4) Arginine standard stock solution (500 µg/mL): 60.50 mg arginine.HCl (Sigma) in 100 mL DI water.
- 5) Arginine standard working solution (100 µg/mL): 5-fold dilution of arginine standard stock solution in DI water.

Procedure

To 1 mL of a solution containing the sample was pipetted 1 mL of sodium hydroxide solution, followed by 1 mL of chloromethylphenol reagent, followed by 1 mL of sodium hypochlorite reagent (1%), and the reagents mixed well. After allowing 10 minutes for colour development the colour produced was read against a reagent blank at 440 nm using a Bausch and Lomb Spectronic-20 Spectrophotometer zeroed with air. The arginine standard curve was prepared with the arginine standard working solution (1-4 mL), with DI water to make up to 5 mL.

3.5.3 Analysis of ammonia and glucose

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

3.5.4 Measurements of cell mass

Bacterial cell mass was measured as optical density (OD) at 420 nm using a Nova Tech Spectrophotometer. Samples were necessarily diluted in each instance, by a dilution factor of at least 25 fold to obtain an OD between 0.2 and 0.6, with DI water, and zeroed against DI water. These readings were compared to a standard curve graph for the particular cell type. The standard curves were created by growing 1000 mL of cells to between late log and early stationary phase as usual, then splitting the results into two equal quantities. The first 500 mL were treated as standard for non-growing cells and diluted to a variety of optical densities. The remainder were vacuum filtered in 25 mL aliquots, dried overnight at 100°C and weighed by difference with their filters to gain a dry cell weight (Liu, 1993). Comparison of the figures accrued, graphically, formed the standard curve.

3.5.5 Measurement of pH

An Orion Research pH meter (Digital Ionalyzer/501) calibrated against buffers of pH 7.00 and 4.01 was used to measure pH.

Chapter 4

The Effect of Glucose on the Initiation of the ADI Pathway Enzymes

4.1 Introduction

Induction of the ADI enzymes by the presence of arginine has been demonstrated in past literature (Simon *et al.*, 1982). Other aspects of the regulation of enzyme synthesis, such as the nature of the carbohydrate source, have had some little investigation. In time course studies showing that glucose and arginine were metabolised concurrently in heterofermentative wine LAB (Liu, 1993) it has been indicated that glucose does not repress the synthesis of the arginine deiminase pathway enzymes. Here, the effect of glucose on arginine catabolism was investigated in non-growing cell suspensions.

4.2 Materials and Methods

4.2.1 Lactic acid bacterial strains

Both CUC-3 and MCW were used in this survey. For further information on these organisms see Section 3.2.1.

4.2.2 Experimental procedure

Cell growth, non-growing cell preparation and experimental organisation to this point were carried out as in Sections 3.2, 3.3 and 3.4. After a steady state had been observed in the solution of non-growing cells suspended in KHT buffer, arginine (5 g/L) was added. Samples were taken in order to be able to test for amounts of arginine, ammonia and for the presence of citrulline. After a noted amount of time and sufficient samples to gain a statistically meaningful amount of data, glucose (1 g/L) was added to the solution. Samples continued to be taken at regular intervals to compare the catabolism of arginine, and the formation of ammonia and citrulline before and after the glucose addition.

Arginine solution was prepared by dissolving 5 g L-arginine in KHT buffer, adjusting the pH to 3.6 with phosphoric acid and then making the buffer up to 100 mL with KHT buffer. The resultant solution was 50 g/L and was added to the experimental mixture in order to give an overall concentration of 5 g/L. Thus, with the MCW solutions 5 mL was added to the 45 mL mixture of cells and KHT while with CUC-3 25 mL was added to the 225 mL suspension of cells and KHT.

Glucose solution was composed of 10 g of glucose in 100 mL DI water. As glucose is a neutral sugar and only small quantities of the solution would be added to the experimental mixture pH alteration was unnecessary. For every mL of cell suspension remaining in the resting cell fermentation at the time of its addition, 0.01 mL of this 100 g/L glucose solution was added to give a final concentration of 1 g/L.

Solutions were equilibrated to 30°C before addition to the cell suspension to avoid any temperature effects on the cells.

4.2.3 Culturing procedures and sampling regime

As stated in Section 3.3, after centrifugation of a 500 mL growth of cells in TBJAG broth MCW was resuspended in 45 mL of 10 mM KHT buffer, whereas CUC-3 was resuspended in 225 mL of a saturated solution of KHT buffer. With the arginine solution quantities added these produced 10 fold and 2 fold concentrations of cells respectively. As the volume of the two experimental solutions varied considerably, so the sample size was necessarily different for each of the two LAB. Reducing the experimental volume too drastically over the period of the experiment could possibly cause variations in the results as well as the basic need to retain enough of the cell suspension in order to continue to take said samples. Therefore, 2 mL samples were obtained from the MCW experiments while 5² mL samples were taken from the CUC-3 experiments.

Samples were taken directly from the experimental mixture and placed in a centrifuge tube in an ice bath to halt any further reaction. They were then centrifuged at 6,500 x g for 10 minutes at 4°C in a Sorvall RC-5B refrigerated centrifuge and the supernatant taken and stored frozen for subsequent chemical analyses.

4.2.4 Chemical analysis of samples

The rate of pH change, catabolism of arginine, production of ammonia and citrulline were measured using the procedures described in Section 3.5.

4.3 Results

4.3.1 Comparison of organisms

Differences between the two organisms were apparent simply from their rates of growth, with CUC-3 being both more robust and growing quickly. This carried over into experimental procedures, where the concentration of cells needed for CUC-3 was 2 fold compared to the 10 fold concentration for MCW. Even at this concentration the activity of the ADI pathway for MCW was less than that for CUC-3. This lesser activity was already indicated by the enzyme assays carried out by Liu (1993, 1995b) and his placement of CUC-3 into the group I wine LAB, while MCW was placed in group II. The rate of arginine degradation and ammonia production were routinely lower in MCW in contrast to CUC-3, however, the comparative effects on said rates by glucose were, overall, distinctly alike. Due to this factor and the faster growth displayed by CUC-3, it was the organism used more frequently for investigating the varied pitfalls of acquiring a workable, reproducible experimental procedure.

