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MALOETHANOLIC DEACIDIFICATION OF HIGH ACID JUICES DURING WINE YEAST ALCOHOLIC FERMENTATION

A thesis presented in partial fufilment of the requirements for the degree of Master of Science in Microbiology at Massey University, New Zealand.

Frances Alison Ryan
February, 1996

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I would like to dedicate this thesis to a very special person who has brought much peace and love to my life.

F.R.

ACKNOWLEDGEMENTS

Thankyou to the Dept. of Microbiology and Genetics for research facilities and to the Molecular Genetics Unit, especially Carolyn Young, for all her time and assistance with karyotype analysis. Thanks also to Dr. Richard Morenzoni who also helped guide this part of my research.

To my supervisor, Dr. Gordon Pilone, a very special thanks is extended. Thankyou for all your patience, time and guidance over the last two years. Also to Anne Pilone, thankyou for the translation of various foreign papers.

I would like to thank Paul Mooney of Mission Vineyards and Kate Radburn of C.J.Pask Winery, and all the management and staff at these wineries, for your participation and cooperation in this project. Thanks also to Paul Monk of Lallemand Industries for providing the yeast strains used in this research.

A grant, for partial research funding from Winegrowers New Zealand is gratefully acknowledged. So too, is a New Zealand Federation of University Womens Scholarship which provided some financial support in the final year of my masterate.

Thanks to my friends and all the staff at Massey. To Karyn, Peter, Lisa, Chris, Anita, Linda, Miranda, Scott, Pete, Tanya and all the rest- thanks for all you kindness and help, and especially for the laughs we all shared.
And to my best friend, Grant, whose understanding, support and love has always been in abundance, a very special thankyou is given.

Finally an enormous thankyou to my mother and father and all my family. Your support and encouragement, especially at the end, has meant so much to me.

TABLE OF CONTENTS

CONTENT	PAGE
Acknowledgements	iv
Table of Contents	v
List of Figures	ix
List of Tables	xii
Abstract	xiii
1.0 Introduction	1
1.1 Objectives	2
2.0 Literature Review	4
2.1 Winemaking	4
2.2 Yeasts	6
2.2.1 Introduction	6
2.2.2 Yeast Diversity	6
2.2.3 Yeast Growth	9
2.2.4 Yeast Metabolism	11
2.3 Deacidification	15
2.3.1 Introduction	15
2.3.2 Physio-chemical	16
2.3.3 Biological	19
2.4 Genetics	29
2.4.1 Mutagenesis	29
2.4.2 Cell Fusion	30
2.4.3 Extrachromosomal Inheritance	30
2.4.4 Transformation	31

3.0 Materials and Methods 34

3.1 Simulated Grape Juice Fermentations 34

3.1.1 Microorganisms 34

3.1.2 Preparation of Microorganisms 34

3.1.3 Fermentation Media 35

3.1.4 Fermentation Procedure 36

3.1.5 Yeast Mannitol (YM) Agar 36

3.1.6 Peptone Water 36

3.2 Industrial Fermentations 37

3.2.1 Preparation of Microorganisms 37

3.2.2 Juice Preparation 37

3.2.3 Fermentation Procedure 37

3.2.4 Perchloric Acid 38

3.3 Laboratory Grape Juice Fermentations 38

3.3.1 Juice Preparation 38

3.3.2 Fermentation Procedure 39

3.4 Sample Analysis 39

3.4.1 Temperature 39

3.4.2 Titratable Acidity 39

3.4.3 pH 40

3.4.4 Cell Count 40

3.4.5 Brix 40

3.4.6 L-malic Acid 40

3.4.7 D-glucose and D-fructose 41

3.4.8 Alcohol 41

3.4.9 Reducing Sugar Test 41

3.4.10 SO_2 41

3.5 Statistical Analyses 41

3.5.1 Acid-Ranking Taste Tests 41

3.5.2 Laboratory Grape Juice Fermentations 42

3.5.3 Standard Solutions 42

3.6 Yeast Karyotype Analysis 42

4.0 Results 44

4.1 Standard Solutions 44

4.2 Laboraory Grape Juice Fermentations 46

4.3 Simulated Grape Juice Fermentations 50

4.3.1 Standard Fermentations 50

4.3.2 Variable Temperature Fermentations 55

4.3.3 Variable Malic Acid Concentration Fermentations 60

4.3.4 Variable Nitrogen Concentration Fermentations 63

4.3.4 Variable pH Level Fermentations 66

4.4 Industrial Fermentations 69

4.4.1 Chardonnay Juice Fermentations 69

4.4.2 Sauvignon Blanc Juice Fermentations 74

4.4.3 Acid-Ranking Taste Tests 76

4.4.4 Karyotype Analysis of Yeasts 78

5.0 Discussion 86

5.1 Comparison of Simulated Grape Juice and Commercial Grape Juice Fermentations 87

5.2 Comparison of Variables 89

5.3 Maloethanolic Performance of Yeast Strains 91

5.3.1 Strain EC1118 91

5.3.2 Strain ACID- 93

5.3.3 Strain 71B 93

5.3.4 Strain D432 94

5.3.5 Strain M 96

5.3.6 Strain 442 96

5.3.7 Acid-Ranking Taste Tests 97

5.3.8 Karyotype Analysis 97

6.0 General Conclusions 100

Appendix A 103

Appendix B 109

Appendix C 114

Appendix D 120

Appendix E 125

Appendix F 130

Appendix G 135

Literature Cited 144

LIST OF FIGURES

FIGURE **PAGE**

2.1 Biological deacidification of malic acid. 20

4.1 Percentage reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented (18°C) with different yeast strains. 52

4.2 Malic Acid (M.A.) and titratable acidity (T.A.) concentrations during fermentations (18°C) of Simulated Grape Juice inoculated with yeast strain M. 52

4.3 Malic Acid (M.A.) and titratable acidity (T.A.) concentrations during fermentations (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. 54

4.4 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice inoculated with different yeast strains. 54

4.5 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented at different temperatures with yeast strains E (EC1118) and D (D432). 57

4.6 Malic Acid (M.A.) and titratable acidity (T.A.) concentrations during fermentations (25°C) of Simulated Grape Juice inoculated with yeast strain EC1118. 58

4.7 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice inoculated with strains E (EC1118) and D (D432). 59

4.8 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented with varying initial concentrations of malic acid (g/L) with yeast strains EC1118, 71B and D432. 62

4.9 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice with different initial concentrations of malic acid (g/L) inoculated

with yeast strains EC1118, 71B and D432. 62

4.10 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented with varying initial concentrations of nitrogen (mg/L) with yeast strains EC1118, 71B and D432. 65

4.11 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice with different initial concentrations of nitrogen (mg/L) inoculated with yeast strains EC1118, 71B and D432. 65

4.12 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented at different initial pH levels fermented (18°C) with yeast strains EC1118, 71B and D432. 68

4.13 pH values of fermentations (18°C) of Simulated Grape Juice at different initial pH levels inoculated with yeast strains EC1118, 71B and D432. 68

4.14 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of wines vinified in duplicate from commercial Chardonnay juice with strains EC1118, ACID-, 71B and D432. 71

4.15 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain 71B. 71

4.16 Initial and final pH values of commercial Chardonnay juice vinified in duplicate with yeast strains EC1118, ACID-, 71B and D432. 73

4.17 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of wines vinified from commercial Sauvignon Blanc juice with strains EC1118, ACID-, 71B and D432. 75

4.18 Initial and final pH values of commercial Sauvignon Blanc juice vinified with yeast strains EC1118, ACID-, 71B and D432. 75

4.19 CHEF electrophoresis of reference strains and isolates removed from fermenting Sauvignon Blanc juice. 79

4.20 CHEF electrophoresis of reference strains and isolates removed from fermenting Sauvignon Blanc juice. 79

4.21 CHEF electrophoresis of isolates removed from fermenting Sauvignon Blanc juice and fermenting Chardonnay juice. 81

4.22 CHEF electrophoresis of reference strains and isolates removed from fermenting Chardonnay juice. 81

4.23 CHEF electrophoresis of isolates removed from fermenting Sauvignon Blanc juice and fermenting Chardonnay juice. 82

4.24 CHEF electrophoresis of isolates removed from fermenting Chardonnay juice and fermenting Sauvignon Blanc juice. 82

4.25 CHEF electrophoresis of isolates removed from fermenting Chardonnay juice and fermenting Sauvignon Blanc juice. 84

4.26 CHEF electrophoresis of reference strains and isolates removed from fermenting Chardonnay juice. 84

LIST OF TABLES

TABLE **PAGE**

4.1 Estimation of reproducablity of some analytical methods used in this this research. 45

4.2 Analyses of wines from replicate laboratory grape juice fermentations. 47

4.3 Differences between initial juice and final wine component concentrations and (percent change) of replicate Chardonnay juice fermentations. 48

4.4 Analyses of fermentations (18°C) of Simulated Grape Juice inoculated with different yeast strains. 51

4.5 Analyses of fermentations at different temperatures of Simulated Grape Juice inoculated with yeast strains E (EC1118) and D (D432). 56

4.6 Analyses of fermentations (18°C) of Simulated Grape Juice with varying initial concentrations of malic acid. 61

4.7 Analyses of fermentations (18°C) of Simulated Grape Juice with varying initial concentrations of nitrogen. 64

4.8 Analyses of fermentations (18°C) of Simulated Grape Juice at different pH levels. 67

4.9 Analyses of fermentations of commercial Sauvignon Blanc and Chardonnay juices inoculated with different yeast strains. 70

4.10 Taste trials of Chardonnay wines produced by different yeast strains. 77

4.11 Taste trials of Sauvignon Blanc wines produced by different yeast strains. 77

ABSTRACT

Malic acid is a major acid found in grapes. In countries with cooler climates, such as New Zealand, this acid is not fully respired from grapes and will impart a sour taste on grape juices. Therefore steps must be taken to ensure deacidification of the juice or wine occurs. Deacidification is the process whereby the acidity of a juice or wine is lowered by physical, chemical or biological means. Biological methods of deacidification such as malolactic fermentation and maloethanolic fermentation involve the degradation of malic acid to other products. Malolactic fermentation is the bacterial conversion of malic acid to lactic acid and carbon dioxide and is commonly used in New Zealand wineries. Maloethanolic fermentation is the simultaneous conversion of grape sugars and malic acid to ethanol by specialized yeast strains and is the focus of this investigation.

This research examines several commercially available yeast strains (Lallemand Lalvin strains 71B, ACID-, D432 and reference strains EC1118 and Red Star Montrachet (M)) and *Schizosaccharomyces* strain 442, for their ability to degrade malic acid during grape juice fermentation under New Zealand conditions. A Simulated Grape Juice medium was used to mimic these conditions, as well as commercial Chardonnay and Sauvignon Blanc juices. Strains 71B and D432 consistently degraded the greatest percentage of malic acid under all conditions and parameters investigated in this research. Respectively, these strains degraded malic acid by 36% and 22% of the initial concentration (7.0g/L) in industrial Chardonnay juice fermentations and by 47% and 36% of the initial concentration (3.7g/L) in industrial Sauvignon Blanc fermentations. Furthermore, in Sauvignon Blanc wines, a significant (P=0.05) difference was found between the wine made with strain 71B and all other wines. However, in Chardonnay wines, a significant difference was found between the wine made with strain D432 and all other wines. In addition, molecular genetic techniques (CHEF chromosomal banding pattern polymorphisms) were utilised to confirm yeast strain identity.

from industrial fermentations. From this, it was concluded that all strains inoculated into the commercial juices were dominant at the most vigorous stage of fermentation.

Factors influencing malic acid degradation were investigated in Simulated Grape Juice fermentations. These included initial concentrations of malic acid and nitrogen and the initial pH level of the juice. It was found that strains 71B and D432 degraded the greatest percentage of malic acid when the initial malic acid concentration of the juice was high (7.5g/L), the initial nitrogen concentration was low (463mg/L with proline) and an initial pH of pH 3.5.

These results indicate that there is an interaction between yeast and grape variety/maturity, and that proper selection of yeast strain can be used as a tool for deacidification.

1.0 INTRODUCTION

Wine is produced by the fermentation of grape sugars into alcohol. The two main sugars, fructose and glucose, constitute the majority of soluble sòlids in grapes. Harvesting of the grapes rich in these sugars occurs after a lengthy growth period. Flowering, pollination and germination occurs in the previous spring, and is followed by a vegetative or first growth cycle. This cycle lasts 3-4 weeks and involves a rapid increase in berry size followed by a 4-6 week cycle of berry cell enlargement. At this stage the berry is still hard and contains large quantities of organic acids. The berry then enters a lag phase (approximately 4 weeks in duration) in which growth slows and the grape seeds complete the majority of their development. The ripening phase, or second growth cycle, then begins and lasts 5-8 weeks. Commencement of this phase is marked at veraison. Berries soften, change colour, respire organic acids, accumulate further sugar and expand in volume (Coombe and Dry, 1992). Hand or machine harvesting follows when a suitable Brix level is reached.

In New Zealand, a predominantly cooler climate exists which reduces the ripening period of grapes. As a consequence, the grapes harvested at sugar (Brix) maturity, are underripe. This means that considerable amounts of organic acids are not respired from the grape. The main types of organic acids retained in the grape are L-malic acid and D-tartaric acid. L-malic acid imparts a tart taste on the wine at levels above 3 g/L. Methods of removing or biochemically converting this acid to a weaker form is often necessary in many New Zealand wines to improve organoleptic properties.

Juice or wine deacidification is achieved by physical, chemical or biological means (Beelman and Gallander, 1979). However, most methods rely on the juice first being fermented. Alcoholic fermentation can either occur spontaneously, or by the introduction of desirable wine yeast strains such as *Saccharomyces cerevisiae*. Most New Zealand wineries use biological methods of deacidification such as bacterial malolactic

fermentation (MLF). Biological methods of deacidification are gaining increased popularity due to consumer demand for grapes and grape products to be free of artificial substances (Coombe and Dry, 1992).

MLF involves lactic acid bacteria (LAB), which convert L-malic acid to L-lactic acid. As a result, a slight increase in pH of the wine occurs and the latter acid imparts a less sour taste on the wine. However, problems with such a method are numerous and include control and inoculation of LAB and an extended period of wine production.

Another biological method to reduce acidity not widely used in New Zealand wineries, is maloethandic fermentation (MEF). MEF involves the use of specialised yeast strains to simultaneously deacidify and ferment the wine. Deacidification is achieved through yeast metabolism, where L-malic acid is taken up into the yeast cell and metabolised to ethanol and carbon dioxide (CO_2). The process reduces acidity and increases pH. As a consequence, the benefits of MEF to the winemaker include easier induction and control of yeast strains compared to bacterial cultures, with less expense as only one microorganism needs to be cultured. In addition, the overall vinification process is shortened since deacidification is simultaneous with alcoholic fermentation instead of following as with MLF.

1.1 Objectives

Research conducted with MEF on New Zealand grape juices would be advantageous to winemakers to establish parameters of use in New Zealand wineries. This study therefore investigates the activity of MEF yeast strains in simulated grape juice and in Chardonnay and Sauvignon Blanc juices. The parameters in which effective malic acid degradation occurs were studied through alteration of the simulated grape juice, whilst Chardonnay and Sauvignon Blanc juice trials were undertaken to examine yeast strain

performance in industrial situations. Both the chemical and organoleptic (where appropriate) effects of malic acid degradation were investigated.

2.0 LITERATURE REVIEW

2.1 Winemaking

Various vinification methods are used to produce wine. The general outline of these procedures is presented below.

Grapes are first destemmed and crushed. Pectic enzymes may be added to aid cell wall deterioration which liberates more juice.

In production of white wines, the pips and skins are removed from the juice whilst being retained in red wines for colour and flavour extraction. The pH of the juice must be able to sustain yeast growth and chemicals may be used to adjust a pH imbalance (e.g. tartaric acid to lower pH, or calcium carbonate to increase pH). Sulphur dioxide (SO_2) is often added (20-100 mg/L) for protection of the juice due to its antioxidant and antimicrobial properties. It is not added if a spontaneous fermentation is desired. Clarification of the juice to remove suspended solids takes place by settling overnight at a low temperature, or by centrifugation in more technologically advanced wineries. At this time bentonite and diammonium phosphate (DAP) may also be added. Bentonite clay adsorbent removes excess proteinaceous materials whilst DAP is a source of nutrients in which yeasts are able to utilize components for growth. The juice is then transferred into a clean tank for alcoholic fermentation.

Red wine production requires a period of skin contact. The skins break off the grape flesh during alcoholic fermentation due to the formation of carbon dioxide (CO_2) gas. The floating skins (cap) are mixed with the fermenting juice to control temperature increase and to induce further colour and flavour extraction.

Alcoholic fermentation generally begins with the introduction of wine yeasts, often in active dry yeast (ADY) form purchased from commercial suppliers. An ADY inoculation of 5 x 10^6 cells/ml is desired for a vigorous and complete fermentation. However, traditional winemakers support spontaneous methods of fermentation where indigenous yeasts on the grape conduct the fermentation [yeasts are discussed further in section 2.2].

Over the course of the fermentation heat is generated. As a result, fermentations need to be controlled by refrigeration. White wine fermentations are controlled at temperatures of 10-18°C and red wine fermentations are controlled between 22-27°C. Temperatures above 30-35°C will kill the yeast cell and harm the wine (Reed and Nagodawithana, 1991). Cooler temperatures of white wine fermentations ensures that the fresh, crisp, varietal flavours are not lost. Temperature control is also important so that colour extraction and ethanol evaporation do not occur (Kunkee and Bisson, 1993). Alcoholic fermentations take between 14-40 days depending on the temperature, variety and yeast strain(s) used. Approximately 10-14% v/v alcohol is formed in most table wines. Due to this high concentration of alcohol, most yeast cells lyse and settle to the bottom of the tank. These dead cells are known as the "lees".

The wine is racked from the lees into another tank, unless deacidification is required (deacidification is discussed further in section 2.3). The headspace of the tank is sparged with inert gas (CO_2, N_2) to replace the air. This prevents oxidation of the wine occuring. Further additions of SO_2 may also be made to protect the wine.

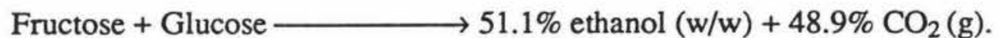
Post fermentation processes of clarification and stabilization are required to prevent chemical change and microbial spoilage. Fining agents such as bentonite, gelatine and caseinate may be added to prevent protein haze formation (Reeves, personal communication). The pH may be chemically adjusted (see above) to a desired level. The wine is then racked either into bottles for immediate consumption, or into wooden barrels or stainless steel tanks for further aging.

2.2 Yeasts

2.2.1 Introduction

In 1818 Erxleben recognised that yeast cells in beers and wines were responsible for the fermentation process (Martin, 1993). However, his work was not taken seriously, and not accepted until the time and work of Louis Pasteur in the late 1800s.

Wine yeasts are responsible for the fermentation of grape sugars which mainly generate alcohol and CO_2 in winemaking (Reed and Nagodawithana, 1991):



Fructose + Glucose ⟶ 51.1% ethanol (w/w) + 48.9% CO_2 (g).