4.3.2 pH monitoring

Although statistically non-viable as a source of data, due to the number of variables affecting it, the constant information gained from the pH meter in the solution proved quite useful. As well as showing the initial settling of the cell suspension to a base line environment where changes due to additions from outside the system could be best observed, it allowed some degree of monitoring of the activities of the cells throughout the experiment. This was particularly useful in the early stages of experimentation, where buffer concentration, cell density, sampling and the time of the experimental run were necessarily altered frequently until the optimal settings for each were accomplished. A disturbance in experimental procedure, such as a higher than expected cell density, necessitating sampling at shorter intervals to avoid depletion of arginine before all were collected, could be identified and dealt with.

Figure 4.1 shows the time course of pH during a typical experimental run. Although the initial pH was expected to be that of the KHT buffer (pH 3.6) it was commonly raised slightly. This was most likely due to some residual phosphate buffer (pH 7) from the wash procedure interacting with the experimental environment. Another common feature was the drop in pH directly after the cells had, presumably, overcome the shock of transfer from repeated 4°C centrifuging and washing into the 30°C environment and lower pH of the KHT buffer. This was attributed to residual glucose in the resting cells being catabolised and forming lactic acid. At addition of the (pH 3.6) arginine solution, after a steady state had been observed in the pH of the solution, the cell suspension dropped to the expected pH of 3.6 and arginine begins to be absorbed and utilised. This continued at a steady rate until addition of glucose, at which point a marked increase in the rate of pH rise was apparent, mirroring to some degree the alterations in rate found in the analyses of the samples.

4.3.3 Arginine analysis

4.3.3.1 Comparison of the Sakaguchi reaction method and Staron Allard method

Both the (Section 3.5.2.1) and the Staron-Allard (S-A) method (Section 3.5.2.2) were used to analyse samples for arginine. Initially the Sakaguchi method was employed, but a number of factors led to employment of the S-A method more commonly. The ease of performing the S-A method in comparison with the very time dependent Sakaguchi method was a definite factor in the decision to switch procedures, but it was also thought that an observed deviation from expected arginine recovery could have been due to complications with the Sakaguchi method. In practice, both methods gave arginine concentration results for the samples which were well within 95% probability levels and frequently exactly the same. This factor, in conjunction with the speed and ease of the S-A method, led it to be employed solely after this fact had been ascertained.

4.3.3.2 Arginine data

Figure 4.2 shows graphically the alteration in the rate of arginine degradation through the ADI pathway. Two lines of best fit are indicated before and after the time of glucose addition to highlight the increase of arginine catabolism. Table 4.2 gives the equations for the lines of best fit and indicates the factor of the increase. This data is from the upper range of that gathered over many experiments, with a more consistent reaction to

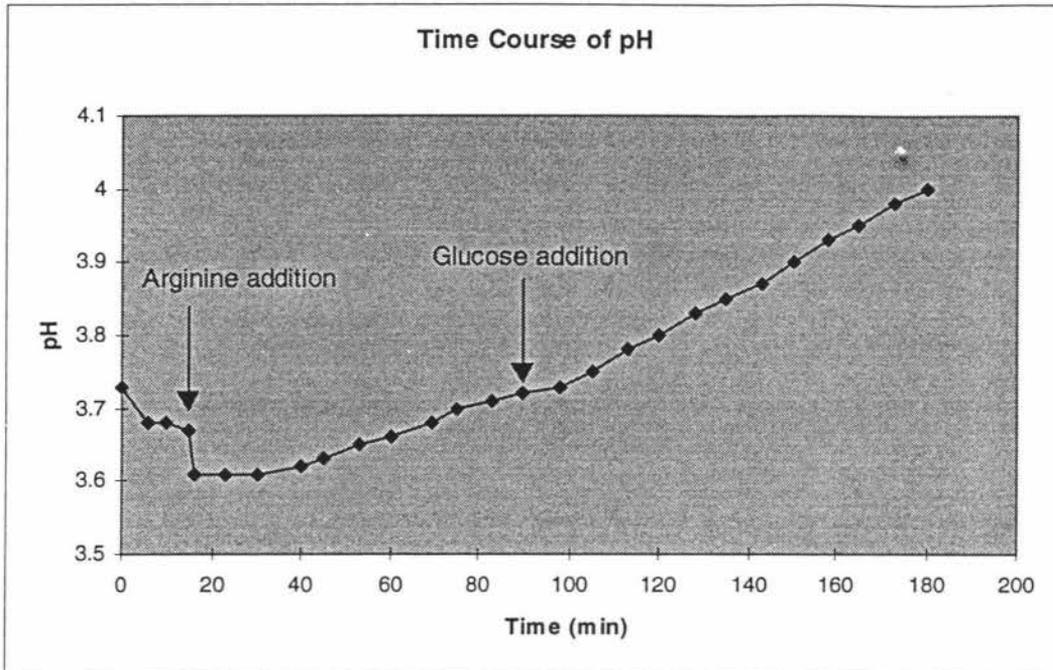


Figure 4.1. pH Overview of Cell Activity Before and After Addition of Glucose

Arginine
Before glucose addition: $y = -0.003071x + 4.373714$, R-squared: 0.866247
After glucose addition: $y = -0.012206x + 6.72913$, R-squared:
Activity rate increase: by factor of 3.97.
Ammonia
Before glucose addition: $y = 2.226095x - 99.627619$, R-squared: 0.937823.
After glucose addition: $y = 4.622857x - 515.952381$, R-squared: 0.92244
Activity rate increase: by factor of 2.08.