2.2.2 Yeast Diversity

Yeasts are present in the vineyard, on the grapes and on winery equipment. Some yeasts are able to survive from year to year on this equipment, whilst others survive in the intestines of bees and wasps, and are transferred during harvest season by fruit flies (Reed and Nagodawithana, 1991). Not all yeasts present are beneficial to the winemaking process. Yeasts initially found on the grapes and after crushing are called "wild" yeasts and are predominantly *Kloeckera, Hansenula, Hanseniaspora, Candida, Pichia* and *Schizosaccharomyces* species. These yeasts are nearly always inhibited by alcoholic fermentation due to low alcohol tolerance and are succeeded by *Saccharomyces cerevisiae* (Reed and Nagodawithana, 1991). *S. cerevisiae* strains are able to become dominant during alcoholic fermentation due to increased SO_2, sugar, CO_2 and alcohol tolerances and a low pH tolerance, which constitutes the harsh wine environment.

S. cerevisiae has been described as the wine yeast of excellence (Martini, 1993), and is more generally isolated from the winery than the grape or vineyard. However, there is

debate over the "birthplace" of this yeast. Some authors believe the winery is the natural home of *S. cerevisiae* where the yeast is able to survive from harvest to harvest in or on the equipment, especially if it is wooden. Others believe that *S. cerevisiae* is transferred from the vineyard to the cellar (Mortimer, 1995). Mortimer (1995) argues derives that not only is *S. cerevisiae* able to be isolated from the vineyard, but also that the strains of *S. cerevisiae* present in spontaneous fermentations can firstly change up to ten times during the course of the fermentation, and secondly that the yeast populations present in the fermentation are characteristic of the must, not the winery. However, at some point in time, commercially grown *S. cerevisiae* would have been used in most wineries, survived and become involved in subsequent fermentations.

Recent developments have enabled genetic techniques to monitor the yeasts present in the fermentation. Previously, winemakers were unsure which yeast conducted the fermentation, even when a commercial inoculum was added. In the past, differentiation was based on morphological, biochemical, sexual reproduction and growth response methods (Kunkee and Bisson, 1993); or on the pattern of the fermentation process (Reed and Nagodawithana, 1991). Recently, molecular genetic techniques such as electrophoretic separation of chromosomes (karyotype analysis) have been utilised to identify yeasts present in the fermentation (Schutz and Gafner, 1993). Shutz and Gafner (1993) found from karyotype analysis that the inoculated yeasts are dominant during and at the end of fermentations. [Karyotype analysis is discussed further in section 4.4.4 and 5.3.8].

Traditional winemakers encourage wines to undergo alcoholic fermentations spontaneously rather than using commercial yeast preparations (Reed and Nagodawithana, 1991). They believe that wines made with wild yeasts give a more complex wine with a better aroma compared with commercially prepared strains (Reed and Nagodawithana, 1991). However, the quality of wines made from spontaneous fermentations cannot be reliably reproduced (Reed and Nagodawithana, 1991).

Furthermore, if ADY were at some point introduced into the winery it may become the dominant yeast in the spontaneous fermentation (Martini, 1993).

Presently, most New Zealand wineries use commercially produced yeasts in ADY form due to simplicity and ease of use. From the 1960s when yeasts became available in this form, increasing acceptance and usage has grown worldwide (Kunkee and Bisson, 1993). However, the origin of most of the strains are unknown (Reed and Nagodawithana, 1991). ADY preparations are not necessarily a pure yeast culture. Karyotype analysis has shown that two different *S. cerevisiae* strains from the same ADY preparation were in competition at the most vigouous stage of alcoholic fermentation (Schutz and Gafner, 1993). Trials by Bakalinsky and Snow (1990) involved the isolation of pure cultures from ADY preparations followed by the comparison of their activity against typical ADY preparations. It was observed that pure cultures did not show any increase in fermentation performance. Comparitively, Wenzel *et al* (1982) observed that pure cultures exhibited a narrower range of malic acid degradation than standard commercial preparations.

There are several types of commercial preparations: traditional, ADY and liquid starter cultures. Commercial yeast starters give the winemaker greater control over the fermentation, and are said to be more economical with respect to fermentation time, style and avoidance of delayed or stuck fermentations (Henick-Kling, 1988). Traditional starters involve the inoculation of a juice with an amount of already actively fermenting juice. Problems arise however with timing and loss of yeast viability (Henick-Kling, 1988). ADY have been previously described and have further advantages including small inoculation sizes, ready-to-use, reproducibility, and have special fermentation characteristics specific to the strain. However ADY cultures are not able to be directly added to the juice; they must first be rehydrated (Henick-Kling, 1988) for bioactivation. Liquid starter cultures are prepared commercially and do not need drying or rehydration. These cultures require specific conditions for growth. When sufficient growth has

occured liquid cultures are added directly to the juice (Henick-Kling, 1988). However, possible contamination can occur during the growth stage.

2.2.3 Yeast Growth

Most wine yeasts are oval or ellipsoid in shape and reproduce asexually by budding. However, sexual recombination has been observed in the laboratory and is presumed to occur in nature (Zoecklein *et al,* 1995). The position of the buds formed on the parent cell provide a means of yeast identification. For example, if multilateral buds are formed the yeast species is likely to be of the *Saccharomyces* genera whilst polar budding indicates *Kloeckera* or *Hanseniaspora* genera. Alternatively, *Schizosaccharomyces* species are cylindrical in shape with hemispherical ends. This genera reproduces asexually but divides by fission rather than budding (Zoecklein *et al,*1995).

Budding commences in *Saccharomyces* species by the duplication of the parental spindle pole body which initiates nuclear membrane division and cell separation (Wheals, 1987). In comparison, *Schizosaccharomyces* species elongate, form cross walls, then separate in a manner similar to bacterial cells (Wheals, 1987).

When used in wine fermentations, yeast population growth occurs in three main stages. According to Ribereau-Gayon (1985) these are:

a) Multiplication. This stage lasts 2-5 days and the population reaches a maximum of 10^7-10^8 cells/ml.
b) Stationary Phase. This is where the viable cell level remains approximately the same, and lasts about 8 days.
c) Decline. During this phase which lasts several weeks, the population numbers fall to around 10^5 cells/ml.

Alcoholic fermentation reaches its most vigorous stage during the stationary phase which is brought about when sugar concentration is still high but nitrogen and other macronutrients have been depleted. Yeast growth stops before all the sugar is metabolized. This is probably due to a lack of energy for biosynthesis because anaerobic conditions only produce 2 molecules of adenine triphosphate (ATP) as compared to 38 ATP aerobically.

Yeast growth during fermentation can be effected by a number of factors. These factors include temperature, SO_2 and juice clarification and composition.

The optimum temperature for maximum yeast cell growth occurs at 20-25°C (Fleet and Heard, 1993). Temperatures above 30-35°C cause cell lysis which harms the wine (Reed and Nagodawithana, 1991). Cooler temperatures however, favour the production and retention of flavour volatiles but may slow the rate of cell growth, which increases fermentation duration (Fleet and Heard, 1993).

The addition of SO_2 to the juice or must may inhibit the growth of many wild yeasts. This prevents the occurence of spontaneous fermentations which are not wanted by winemakers who prefer ADY fermentations. Most ADY strains are resistant to the additions of SO_2 used to kill wild yeasts (Fleet and Heard, 1993).

Clarification of the juice may remove indigenous yeast populations. This may be desirable in wineries wanting selective yeast growth, such as the subsequent addition of ADY (Fleet and Heard, 1993).

Juice composition effects the rate of yeast cell growth, the fermentation and the quality of the resultant wine. Juices lacking in some constituent (e.g. nitrogen) may deletoriously effect yeast growth giving a sluggish fermentation which may eventually become "stuck". Other important constituents in the juice to effect cell growth include initial sugar and acid

concentrations, dissolved oxygen and the presence of vitamins. In addition, chemical and insecticide spray residues remaining on the grape can prevent the onset of fermentation.

2.2.4 Yeast Metabolism

The three main sugars in grapes; glucose, fructose and mannose, are transported into the yeast cell via facilitated diffusion. This process is complex in *Saccharomyces* due to the involvement of multiple carriers (Fleet and Heard, 1993). Although not a lot is known about these carriers, a high affinity putative glucose carrier has been cloned and sequenced on the SNF3 gene of *S. cerevisiae* (Kunkee and Bisson, 1993). Once sugar has been integrated into the yeast cell, it is metabolised through the glycolytic pathway. This pathway produces pyruvate which can be converted to the waste product ethanol or used in the creation of energy or cell components via the Krebs cycle.

Further products are formed by yeasts during alcoholic fermentation. Higher alcohols, esters and other important flavour compounds are formed from the secondary metabolism of pyruvate. Higher alcohols or fusel oils, are quantitatively the most important group of flavour compounds formed (Reed and Nagodawithana, 1991). The major fusel oils formed are n-propanol, isobutanol, active amyl alcohol, isoamyl alcohol and phenethyl alcohol. Aerobiosis increases the level of fusel oil production, which may lead to increased temperatures and pH (Reed and Nagodawithana, 1991) and possible spoilage.

Ester production is greater in wines from musts with higher sugar concentrations and higher fermenting temperatures. The yeast strain used also contributes to ester formation. The fruity aroma of wines is due to isoamyl acetate and n-hexyl acetate, whilst the more 'heady' aroma's are due to ethyl octanoate, ethyl decanoate and 2-phenethyl acetate formation (Reed and Nagodawithana, 1991).

Other important flavour compounds are formed and include diacetyl, acetoin and butanediol (Reed and Nagodawithana, 1991). However, bacteria rather than yeasts generally cause excess formation of these products.

Glycerol is nonvolatile and formed by the reduction of dihyroxyacetone phosphate (Zoecklein *et al,* 1995). In some wines glycerol is produced in greatest abundance after ethanol and water (Rankine, 1993). Glycerol does not contribute to a wines aroma, but rather to its smoothness and viscosity. It is found in wine at concentrations between 1 and 10 g/L. Factors which influence glycerol production include; inoculation populations (10^8 cells/ml produce greatest amounts of glycerol), yeast strain used, aeration, agitation, increased temperature and the maturity of the grapes at harvest. Mature grapes contain more sugar, so more glycerol is produced when *S. cerevisiae* is under solute stress. At six degrees Brix and below, glycerol content becomes stable (Gardener *et al.*, 1993).

Organic acids may be produced or decomposed during alcoholic fermentation. Excretion of organic acids is due to disturbance of the intracellular pH, which affects the cells enzyme levels and control mechanisms, which in turn affects the level of excretion (Whiting, 1975). In countries with hotter climates, greater amounts of organic acids are respired from the grape into the atmosphere, which effects the intracellular pH. Additions of organic acids to help the acid balance, and prevent bacterial growth, take place in such climates (Wagener, 1971).

Succinic acid is the main acid produced by wine yeasts at levels of between 0.2-1.7 g/L. It is only produced during the exponential growth phase, but amounts of the acid produced depend on yeast strain, the composition of the nitrogen source (up to 500 mg/L nitrogen increase amounts of succinic acid) and initial levels of malic acid present (Heerde and Radler, 1978). Succinic acid is formed by the reduction of malate to pyruvate, to succinic acid (Radler, 1986); and also by amino acids precursors. Precursors are

deaminated to α-ketoglutaric acid or oxaloacetic acid which enter the Krebs cycle and are reduced to succinic acid by enzyme reduction (Heerde and Radler, 1978). In addition, a linear relationship exists between the fermentation of glucose and the production of succinic acid, but only up to a concentration of 8% glucose (Heerde and Radler, 1978). Nitrogen sources of glutamate and proline also increase succinic acid production. Oxidation of glutamate by fermenting yeasts to succinic acid is the main pathway by which the acid is formed (Heerde and Radler, 1978).

To a lesser extent, other organic acids may be excreted by yeasts during fermentation. Pyruvic, citramalic, lactic, gluconic, tartaric, acetic acids are formed in wine under certain fermentation conditions (Radler, 1993).

Formation of malate by wine yeasts has been reported since 1924 (Whiting, 1975). Malate is formed by the fixation of CO_2 by pyruvate to form oxaloacetic acid then subsequent reduction to malate by the yeast cell. Research has shown that approximately 2g/L malate was formed by *S. uvarum* strains (Radler and Lang, 1982) and approximately 1g/L malate was formed by*S. cerevisiae* in certain defined media (Subden, 1987). Factors which increased malate formation include sugar concentrations of 20-30%, pH ~ 5, the presence of CO_2 and a requirement of 100-250 mg/L nitrogen (Radler, 1993). Farris *et al* (1989) observed that most malate was formed at pH 4.2 and at a fermentation temperature of 25°C but was also dependant on the yeast strain used.

Castellari (1992) looked at cold tolerant *Saccharomyces* yeast strains and observed that lower quantities of acetic and volatile acids were formedwhen a greater production of malic acid (3.0 g/L to 4.58 g/L) was produced. Further work by the author and co-workers in 1994 showed that in cold resistant strains of *S. cerevisiae, S.uvarum* and *S. bayanus*, malic acid was again produced. Higher levels of glycerol and succinic acid were also formed which lowered the yield of ethanol produced. These strains were active from 6 to 30°C (Castelburi *et al.*, 1994). The benefit of these strains would be to

produce a more balanced wine by biological methods rather than chemical or physical means.

Malic acid can be degraded by maloethanolic fermentaion (MEF) and malolactic fermentation (MLF). [MLF is discussed further in section 2.3.3]. During MEF maloethanolic yeast strains firstly oxidatively decarboxylate malic acid to pyruvate by an NAD-dependent malic enzyme. This pyruvate is then decarboxylated to acetaldehyde which is then reduced to ethanol. In addition, some malate may be transformed to succinate via fumarate (Radler, 1986).

Schizosaccharomyces pombe is reported to consistently degrade most, if not all the initial malic acid present in grape juices (Maconi *et al,* 1984). Although many different yeasts have enzymes necessary for degradation of malic acid, it is thought that *S. pombe* has a very high substrate affinity of its malic enzyme, and an active carrier protein assisting transport across the yeast cell membrane. Grobler *et al* (1995) recently identified and cloned the gene partially responsible for L-malate uptake by this yeast. The *mae* 1 gene encodes a permease involved in the uptake of l-malate, succinate and malonic acid. Research performed by Queiroz *et al* (1990) suggests that the monoanionic form of malic acid is the preferred form for transport into the*S. pombe* cell. In addition, malate transport in *S. pombe* is pH and temperature dependent (Osothsilp and Subden, 1986).

Conversely, malate enters *S. cerevisiae* by simple diffusion, and its metabolism may be under catabolite repression where glucose is depleted before malate is utilized. The glucose in the medium may act as an inducer for the expression of malate transport gene(s) (Osothsilp and Subden, 1986). Transport of malate in *S. bailii* is by way of an inducible carrier protein (glucose dependent). The effect of this carrier protein is to supply the cell with a higher intracellular concentration of malate than that of the level found in *S. cerevisiae* (Kuczynski and Radler, 1982).
[MEF is discussed further in section 2.3.3].

Fumaric acid constitutes about 1% of the organic acids in grapes. It is found in the Krebs cycle and is involved in yeast metabolism. In hotter countries where wines of low acid are generally produced, the addition of fumaric acid was considered due to its low cost and apparent biological stability conferred on the wine. However, trials have shown that fumaric acid can be degraded by yeasts conducting alcoholic fermentation, and should not be added prior to alcoholic fermentation (Pilone *et al.*, 1973). Initial pH and temperature also influence the amount of fumaric acid degraded. At pH 3.0, 78% of the fumaric acid was degraded, whilst only 28% was degraded at pH 4.2. At 25°C greater amounts of fumaric acid were degraded than at 15°C (Pilone *et al.*, 1973).

2.3 DEACIDIFICATION

2.3.1 Introduction

Milisavljevic stated in 1971 that acidity was the most important function of all the components in a wine (Beelman and Gallander, 1979). Acidity influences the colour, clarity, stability and the sensory qualities and characteristics of the wine. If too much acid is present, a sour taste predominates. If too little acid, the wine is regarded as flat or insipid (Beelman and Gallander, 1979). The optimum total acidity in table wines range from 0.55-0.85% where the lower percentage is a better level for red wines, whilst the higher percentage is better for white wines (Beelman and Gallander, 1979).

The process of deacidification in countries such as New Zealand is of critical importance due to the cooler climate where grapes are typically harvested under-ripe. The acid content in grapes is high as little respiration of acids occurs. Deacidification results in a wine which is less tart and sour tasting and thus easier to drink. In New Zealand grapes,

the main acid levels are typically 5 g/L tartaric acid and 5 g/L malic acid. Deacidification by removal of malic acid is more common than removal of tartaric acid due to the bitter, sour taste malic acid imparts (Fowles, 1992). The two main ways in which deacidification are achieved are by physio-chemical and biological methods.

2.3.2 Physio-chemical

a) Amelioration

Amelioration is the practice of adding sugar and water to the must or fermenting wine. In New Zealand the following sweetners are legally allowed to be added to the must or fermenting wine; fructose, glucose, glucose syrup, sugar, and sugar by products. Drinking water may also be added to a wine, but only in a proportion not exceeding 50ml/L of wine (Beattie, 1984).

Amelioration causes an increase in wine volume and hence dilution of the juice will result. This in turn will affect the final colour, body, flavour and aroma of the wine and so regulations are necessary. Malic and tartaric acid levels are diluted equally by this process, which deacidifies the wine. However, many authors consider amelioration an adulteration or falsification of wine (Beelman and Gallander, 1979).

(b) Neutralisation and Precipitation of Tartrate

In the 1970s neutralisation and precipitation of tartrate was the primary method of reducing acid in high acid musts and wines. Acidity is neutralized by one of several mineral salts. Calcium carbonate ($CaCO_3$) is the most common salt employed and in the presence of excess tartrate the following reaction occurs (Beelman and Gallander, 1979):

$$CaCO_3 + H_2T \text{ (excess)} \longleftrightarrow CaT(s) + H_2O(l) + CO_2(g)$$

Tartrate precipitates which lowers the acidity of the wine. The pH must not be raised above pH 3.6 during this procedure, so that colour and relative bacterial stability is maintained (Mattick *et al*, 1980). Treatment with $CaCO_3$ is used either directly or in a slurry form (Mattick *et al*, 1980). $CaCO_3$ treatment produces a good reduction in acidity but with minimal pH shift, hence, it is preferred over other chemicals which may also be able to precipitate tartrate. The main problems associated with this method is the delayed precipitation of calcium tartrate and calcium ions that remain in solution and impart a salty flavour to the wine (Beelman and Gallander, 1979).

Potassium carbonate ($KHCO_3$) can also be used in this process. However, it precipitates malate as well as tartrate, which increases the potential amount of deacidification. Although $KHCO_3$ can be used as an additive in New Zealand wines, it often is not due to excessive precipitation of acids causing a substantial pH increase. In European countries such as Germany and Switzerland, the minimum tartrate concentration allowed is 0.05-0.15% (Beelman and Gallander, 1979). Treatment with $KHCO_3$ may lower the tartrate concentration below this level.