Table 4.1. Regression Analyses for Figure 4.2 Graphs

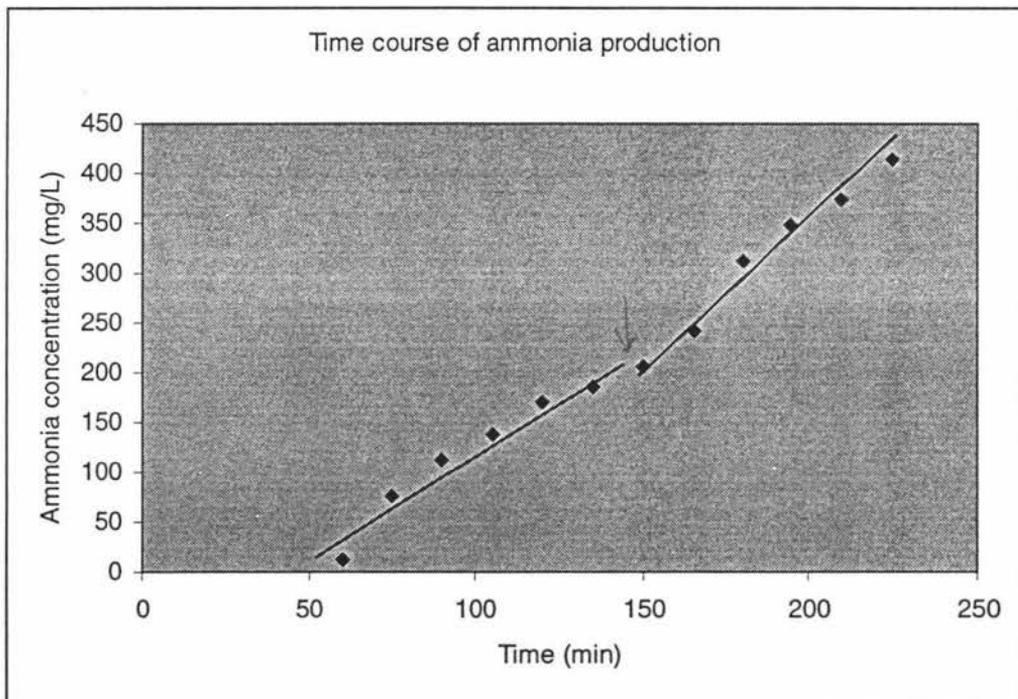
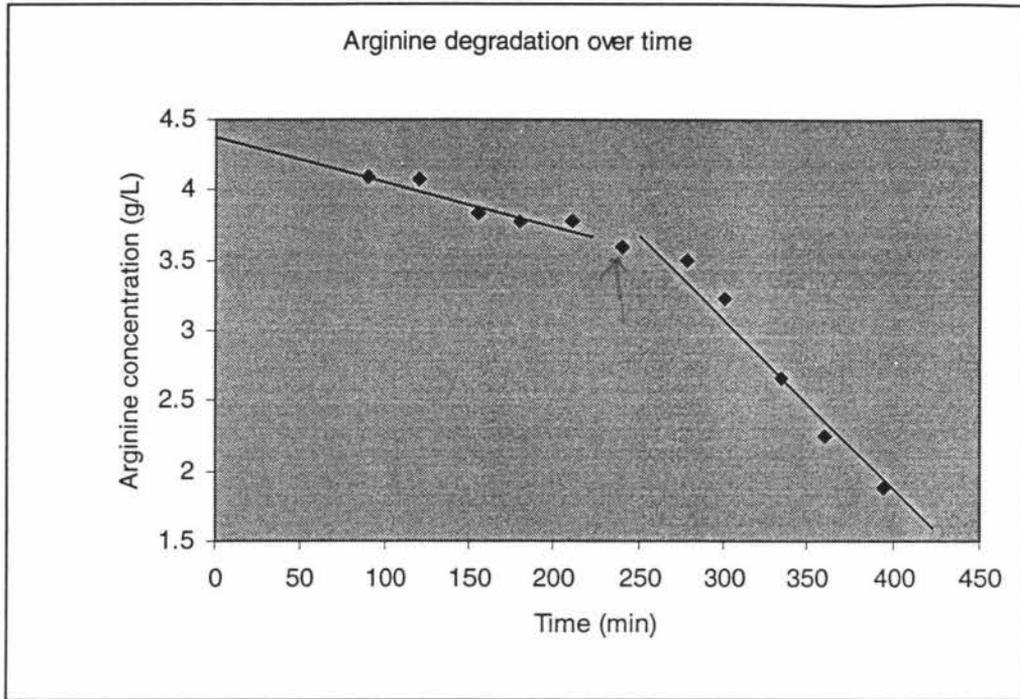


Figure 4.2 Rates of Arginine Degradation and Ammonia Production Over Time Before and After Glucose Addition

glucose being an increase of approximately 100% in activity, as the ammonia graph also in Figure 4.2 presents.

4.3.3.3 Arginine recovery

Due to the fact that it was the examination of the comparative rates of arginine catabolism by the wine LAB that was of interest it was unnecessary to worry unduly about the less than expected amounts of arginine observed in our samples. Nonetheless, this inconsistency was considered to some degree. Although the initial quantities of arginine added to the experimental solution was certainly 5 g/L there always appeared to be a significant drop off, of approximately 20% of expected values, in the time between inoculation of the arginine into the solution and the first sample. Even sampling directly after inoculation showed this irregularity. However, throughout the period of experimentation standard solutions produced the results expected of them each time the arginine assay procedures were performed, giving expected values on the standard curve. A possibility that the arginine free base being used to make up the solutions inoculated into the experimental mixture may have been lower in concentration than the arginine.HCl standards due to water pick up was considered. Experiments run with arginine.HCl producing the original concentration of 5 g/L arginine rather than free base arginine proved to produce similar results, however. Arginine solutions (50 g/L) were made with 3.0238 g arginine.HCl in 50 mL KHT buffer on occasion afterwards to compare and also due in part to reduced stocks of arginine free base.

Test runs with greater quantities of arginine added to the experimental solution were performed to investigate the possibility of a limiting factor due to the amount of arginine available in the experimental system. As quantities in the wine environment would be unlikely to exceed even 5 g/L, and since care was taken to take samples while arginine was still being metabolised, this seemed unlikely, but it was also of interest in regards to the expected recovery of arginine. The standard experimental procedure was carried out with a three fold increase in the quantity of arginine added to the cell suspension, by making a 150 g/L standard solution rather than the usual 50 g/L. These high levels of arginine did not produce any marked difference in the reaction mixture, leading to the conclusion that arginine availability was not a significant limiting factor to the resting cell suspension. The initial retrieved value of arginine was again reduced from expected

values by approximately 20%, suggesting that a calculation error was in effect, though none could be identified.

4.3.4 Ammonia data

The simple and easily reproducible enzymatic ammonia assay consistently produced results such as those seen in Table 4.1 and Figure 4.2. An initial rate of production of ammonia invariably increased output of the ADI pathway byproduct by a factor of approximately 100%, keeping it in line with the effects on arginine rates.

4.3.5 Citrulline Output

Measures of citrulline were taken on only a few samples, mostly as an indicator of its presence only, as a check on the actions of the ADI pathway. Where arginine degradation and ammonia production occurred, citrulline was also present, as expected.

4.3.6 Starved and Non starved Cells

Originally there was concern about the drop off in pH early in the experimental time frame. This was attributed to the presence of internal glucose remaining within the non-growing cells, which was being metabolised and producing a reduction in pH through byproducts such as lactic acid. The use of starved cells (Section 3.4) was initiated to eliminate the interference of any possible internal glucose on the results. It was thought that induction of the ADI pathway enzymes could be effected by this internal glucose and thus skew the expected increase in arginine degradation when glucose was introduced into the experimental system. After considerable testing with both CUC-3 and MCW it was found that starving cells before the main section of the experiment proved to have no measurable beneficial effect on the behaviour of the system other than to remove the initial pH drop and initiate a steady state more quickly. Conversely, cells tended to act somewhat more erratically, giving less than optimal reproducibility of results when they had been starved. This may have been due to the increased handling and time that the cells were subjected to during the starvation process in comparison to non-starved cells. It also could be attributable to a degree of autolytic activity within the starvation process.

4.4 Discussion

This study has demonstrated the use of non-growing cells in a buffered medium to investigate the effects of glucose on the enzyme initiation of the arginine deiminase pathway. As indicated by Liu (1990), the absence of sugars in a medium can limit the utilisation of arginine. Glucose and arginine were metabolised concurrently, indicating no repressive effect of the carbohydrate on the ADI pathway as reported. Indeed, glucose addition apparently enhances activity of the arginine deiminase pathway in the non-growing cell suspensions. Rate increases of 100% or more in both arginine degradation and ammonia production were seen to occur after glucose introduction to the system. This data suggests that the presence of the fermentable substrate glucose is a positive regulator in the initiation of the ADI pathway enzymes in these wine lactic acid bacteria.

As the ADI pathway is known to be regulated through the availability of ATP in some bacteria (Mercenier *et al.*, 1980; Simon *et al.*, 1982; Cunin *et al.*, 1986), it is likely that the method of regulation is linked to the ATP supplied by glucose fermentation. Glucose has been observed to be a preferred sugar of LAB (Liu *et al.*, 1995a) and thus its fermentation is likely to produce effects on the pathways involved in the organisms life cycle with ease.

Chapter 5

The Effects of Inhibiting Glycolysis on the Observed Rate of Arginine Degradation

5.1 Introduction

In order to further investigate the effect of glucose on arginine catabolism, previous work (Chapter 4) was duplicated somewhat in reverse. With both glucose and arginine in the experimental solution, the effects of glycolysis were halted after a time with fluoride, thereby effectively making glucose inaccessible to the non-growing cells. Fluoride was chosen as the inhibitor due to the low quantities required for glycolytic inhibition at low pH (see Section 2.5) and its proven efficacy in this manner. Carrying on from the hypothesis of Chapter 4, that enhancement of ADI pathway enzyme activity in the presence of glucose is linked to the ATP production from its glycolysis, the expected result would be to see a drop off in arginine degradation after the addition of fluoride.

5.2 Materials and Methods

5.2.1 Lactic acid bacterial strains

Both CUC-3 and MCW were used in this survey. For further information on these organisms see Section 3.2.1.

5.2.2 Experimental Procedure

As with the previous experiment (Chapter 4), cell growth, non-growing cell preparation and experimental set up to this point were carried out as in Sections 3.2, 3.3 and 3.4. After a steady state had been observed in the solution of non-growing cells suspended in KHT buffer, glucose (1 g/L) and arginine (5 g/L) were added. Arginine and glucose solutions were prepared and inoculated into the cell suspension in quantities as indicated in Section 4.2.2. Samples were taken in order to be able to test for amounts of arginine, ammonia and for the presence of citrulline. After a noted amount of time fluoride (1 mM) was added to the solution and samples continued to be taken at regular intervals

compare the catabolism of arginine, and the formation of ammonia and citrulline once glycolysis had been inhibited.

Fluoride stock solution was composed of 4.199 g NaF in 100mL KHT buffer, giving a 1.0 M concentration, and frozen. Standard solution was prepared fresh from 10 mL stock solution and 90 mL KHT buffer to give a 100 mM concentration. Similarly to the glucose addition of the previous experiment (Chapter 4), for every mL of cell suspension remaining in the resting cell fermentation at the time of its addition, 0.01 mL of this 100 mM fluoride solution was added to give a final concentration of 1 mM.

Originally the fluoride strength was set at an arbitrary value of 4 mM, but this was found to inhibit the ADI pathway as well as glucose utilisation by glycolysis. A reduction to a 1 mM concentration of fluoride proved to allow glycolytic inhibition to take place as well as have no unwanted inhibitory effects on the cells and ADI pathway enzymes.

5.2.3 Culturing procedures and sampling regime

As laid out in Section 4.2.3.

5.2.3 Analysis of samples

The rate of pH change, catabolism of arginine and glucose, production of ammonia and citrulline were measured using the procedures described in Section 3.5

5.3 Results

5.3.1 pH monitoring

Figure 5.1 shows the time course of pH during a typical experimental run. After steady state had been observed and the glucose and arginine solution inoculated into solution at the 25 minute point the concurrent metabolisation of the amino acid and the carbohydrate began. This continued at a steady rate until addition of fluoride, at which point a marked decrease in the rate of pH rise was apparent, mirroring to some degree the alterations in rate found in the analyses of the samples.