(c) Double Salting

This is a German process developed to solve the problems associated with the above procedures. In the double salt procedure, $CaCO_3$ is added to a portion of the must until the pH is increased to pH 4.5 or above. At this pH, a double salt is formed followed by overnight settling and filtration. The treated portion is then mixed in with the untreated portion which increases the pH of the whole (Beelman and Gallander, 1979). In some cases 1% calcium double salt (Acidex™) is added in addition to $CaCO_3$ to encourage nucleation and crystal formation for faster and more complete precipitation of the double salt (Beelman and Gallander, 1979). Acidex™ is well suited to lowering high acid musts and wines as it is able to lower the total acidity by 1.0-1.2% if enough tartrate is present for precipitation (Beelman and Gallander, 1979). The procedure allows removal of nearly equimolar quantities of both malate and tartrate (0.13 g/100 ml tartaric acid is

required to precipitate 0.1 g/100 ml malic acid), and wine quality is said to improve (Beelman and Gallander, 1979).

(d) Ion Exchange

The New Zealand Wine Industry has never approved the method of ion exchange in this country. The French also do not approve of this method, but the United States use ion exchange in preference to $KHCO_3$ treatment. In countries which use ion exchange, regulations are imposed on resin types and operating procedures and principles (Beelman and Gallander, 1979).

Ion exchange was first used in the 1950s. There are two main types of ion exchange:

Cation exchange

The main objective of this process is to gain tartrate stability. As wine is a supersaturated solution of potassium bitartrate, unless excess salt is removed, crystallisation of potassium bitartrate will occur after bottling. Cation exchange allows the exchange of potassium ions (cations) with sodium salts which are soluble in wine. During this reaction a slight reduction in acidity occurs. However, health aspects relating to increased amounts of sodium in wine does not seem to support its use (Beelman and Gallander, 1979).

A variation of the above method is practised in the United States. In the U.S. potassium ions (cations) are exchanged with hydrogen ions in wine to increase pH and lower acidity.

Anion exchange

This method involves the replacement of tartrate ions (anions) with hydroxyl ions to remove both hydrogen ions and bitartrate. This causes a reduction in wine acidity, but a reported change in sensory quality of the resultant wine occurs (Beelman and Gallander, 1979).

Benefits of chemical over biological methods include greater precision and chemical control but the resultant pH is not easily regulated. (Mattick, 1983). In addition, the wines produced by the above methods may not be of good organoleptic quality.

2.3.3 Biological

Biological methods of deacidification are becoming more popular due to consumer demand that grapes and grape products be free of artificial substances (Coombe and Dry, 1992). Several biological methods are available.

(a) Carbonic Maceration

Intact bunches of grapes are held in storage tanks filled with CO_2 for a period of time prior to crushing or pressing. Intracellular reactions or intracellular fermentations occur where malic acid is degraded mainly to ethanol. SO_2 is also added to prevent excessive intracellular fermentations and to control possible spoilage. Intracellular reactions will only take place in an environment where the oxygen concentration falls below 5% or if the CO_2 concentration is increased to approximately 50% (Beelman and Gallander, 1979). Consequently, the wines produced are of lower total acidity and higher pH. However, the process of degrading malic acid to ethanol may produce some metabolites of malic acid, nitrogenous substances, inorganic matter and polyphenols may also be formed which affects sensory quality (Beelman and Gallander, 1979).

(b) Malolactic Fermentation

Specialized wine bacteria known as wine lactic acid bacteria (LAB) have a malolactic enzyme which catalyses the direct decarboxylation of the dicarboxylic acid L-malic acid to the weaker monocarboxylic acid L-lactic acid. This reaction is represented in figure 2.1, Malolactic fermentation (MLF) is an enzymatic conversion producing a pH increase from

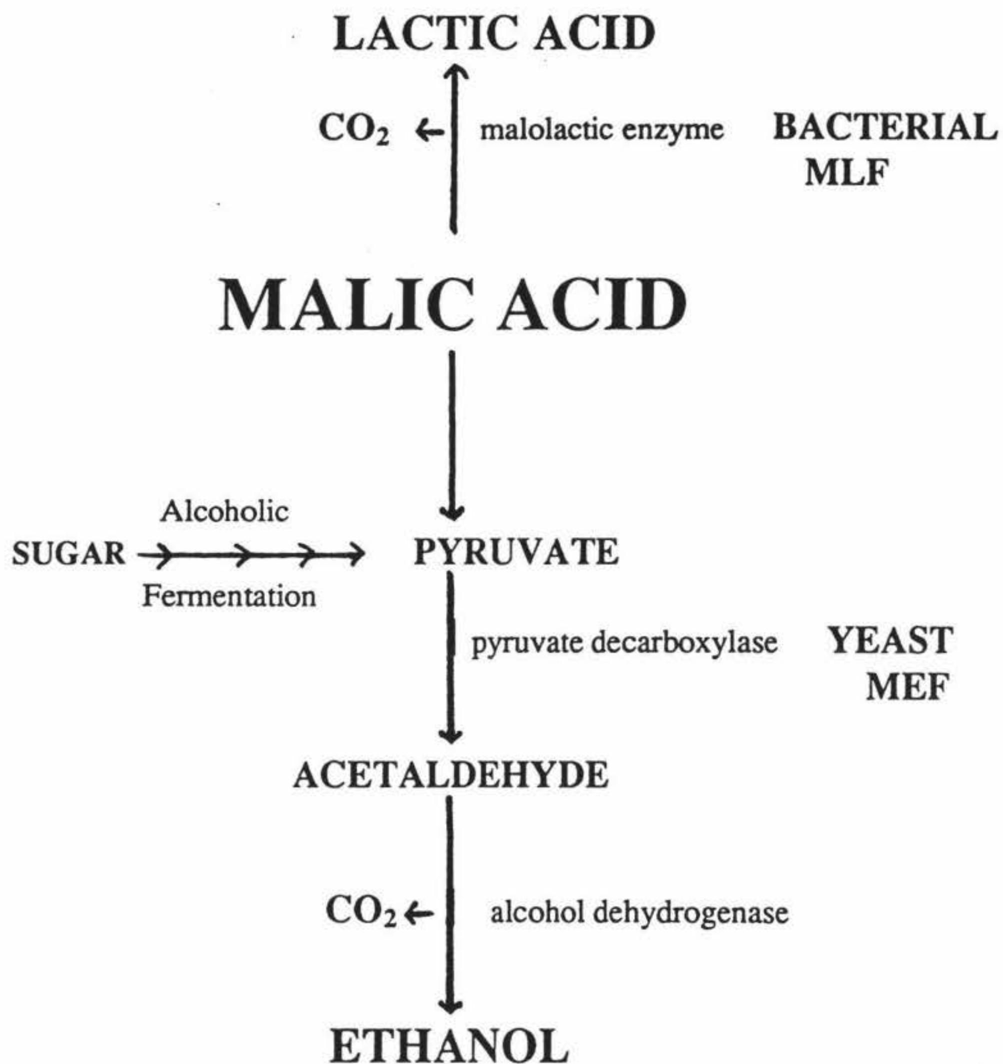


Fig.2.1 Biological deacidification of malic acid.

0.1 to 0.3 pH units (Davis *et al.*, 1985) and decrease in acidity. MLF is required on almost all low pH red wines and some white wines such as Chardonnay. MLF is not desirable in most white wines where the process may strip the delicate fruit flavours and aromas (Reeves, personal communication).

MLF occurs spontaneously or is induced by inoculation of the wine with LAB. Spontaneous fermentations usually involve the growth of three main genera: *Leuconostoc, Lactobacillus,* and *Pediococcus*. *Lc. oenos* often becomes the dominant bacterium conducting the most vigorous part of the fermentation. However, spontaneous fermentations are not always reliable. Usually there is a long lag phase in growth of *Lc. oenos* leading to delays in the winemaking process (Pilone, 1995) and possible competition by other microorganisms that may spoil the wine. Therefore to guide and control MLF, inoculation of commercially prepared wine LAB occurs. Previously, frozen or freeze-dried starter cultures requiring rehydration and bioactivation have been used. However, freeze-dried wine LAB strains that do not need bioactivation and an adaptation period to the harsh wine environment have recently been developed. These strains are basically "ready to use" for direct vat inoculation (Pilone, 1995). New Zealand trials have shown that these strains degrade all or part of the malic acid present.

A decrease in acidity and pH increase are not the only benefits of MLF. If MLF has occurred by either spontaneous or induced fermentation, the wine is less likely to undergo MLF in the bottle, which confers some microbial stability (Davis *et al.*, 1985). Also, flavour modification due to the metabolism of the wine LAB at such high cell numbers (10^7 - 10^8 cells/ml) occurs. These flavour changes are often welcomed by winemakers and consumers, but debate on this issue continues. Furthermore, the lactic acid produced is said to give the wine a much 'softer' palate. The flavour compounds produced include acetaldehyde, acetic acid, ethanol, diacetyl, acetoin and 2, 3-butanediol. The last three are important to the flavour profile of the wine (Davis *et al.*, 1985). Diacetyl at a concentration of 1- 4 mg/L is often thought to add complexity to a wine

(Davis *et al.*, 1985). Esters produced by LAB may affect the bouquet of the wine. Comparatively, Castino *et al* (1975) described MLF as having little if any affect on the sensory quality in Italian red wines. Kunkee *et al* (1964) supported this view, stating that in trails with Pinot Noir, the sensory characteristics of the wine were not significantly changed by MLF.

However, many disadvantages with MLF exist. Total or partial failure of MLF may occur as a result of the LAB used (Tortia *et al.*, 1993). In addition, if MLF occurs at a pH greater than pH 3.5 some tartaric acid is able to be degraded (3-30%). This degradation can produce off flavours, known as tourne (Radler, 1986). Also, high levels of lactic acid are believed to mask the fresh fruit characteristics of white wines (Beelman and Gallander, 1979). Furthermore, MLF may produce excess diacetyl imparting a buttery character, on the wine (Henske, 1993). Amine formation also occurs as LAB are known to decarboxylate amino acids for growth. Formation of histamine by *Pediococcus* species in wines with high pH is deletorious to the wine (Davis *et al.*, 1985). A further disadvantage of MLF is the risk of prophage attack. Prophage may exist in the wine and will kill the LAB, ultimately ceasing the fermentation.