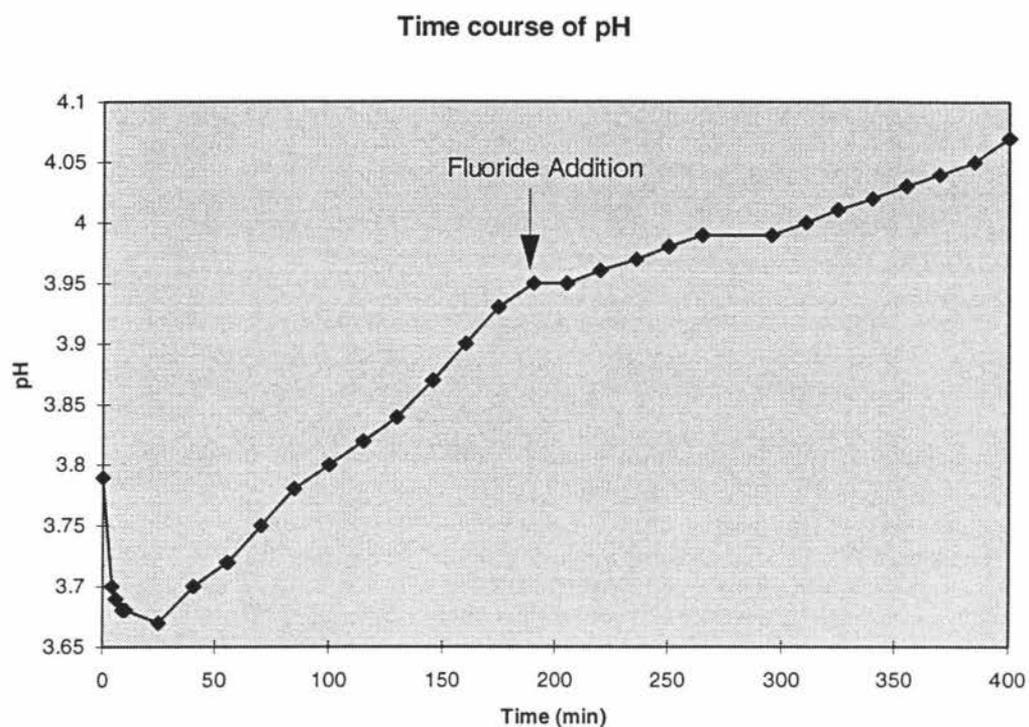
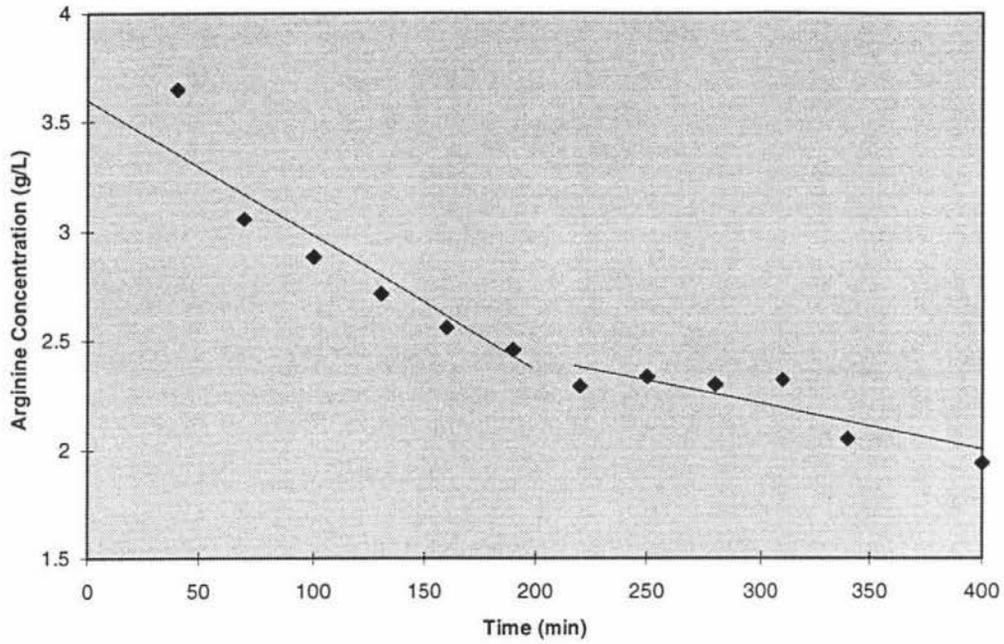


Figure 5.1. pH Overview of Cell Activity Before and After Addition of Fluoride

Arginine	
Before fluoride addition:	$y = -0.007314x + 3.844476$, R-squared: 0.969924.
After fluoride addition:	$y = -0.002233x + 2.8.8333$, R-squared: 0.932848.
Activity rate decrease:	by factor of 3.28.
Ammonia	
Before fluoride addition:	$y = 0.830833x + 11.802381$, R-squared: 0.972953.
After fluoride addition:	$y = 0.582143x + 40.964286$, R-squared: 0.985952.
Activity rate decrease:	by factor of 1.43.