In addition, the carcinogen ethyl carbamate can be formed by some LAB. Studies have shown that arginine in wine is catabolized by some LAB and citrulline is excreted. As citrulline is a precursor of ethyl carbamate, the wine LAB producing citrulline is undesirable for MLF. The arginine deiminase pathway produces precursors of ethyl carbamate is absent in all homofermentative lactobacilli and pediococci, so careful strain selection is recommended (Liu *et al.*, 1995).

In hotter climates with grapes picked at maturity most of the malic acid has been respired. Thus, spontaneous MLF must be prevented to stop the risk of spoilage (Davis *et al.*, 1985). Control in these conditions is mainly achieved by additions of SO_2. Choice of yeast strain may also help to prevent MLF as some strains are inhibitory to LAB growth

(Davis *et al.*, 1985). Hygienic practices and regular microbial monitoring of the wine also prevent MLF.

(c) Maloethanolic Fermentation

Maloethanolic fermentation (MEF) utilises yeast strains which induce simultaneous alcoholic fermention and deacidification. Yeast strains of *Saccharomyces, Zygosaccharomyces* and *Schizosaccharomyces* genera are able to perform MEF.

MEF (see Fig.2.1) involves the transport of malate into the yeast cell where it is oxidatively decarboxylated to pyruvate by an NAD-dependent malic enzyme. Pyruvate is then decarboxylated to acetaldehyde which is then reduced to ethanol (Radler, 1986).

The advantages yeasts have over bacteria which make them preferential to use for deacidification purposes are as follows: easier inoculation and control, generally greater resistance to SO_2, tolerance to lower pHs, yeasts have simple growth requirements which are usually satisfied in grape juice composition, and have no threat of phage attack (Thornton and Rodriguez, 1988).

Previously regarded as spoilage microorganisms, *Schizosaccharomyces* species are strong malic acid degraders, and were first isolated by Linder in 1893 in millet beer. Trials by Dharmadhikari and Wilker (1994) showed that *S. pombe* degraded 94% of the malic acid in 23 days. In addition the total acidity had fallen from 13.2 g/L to 6.1 g/L, with a pH increase from 3.08 to 3.50, although 7 g/L residual sugar remained. Acetaldehyde was formed at 80 ppm. A relationship exists between the amount of acetaldehyde formed and the production of off odours. No hydrogen sulfide odour was formed, but taste tests of the resultant wine were not met with favour.

In other experiments, wines produced from *S. pombe* which had most or all of the initial malic acid degraded, were judged as flat, bitter, and unbalanced with herbaceous flavours (Carre *et al.*, 1983). Similar opinions have also been made on wines produced by *S. pombe* which were blended with control wines at 10%, 20% and 50%. In addition, some *S. pombe* strains have also been found to produce relatively high amounts of histamine and 2, 3-butanediol. These products may be responsible for amertume effect (Carre *et al.*, 1983). Furthermore, pyruvic acid and acetaldehyde are also produced with less glycerol and volatile acid production by *S. pombe* (Carre *et al.*, 1983).

The addition of *Schizosaccharomyces* to the wine at the end of alcoholic fermentation has been suggested as the most convenient way of using *Schizosaccharomyces* species for deacidification (Carre *et al*, 1983). This recommendation is also given by Tortia *et al* (1993) who also support procedures where *Schizosaccharomyces* cells are entrapped within calcium-alginate beads at this time for greater control (Tortia *et al.*, 1993).

Blends of *Schizosaccharomyces* and *S. cerevisiae* strains at an initial inoculum ratio of 1:1 have been trialled by Charpentier *et al* (1985). An almost complete malic acid degradation occurred in 48 hours even though the *S. cerevisiae* population was 6-8 times greater than the *Schizosaccharomyces* population at the end of this time period. *Schizosaccharomyces* strains are generally slow growers, having high temperature requirements for growth, hence compete poorly against *S. cerevisiae* (Tortia *et al.*, 1993). When inoculations of *Schizosaccharomyces* occurred at the middle or at the end of alcoholic fermentation, 30-50% of the malic acid was degraded. An optimum inoculation of *Schizosaccharomyces* cells was 3×10^6 cells/ml (Charpentier *et al.*, 1985). Taste trials of the wines produced were not reported.

A new yeast generated by spheroplast fusion between *S. pombe* and a wine yeast strain of *S. cerevisiae* has been developed by Carrau *et al* (1994). This yeast is capable of

degrading malic acid during alcoholic fermentaion showing potential in future industrial situations [discussed further in section 2.4.2].

S. malidevorans strain 442 degrades up to 90% of the malic acid present in juice (Rankine, 1966). Thornton and Rodriguez (1988) isolated a mutant (mutant #11) from strain 442. This mutant is dependent on malic acid for growth, and is capable of degrading high malic acid levels (10 g/L in New Zealand Chardonnay juice and 6.6 g/L in New Zealand Semillon juice). Mutant #11 only utilized 25% of the glucose as compared to 80% utilization by strain 442. No objectionable flavours or aromas were reported which suggests the potential for use in the wine industry. The same authors also isolated mutants of *S. cerevisiae* strain AH22. However, no increase in malic acid degradation was observed between mutants and parental strains, thereby providing no benefit to industry.

Schizosaccharomyceae strain I.C.V-M holds the most potential in winemaking as it is able to degrade 90-95% of the initial malate if between 4-8 g/L and 80% if the initial concentration is 16 g/L. Strain I.C.V-M utilizes more sugar compared with other *Schizosaccharomyces* and performs better at pH extremes (< 3 and > 6). In addition, no lag phase in growth was observed (Auriol *et al.*, 1987). However, it must be noted that I.C.V-M has had no taste tests performed on the wines it has fermented.

Schizosaccharomyces are not presently used in industry for three reasons. Firstly, there is a lack of knowledge surrounding their reaction kinetics, and secondly the diversity of strains and the different capabilities of each strain has not yet been studied extensively (Auriol *et al.*, 1987). Thirdly, complete degradation of malic acid is often not desired in New Zealand wines (Pilone, 1995).

Zygosaccharomyces yeasts are close relatives of *Saccharomyces* yeasts. It is a genus minorly responsible for food and beverage spoilage (Romano and Suzzi, 1993). This

genus occurs naturally in juice and is occasionally present in the resultant wine (Romano and Suzzi, 1993). Some strains possess good winemaking abilities indicating potential industrial application. Such strains include *Z. bailii* and *Z. fermentati*. *Z. bailii* can conduct refermentation of beverages due to tolerance of SO_2, pH, alcohol, and high sugar concentrations, hence its reputation as a spoilage yeast. However, it is also able to degrade up to 70% of malic acid present in must. In addition, it is fructophillic and presently encouraged to grow on Spanish type sherry as "flor" yeast (Romano and Suzzi, 1993).

In *S. cerevisiae,* the level of malate degraded is highly variable. Most researchers have found that between 5 and 40% of the initial malate is degraded (Tortia et al., 1993). The factors influencing malic acid degradation include initial sugar concentration, temperature, oxygen level and thiamine addition. The initial malate concentration affected only the amount of malic acid degraded and not the percentage degraded, which was generally constant (Tortia et al., 1993).

Tortia *et al* (1993) found that *S. cerevisiae* strain D432 degraded more than 50% of the malic acid present in juices from trials performed during 1964 and 1966 vintages. This high level of malic acid degradation has not been observed by the authors since. The authors suggested that the level of degradation was due to very high initial sugar concentrations in the grapes. Results of 1987 and 1988 vintages show a 3 fold greater reduction in malic acid using strain D432 compared to spontaneous controls (Tortia *et al.*, 1993). Stain D432 shows potential for use in industry due to its degradative ability and as it forms up to 16.6% alcohol, whilst volatile and acetic acid production remains moderate.

(d) Other Yeasts

Other yeasts can degrade acids in musts and wines, but not by the conventions mentioned above.

Corte-Real *et al* (1990) generated mutant strains of *Hansenula anomala* which only metabolised glucose once all the malic acid was consumed. Malate transport into the cell was repressed by glucose in the parent strain (Tortia *et al.*, 1993). However, trials with these mutant strains are preliminary, and further research is necessary.

Candida and *Pichia* strains are able to grow oxidatively as films on the top of some musts. In this capacity, these yeasts are able to oxidatively degrade malic acid (Rankine, 1966; Whiting, 1975). *Candida* and *Pichia* strains are generally regarded as spoilage microrganisms and grow when conditions such as poor hygiene and poor control over the winemaking process occur.

Another oxidative yeast species, isolated in 1977 and named *Exophiala jeanselmei* var *heteromorpha* or black yeast, is able to proliferate in unhygenic conditions. This yeast causes spoilage by oxidising tartaric acid in grape juice. *E. jeanselmei* var *heteromorpha* is very resistant to antiseptics generally employed in the winery, and high levels of SO_2 are required to kill this yeast. This yeast is well integrated into the regional microflora of Nantes, France, and wineries in this province strongly sulfite, then rack the wines made with Botrytis infection where *E. jeanselmei* var *heteromorpha* harbours (Poulard *et al*, 1983).