Table 5.1. Regression Analyses for Figure 5.2 Graphs

Time course of Arginine Degradation



Time Course of Ammonia Production

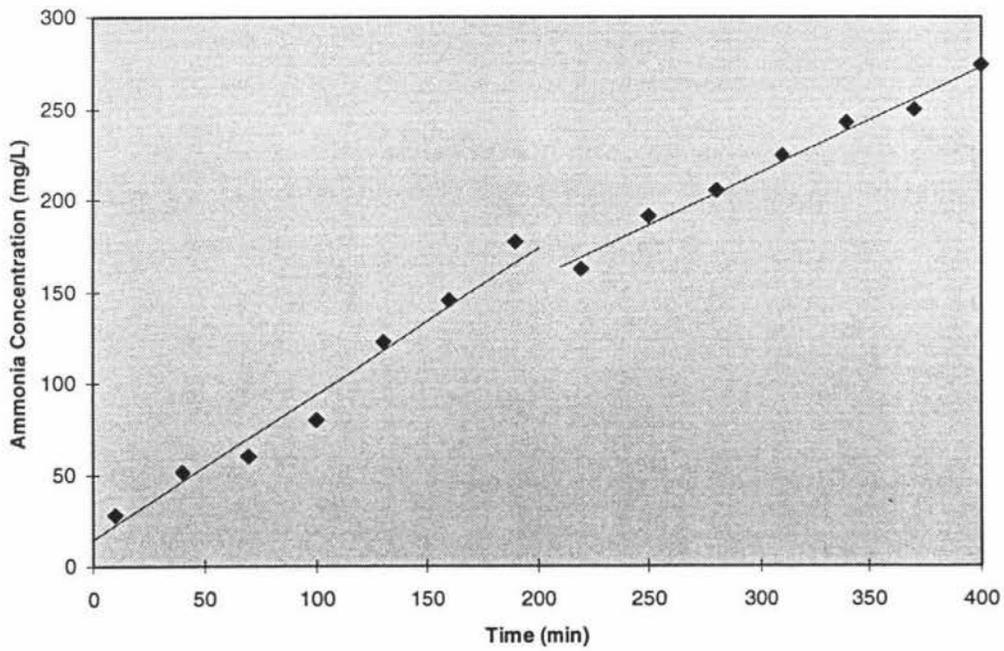


Figure 5.2 Rates of Arginine Degradation and Ammonia Production Over Time Before and After Fluoride Addition

5.3.2 Arginine data

Figure 5.2 shows graphically the alteration in the rate of arginine catabolisation by the ADI pathway. Two lines of best fit are indicated, before and after the time of fluoride addition to highlight the decrease in the rate of arginine catabolism. Table 5.2 gives the equations for the lines of best fit and indicates the factor of the increase. This data is a typical sample from the experiments, which gave highly reproducible results.

5.3.3 Ammonia data

Table 5.1 and Figure 5.2 show typical data from the series of experiments. The rate of ammonia production was not as drastically reduced as that of arginine, though a reduction of approximately 50% was observed repetitively. It may be noted that samples for ammonia gave a distinctly slower decrease in rate over time, but that the decrease continued to become more pronounced.

5.3.4 Citrulline Output

Measures of citrulline were taken on only a few samples, mostly as an indicator of its presence only, as a check on the actions of the ADI pathway. As with the previous experiment, as arginine degradation and ammonia production occurred, citrulline was also present.

5.3.5 Fluoride effects on glucose catabolism

Glucose use in the cells decreased by 5.42 fold after addition of 1 mM fluoride.

5.4 Discussion

This study continued from the previous experiment in investigating the effect of glucose on the expression and activity of the arginine deiminase pathway enzymes. Both glucose and arginine were initially present in the experimental solution, allowing increased activity of the ADI pathway as noted in the previous experiment (Chapter 4). The ATP producing effects of glucose were halted by the addition of fluoride, inhibiting glycolysis. The rate of glucose usage in the cell dropped at this stage by a factor of 5.42 fold and decreases in both ammonia production and arginine degradation rates occurred at this point also. The theory that enhancement of ADI pathway enzyme activity in the

presence of glucose is linked to the ATP production from its glycolysis (Chapter 4) appears to be borne out.

The lesser reduction in ammonia production rate compared to arginine degradation suggests the possibility that one or more of the latter ADI pathway enzymes retain the induction effects of glucose. This could be attributed to build up of citrulline by the ADI enzyme and continued use of this resource by OTC and then CK, but levels of citrulline in solution did not indicate a drop in its level before and after fluoride addition. The continued observations of co-inducibility of the enzymes and their probable genetic clustering (Section 2.4.3) may suggest that this is unlikely, however.

Chapter 6

The Effects of Fructose Addition on the Observed Rate of Arginine Degradation in Type II Wine LAB

6.1 Introduction

In order to investigate the effect of fructose on arginine catabolism, a system similar to that applied in previous work (Chapter 4 and 5) was used. With both glucose and arginine initially present in the experimental solution, producing an optimal degradation of arginine, fructose was added and the effects observed.

6.2 Materials and Methods

6.2.1 Lactic acid bacterial strains

Only the *Leuconostoc* MCW was used in this survey, it being a Type II LAB (Section 2.1, Table 2.1) and these strains reported to have the ADI pathway inhibited by fructose (Section 2.2). For further information on this organism see Section 3.2.1.

6.2.2 Experimental Procedure

As with the previous experiments (Chapter 4 and 5), cell growth, non-growing cell preparation and experimental set up to this point in the solution of non-growing cells suspended in KHT buffer, glucose (1 g/L) and arginine (5 g/L) were added. Arginine and glucose solutions were prepared and inoculated into the cell suspension in quantities as indicated in Section 4.2.2. Samples were taken in order to be able to test for amounts of arginine, ammonia and for the presence of citrulline. After a noted amount of time fructose (20 g/L) was added to the solution and samples continued to be taken at regular intervals compare the catabolism of arginine, and the formation of ammonia once fructose had been introduced into the system.

Fructose was added to the cell suspension in solid form at quantities of 20 mg/mL of experimental solution remaining, in order to produce an overall concentration of 20 g/L. Earlier studies (Liu, 1995b) observed a concentration of 20 g/L causing inhibitory

effects. This method of introduction was employed to minimise volume increase in the system with such a high concentration of the substrate being employed. Extra time was included for the fructose to dissolve into solution and bring about any effects before samples were taken.

6.2.3 Culturing procedures and sampling regime

As laid out in Section 4.2.3.

6.2.4 Analysis of samples

The rate of pH change, catabolism of arginine and production of ammonia were measured using the procedures described in Section 3.5

6.3 Results

6.3.1 pH monitoring

Figure 6.1 shows the time course of pH during a typical experimental run. After steady state had been observed and the glucose and arginine solution inoculated into solution at the 40 minute point the concurrent uptake and use of the amino acid and the carbohydrate began. This continued at a steady rate until addition of fructose, at which point a slight decrease in the rate of pH rise was apparent for a period, but nothing so obvious as with former experiments (Chapter 4, Chapter 5). This is not surprising, as the addition of another carbohydrate known to be a substrate of the LAB is unlikely to cause any overt difference in the, already many factored, pH signature.