(e) Immobilized Cells

Immobilized cells or enzymes (e.g. LAB or *Schizosaccharomyces*) are pregrown under optimal conditions using inexpensive substrates. The wine is then passed through a system containing a high density of these cells or enzymes where deacidification occurs. Wine throughput is generally high because the cell growth phase does not usually interfere with deacidification (Divies, 1993). Research performed by Yokotsuka *et al* (1993) used *S. cerevisiae* strain W3 and *S. pombe* strain IF00358 entrapped separately in double-layer calcium-alginate fibres to deacidify white wine. The authors found that

allowing the *S. pombe* fibres to conduct malic acid degradation to a desired level, then removal of these fibres and replacement with the *S. cerevisiae* fibres to complete the fermentation, was most advantageous in terms of wine quality. Simultaneous use of both *S. pombe* and *S. cerevisiae* fibres gave the greatest production of off flavours, and least malic acid degradation (Yokotsuka *et al.*, 1993). However, this process is technical, expensive and time consuming.

Other authors have also used *S. pombe* immobilized cells, but there is debate on the sugar requirement of cells. Rosini and Ciani (1993) found that sugar was needed for the preservation of the malate deacidification activity of *S. pombe*. Activity disappeared after approximately 100 hours in batch conditions after glucose supply had ended (Rosini and Ciani, 1993). Comparitively, Tallandier *et al* (1988) found that *S. pombe* immobilized cells decomposed malate added to the medium before or after sugar exhaustion, and that in wines made solely by *S. pombe*, malate was degraded even before the start of sugar uptake. Magyar and Panyik (1989) among others, also support this finding.

Advantages of immobilised cells include relatively easy removal of *S. pombe* entrapped cells from the wine (Yokotsuka *et al.*, 1993) and a reduction in off flavours and odours that otherwise occur (Rosini and Ciani, 1993). Undesirable effects were only noticed if the cells were left in contact with the fermentation for a longer time period than required (Magyar and Panyik, 1989). Disadvantages include cell leakage and destruction of the gel structure, which may be due to high sugar concentrations and elevated levels of cell growth (Magyar and Panyik, 1989; Yokotsuka *et al.*, 1993; Sousa *et al*, 1993). Because of this, commercial application is impractical at the present time.

A cell cycle malolactic fermentation bioreactor has been developed by Fleet and Costello. The bioreactor is used by the following principle : the system is composed of a wine reservoir from which wine is pumped into the bioreactor which contains a high density of *Leucostonoc oenos* cells (1 x 10^{10} cells/ml). These immobilized *Lc. oenos* cells are

trapped within beads of polyacrylamide alginate or carragean which are supported in columns. Wine is continuously passed through this bed of beads, where malic acid is converted to lactic acid. The wine and bacterial cells are then separated by a membrane filter enabling the return of cells to the reactor. A series of values control the rate of wine into and out of the system. The amount of internal recycle of wine controls the extent of deacidification achieved. This is because greater recycle is needed to achieve a high rate of acid reduction as the activity of the cells fall (Divies, 1993). In trials by the author, 90% of initial malate in grape juice was degraded after only 15 days of operation. Compared with the best batch methods bioreactors provide a rapid malate conversion rate. However, loss in activity of cells in the reactors remain an obstacle to their large scale commercial application. In most studies, activity is lost within 2-7 days (Divies, 1993) therefore requiring addition of further cells. Divies (1993) also reports that a similar system has been described which has a half life of 46 days which would be a more practical alternative for the wine industry.

2.4 GENETICS

Recently, there has been attempts to apply genetic manipulation techniques to the microorganisms involved in the winemaking process.

2.4.1 Mutagenesis

Since 1970 clones of mutagenised yeast strains have been isolated. Ultraviolet irradiation has been widely used to create mutagens with altered phenotypes such as alcohol production and tolerance (Subden, 1987). Mating of desirable genotypes from the isolated clones routinely takes place. However, in this method of genetic alteration little control exists. Subsequently, other genetic manipulation techniques have been used.

2.4.2 Cell Fusion

Spheroplast fusion between *S. pombe* and a wine yeast strain of *S. cerevisiae* has been reported (Carrau *et al*, 1994). The purpose of the fusion was to integrate the fermentation characteristics of *S. cerevisiae* with the high malate degradative ability of *S. pombe*. The yeast created was effective at partial malic acid degradation during alcoholic fermentation but a large inoculum (at least 100 x 10^5 cells/ml) was required. The fission yeasts have not yet been involved in inustrial trials but show enormous potential for future application in the winery.

2.4.3 Extrachromosomal Inheritance

Wild and wine yeast killer strains contain protein-encapsulated virus like particles which contain genetic material (dsRNA or DNA) that can synthesize toxins. These toxins (proteins or glycoproteins) are able to kill sensitive yeast strains. The benefits of a yeast with killer properties include the ability to kill other specific yeast strains in the fermenting juice (Fleet, 1993). Commercially available yeast strains are able to gain resistance to toxins by the transferral of genetic material from killer strains (Subden, 1987). It may be possible in future research to effectively transfer additional genetic information in this manner such as gene(s) involved in malic acid degradation.

In addition, autonomously replicating 2μm circular DNA plasmids are thought to exist in the nuclei of wine yeasts. The plasmids may be of benefit if used as vectors encoding gene(s) involved in the decomposition of malic acid, but further research is required (Subden, 1987).

2.4.4 Transformation

Much of the research involving genetic transformations centres around the Malolactic enzyme found in LAB and the reaction it catalyses in wine (see section 2.3.3).

Caspritz and Radler purified this enzyme from *Lactobacillus plantarum*. They found it consisted of two identical subunits, which suggested that the genetic information for the activation of malolactic activity is carried on a single gene (Williams *et al.*, 1984). By use of genetic manipulation it was hoped that this gene would be cloned and then transformed into a wine yeast, the benefit of which would be a simultaneous alcoholic fermentation and deacidification by the one microorganism (Williams *et al.*, 1984).

Using recombinant DNA techniques, researchers were able to isolate and purify DNA from *Lactobacillus delbrueckii* . *L. delbrueckii* was used as it was easier and more convenient to use in the laboratory. The DNA was then cut with *Sal* I restriction enzyme into several fragments and then ligated into plasmid pBR322. This plasmid was then transformed into *Escherichia coli* K-12 RR1. Transformants were selected by resistance to ampicillin and sensitivity to tetracycline. Only two *E. coli* transformants actually produced L-lactic acid from L-malic acid after growth in minimal medium, and assays showed that approximately 30% as much L-lactic acid was produced as the *L. delbrueckii* controls (Snow, 1985).

From these experiments it was found that a 5kb insert was responsible for the malolactic activity found in the *E. coli* transformant. However, a lot of additional DNA was also present in this fragment, which was taken up in plasmid pBR322 (Williams *et al.*, 1984). It was then hoped that this 5kb insert would be expressed if transferred into the shuttle vector pRC3 which can replicate in both *E. coli* and yeast strains.

The insert was expressed in *E. coli,* where more malate was metabolized in the transformed cells than the control cells. When transformed into a laboratory yeast strain, and these transformed yeast cells grown up in wine, only approximately 1.5% of L-malate initially present in the must was converted to L-lactate. This shows that the malolactic gene in yeast was expressed at a very low level (Snow, 1985).

Another reseach group transformed plasmid vector pTR 262 into *E. coli*. Transformants were selected by resistance to tetracycline. Clones were identified as possessing malate assimilating activity, but their growth on selective media was poor, as was their regeneration (Subden, 1987).

The problems researchers came up against were numerous. The expression of the malolactic gene could have been effected at transcriptional or translational level, or could have been due to the instability of the plasmid, the mRNA transcript, or the protein product in the host cell (Williams *et al.*, 1984). The additional DNA (codons) in the 5kb fragment which coded for the malolactic gene are rarely used by yeast, and could have reduced the translational efficiency. Furthermore, this additional DNA may have caused the phase between the gene and promoter sequence to be disrupted, which again would have caused low expression.

Snow (1985) has suggested these problems may be overcome if the transfer of the malolactic gene into a plasmid occurs when the plasmid contains a cloned yeast centromere (this will increase stability on *E. coli* yeast vectors in mitotic and meiotic divisions). Another possibility is to attempt to integrate the malolactic gene into the yeast centromere, which would essentially make it part of the yeast genome.

The problem genetic engineering faces is not the cloning of the desired genes, it is rather the problems that cause eventual expression of the cloned and transformed gene to not be as productive as in the original organism (Snow, 1985). Radler suggested that the above

techniques have not been successful in *S. cerevisiae* yeast strains due to an additional specific transport system for malate being required.

A considerable amount of genetic research has been undertaken to try to increase efficiency and control over the winemaking process. Microbial strain selection and purification have allowed for greater control in the fermenting juice. Genetic research however, has brought about little change to the overall vinification process. Many wineries still use conventional vinification methods. This research presents a deacidification method (MEF) not presently used in New Zealand wineries.

3.0 MATERIALS AND METHODS

3.1 Simulated Grape Juice Fermentations

3.1.1 Microorganisms

Six wine yeasts were used in this study. *Saccharomyces cerevisiae* Lalvin strains ACID-, D432, Montrachet (M), B71 and *Saccharomyces baynus* Lalvin strain EC1118 were obtained from Dr. Paul Monk (Lallemand PTY, Ltd., North Adelaide, South Australia). All yeasts were received in vacuum sealed containers in Active Dry Yeast (ADY) form. *Schizosaccharomyces malidevorans* strain 442 was obtained from the Australian Wine Research Institute (Adelaide, South Australia), cultured on Yeast Mannitol (YM) agar.

ADY were aseptically transferred from vacuum packed containers to YM agar plates for culturing, and to sterile peptone water (0.1%) for resuspension and subsequent microscopic identification to check for purity. Microscopic examination of strain 442 colonies were also made. Once positive identification of the yeast strains had been confirmed, isolate colonies were transferred to YM agar every 2-3 months.