6.3.2 Arginine data

Figure 6.2 shows graphically the alteration in the rate of arginine use by the ADI pathway. Two lines of best fit are indicated, before and after the time of fructose addition to highlight the decrease in the rate of arginine catabolism. Fructose effects on the ADI pathway can be observed in Table 6.1 and Figure 6.2. This data is a typical sample from the experiments, which gave highly reproducible results. Analysis showed a reduction of approximately 50% in the rate of arginine degradation.

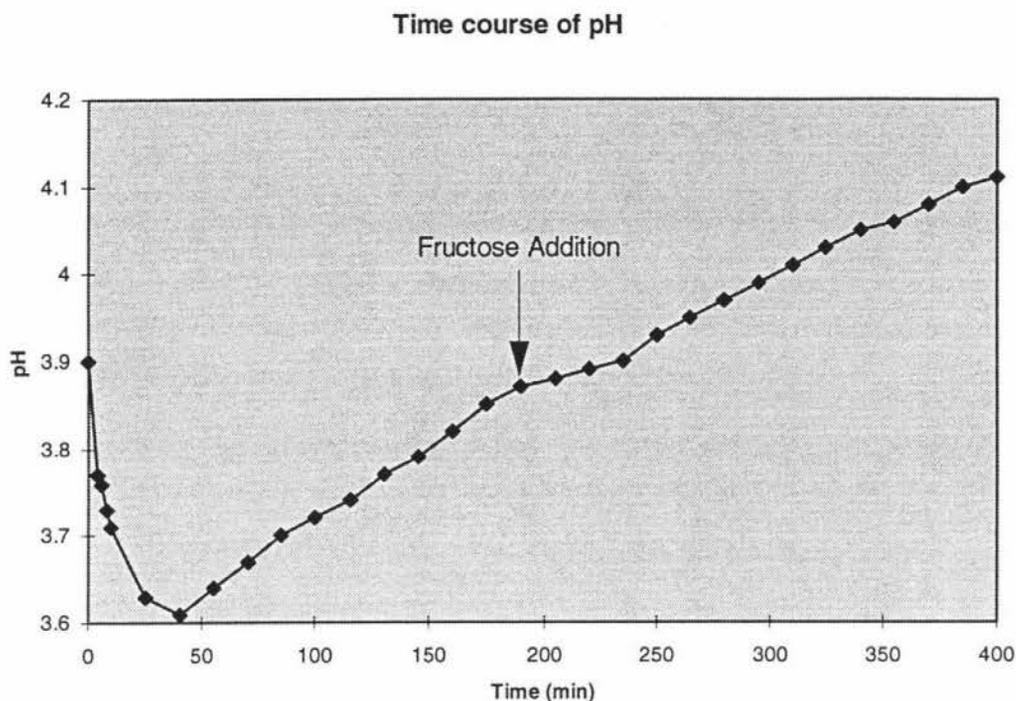
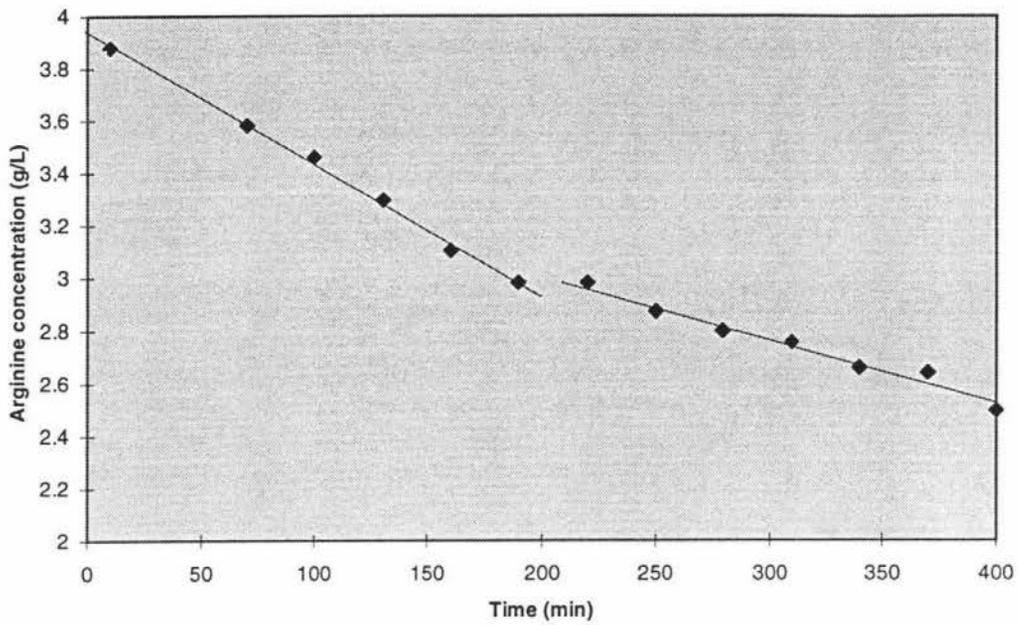


Figure 6.1. pH Overview of Cell Activity Before and After Addition of Fructose

Arginine
Before fructose addition: $y = -0.005076x + 3.942588$, R-squared: 0.996307.
After fructose addition: $y = -0.002393x + 3.4875$, R-squared: 0.977759.
Activity rate decrease: by factor of 2.12.
Ammonia
Before fructose addition: $y = 1.485449x + 40.989239$, R-squared: 0.937438.
After fructose addition: $y = 0.794048x + 93.511905$, R-squared: 0.895889.
Activity rate decrease: by factor of 1.87.

Table 6.1. Regression Analyses for Figure 6.2 Graphs

Time Course of Arginine Degradation



Time Course of Ammonia Production

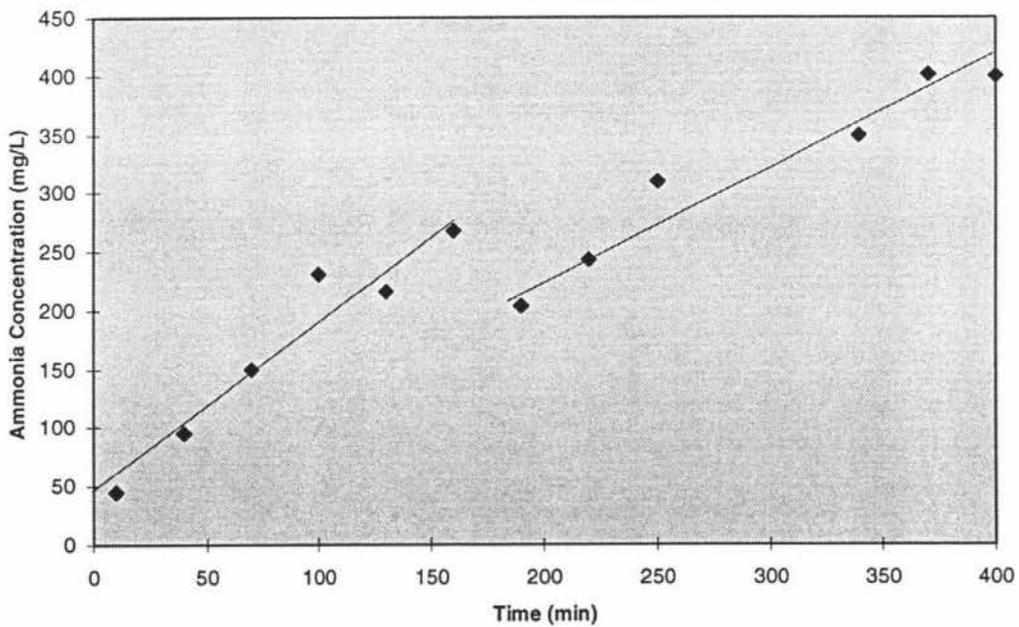


Figure 6.2 Rates of Arginine Degradation and Ammonia Production Over Time Before and After Fructose Addition

6.3.3 Ammonia data

Table 6.1 and Figure 6.2 show typical data from the series of experiments, producing much the same 50% decrease in rate of ammonia production as was observed in with arginine.

6.3.4 Citrulline Output

Measures of citrulline were taken on only a few samples, mostly as an indicator of its presence only, as a check on the actions of the ADI pathway. As with the previous experiments, as arginine degradation and ammonia production occurred, citrulline was also present.

6.4 Discussion

Fructose was shown to affect the activity of the ADI pathway enzymes in this Group II *Leuconostoc*. A reduction in the rates of arginine degradation and ammonia production of approximately 50% was seen once it was inoculated into the experimental mixture. During heretofore fermentation fructose can be reduced to mannitol and thus act as a hydrogen acceptor for the reoxidation of NADH and NADPH (Kandler, 1983). Additional ATP can then be produced from the acetyl phosphate formed from the phosphoketolase reaction, along with acetate, instead of being reduced to ethanol. This extra ATP per mole of substrate could be the reason for the repressive effect of fructose on the ADI pathway. Differences in mechanism or rate of transport of glucose and fructose are other possible reasons for the repressive effect

7.0 Summary and Conclusions

Throughout the experimental procedures there was always some activity of the arginine deiminase pathway when arginine was present in the system. Having been grown in a complex medium with added arginine before experimental procedures began the ADI pathway enzymes of the wine LAB had been active previously, halted in washing procedures as they were prepared as resting cells. Neither of the wine LAB used appeared to require induction of the ADI pathway by any substrate other than arginine itself. This can be identified by the degradation of arginine after its inoculation into the medium when even internal glucose was known to have been entirely catabolised. The investigations into glucose and fructose effects on enzyme induction were thus involved with regulation rather than complete initiation of the pathway.

This study has demonstrated the use of non-growing cells in a buffered medium to investigate the effects of glucose and fructose on the enzyme initiation of the arginine deiminase pathway. Glucose and arginine were metabolised concurrently, indicating no repressive effect of the carbohydrate on the ADI pathway. Indeed, glucose addition apparently enhances activity of the arginine deiminase pathway in the non-growing cell suspensions. Rate increases of 100% or more in both arginine degradation and ammonia production were seen to occur after glucose introduction to the system, while removal of the glycolytic activity of glucose resulted in a drop of the rate of arginine degradation of 50%. This data suggests that the presence of the fermentable substrate glucose is indeed a positive regulator in the initiation of the ADI pathway enzymes in these wine lactic acid bacteria. As the blocking of glycolysis reversed the effects of glucose on the system, the availability of ATP seems likely to be the method of regulation supplied by glucose fermentation.

Fructose was shown to affect the activity of the ADI pathway enzymes in the Group II *Leuconostoc*, MCW. A reduction in the rates of arginine degradation and ammonia production of approximately 50% was seen once it was inoculated into the experimental mixture. Continuing on from the likely method of glucose regulation, the extra ATP per mole of substrate could be the reason for the repressive effect of fructose on the ADI

pathway, lessening the need for the cells to have multiple energy gaining cycles in action.

Future work in this area would likely involve a broadening of the range of wine LAB involved - testing a number of group I and II organisms. The parameters of the experiments could undergo a variety of alterations to more fully investigate the factors involved. The effects of differing levels of glucose and fructose on the system could be investigated. The amount of glucose seems unlikely to have been a rate limiting factor in these experiments, considering the amount still in solution at their terminus, however increases as well as decreases in its concentration in solution could be investigated. Alterations in fructose levels would serve a similar purpose, while determining a Linweaver-Burke plot for fructose as a potential inhibitor at various rate limiting concentrations of arginine would also be of interest. Including factors such as differing amounts of ethanol to simulate the wine environment more closely, or temperature variations, such as occurs after primary fermentation, could give a deeper insight into the ramifications for the arginine as an alternate substrate in the commercial wine environment.

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