3.1.2 Preparation of Microorganisms

ADY strains were rehydrated in 100ml peptone water (0.1%) at 37°C before addition to the fermentation vessel. The weight of the yeast added to the 100ml peptone water was calculated from viable cell determinations (methylene blue method) to give approximately $1x10^6$ viable cells/ml.

Strain 442 was first grown in glucose limited Triple M broth (see below) for five days (30° C) and then added to fermentations to give $1x10^6$ viable cells/ml.

3.1.3 Fermentation Media

A modified Simulated grape juice (Triple M medium) was made by combining three separate media preparations (Kunkee, personal communication) . Medium AAA was made up in 500ml Deionized (DI) water and contained: D-fructose, 110g; D-Glucose, 110g; Ergo-stock, 4ml. Ergo-stock (stored refrigerated) contained 62.5mg ergosterol (Sigma) and 6.25ml Tween 80 (Difco) made in 25ml 95% ethanol. Medium BBB was made up in 250ml DI water and contained: D-tartaric acid, 5g; L-malic acid, 5g; and citric acid, 0.5g. The mixture was titrated to pH 3.5 with conc. NH_4OH. Medium CCC was made up in 250ml DI water and contained: Yeast Nitrogen Base (Difco) (without amino acids and without ammonium sulphate), 1.7g; Vitamin-free Casamino Acids (Difco), 2.0g; myo-inositol, 6.0mg; $CaCl_2$, 0.2g; L-arginine-HCl, 0.8g; L-proline, 1.0g; L-tryptophan, 0.1g. Medium CCC was titrated to pH 3.5 with 1 N phosphoric acid. Each component medium was sterilised by autoclaving at 121°C for 15 minutes. After cooling, the three components were combined aseptically to give 1 litre of Triple M medium.

Triple M medium was prepared as above for strain 442 culture, except component media AAA only contained glucose as the sole carbohydrate source.

Changes to the chemical composition of Triple M media took place for individual experiments (refer to figures).

3.1.4 Fermentation Procedure

One litre of Triple M Media (in a 2 litre bottle) was inoculated with rehydrated ADY (see above) to give $1x10^6$ cells/ml. Strain 442 was inoculated using a culture pregrown in Triple M media (see above). Fermentations were carried out in a forced air draft incubator at 18°C. Once visible signs of fermentation had begun, fermentation traps were placed on the fermentation vessel. Samples of 20ml were taken daily from the vessel and temperature, Brix, pH, T.A., and cell counts (where necessary) were determined immediately. The remainder of the sample was frozen (-30°C) for subsequent malic acid, glucose and fructose, and alcohol determinations. Organoleptic testing did not take place due to the chemical composition of the media. Fermentations were continued until complete as determined by reducing sugar test (Clinitest).

3.1.5 Yeast Mannitol (YM) Agar

Yeast Mannitol Agar contained the following per litre: YM Broth (Difco), 21g; Agar (Davis), 15g; DI water 1000ml. The media was sterilised by autoclaving for 15 minutes at 121°C.

3.1.6 Peptone Water

Stock solutions (1%) of Difco Bacto-Peptone water were prepared in Milli-Q water. The solutions were sterilised by autoclaving for 15 minutes at 121°C.

3.2 Industrial Fermentations

3.2.1 Preparation of Microorganisms

Strains EC1118, ACID- and 71B were added at 25g/hL of juice. These yeasts were first resuspended according to manufacturers specifications as follows: the amount of yeast to be added to the juice was resuspended in ten times their weight in warm water for no longer than 20 minutes, then added to 10% of the juice before final inoculation. Strain D432 was also resuspended according to manufacturers specifications as follows: the amount of yeast needed for a direct inoculation of the entire fermentation was grown up in 10% of the juice (after resuspension in water as above). It was left for 2-3 days to gain a ten-fold higher supplementation rate due to its relatively poor viability.

3.2.2 Juice Preparation

Two different juice varieties were used in the trials. Sauvignon Blanc juice was used at Pask Winery, and Chardonnay juice was used at Mission Vineyards. Additions of a stock solution of SO_2 (10%) were made to both juices to give a molecular SO_2 level of approximately 800μg/l. The additions were made to prevent oxidation and undesirable microbial growth.

3.2.3 Fermentation Procedure

Stainless steel beer kegs (50L) partially filled with Chardonnay juice were inoculated in duplicate with the four yeasts at Mission Vineyard. At Pask Winery, tanks of 1,360L, 1,400L, 2,200L and 10,800L were partially filled with Sauvignon Blanc juice and each was inoculated with one of the four yeasts. At both wineries the juices were inoculated with rehydrated ADY (see above) to give approximately $1x10^6$ viable cells/ml. Fermentations were not conducted under aseptic conditions (using sterile juice), as this

would not take place in normal industrial situations. Samples of approximately 200ml were taken daily from the kegs and tanks. Temperature, pH, Brix, titratable acidity, and cell counts (where necessary) were determined immediately. Five ml of the sample was frozen on certain days, and a further 5ml of sample was added to 5ml perchloric acid for later malic acid and alcohol determinations. Additions of sugar and diammonium phosphate (DAP) to some of the fermentations are standard winemaking procedures, and these were noted.

Fermentations were continued until complete as determined by reducing sugar test (Clinitest). When complete, each of the wines were racked off into 50L kegs. SO_2 was added to each keg so as to give a molecular SO_2 level of 800 *u*g/L for protection from oxidation and undesirable microbial growth. The kegs were completely filled, again to prevent oxidation, and were then sealed (rubber bung). This seal was only broken for further SO_2 additions, and for topping up the kegs.

3.2.4 Perchloric Acid

One volume of perchloric acid (1M) was added to one volume of sample to halt fermentation reactions. These were kept for later analyses.

3.3 Laboratory Grape Juice Fermentations

3.3.1 Juice Preparation

Addition of a stock solution of SO_2 (10%) was made to Chardonnay juice (supplied by Mission Vineyards) to give a molecular SO_2 level of approximately 800μg/l. The sterilant, dimethyl dicarbonate was also added to the juice. Additions were made to

prevent oxidation and undesirable microbial growth. The juice was then refridgerated at 4°C until required.

3.3.2 Fermentation Procedure

Erlenmyer flasks (2 litre) were half filled with Chardonnay juice and inoculated in triplicate with one of five wine yeasts. Yeasts (EC1118, ACID-, 71B, D432 and M) were inoculated in resuspended form (see above) to give approximately $1x10^6$ viable cells/ml. Samples of approximately 200ml were taken initially (Day 0) and on the completion of the fermentation as determined by reducing sugar test (Clinitest). Brix, titratable acidity, and pH were determined immediately and the remainder of the sample was frozen (-30°C) for subsequent malic acid and alcohol analyses.

3.4 Sample Analysis

3.4.1 Temperature

Fermentation temperatures in degree Celsius (°C) were determined by mercury thermometers (Smith Biolab).

3.4.2 Titratable Acidity

Titratable acidity (T.A.) of degased samples was measured by titration with standardised NaOH (0.1N) and expressed in g/L . Samples were degased by shaking for three minutes under vacuum. This was done to eliminate error from CO_2 gas generated during the fermentation. Five ml of the degased sample was added to 200ml neutralised boiling water and titrated to a phenolphthalein end point (Amerine and Ough, 1980).

3.4.3 pH

A pH meter (Orion Research, model 501 ionalyser) was used to measure pH. The meter was calibrated with buffer solutions of 4.0 and 7.0 (Mallinkroft).

3.4.4 Cell Count

Total yeast cell counts were made with a Haemocytometer Counting Chamber (Zintl) of 0.1mm depth. Viable counts were determined from total counts after obtaining the percentage live cells using the vital methylene blue method (Pierce, 1970).

3.4.5 Brix

Brix is a measurement of the soluble solids concentration expressed in g/100g juice. Brix was determined using a temperature correcting Brix Refractometer (Atago) as well as a Brix hyrodrometer (Kessler Instruments Inc.). Hydrometer readings were corrected to 20° C using tables from Amerine and Ough (1980).

3.4.6 L-malic Acid

L-malic acid (M.A.), expressed in g/L, was determined enzymatically using Boehringer Mannheim L-malic acid test kits (Boehringer Mannheim GmbH, Germany). This method measures the amount of NADH formed in the reaction which is stiochiometric to the amount of L-malate. A spectrophotometer (Novatech) was used to measure the absorbance of reduced NADH (340nm wavelength).

3.4.7 D-Glucose and D-Fructose

D-fructose and D-glucose, expressed in g/L, was determined enzymatically using Boehringer Mannheim D-glucose/D-fructose test kits with a spectrophotometer as above.

3.4.8 Alcohol Analysis

Alcohol was determined by distillation and dichromate oxidation as outlined in Pilone *et al.* (1973).

3.4.9 Reducing Sugar Test

Sugar pills (Clinitest) were utilised to measure low levels of reducing sugar which indicates the completion of the fermentation (Amerine and Ough, 1980).

3.4.10 SO_2

Free SO_2 was measured by aspiration method, after Amerine and Ough (1980).

3.5 Statistical Analyses

3.5.1 Acid Ranking Taste Tests

Differences in acid taste from wines produced in Industrial trials were statistically analysed following methods outlined in Amerine and Roessler (1976).

3.5.2 Laboratory Grape Juice Fermentations

Fermentation data was entered on computer programme Stat View™ 512+ (Brainpower, Incorporated) for statistical analysis.

3.5.3 Standard Solutions

Analytical tests were performed on standard solutions to determine the analytical variation in the method. Standard solutions were made up in DI water as follows; 0.1g/L malic acid (or as supplied in L-malic acid reagent test kits), 15g/L tartaric acid, 1.0g/L fructose and 1.0g/L glucose. Repeated alcohol analyses were run on the same wine and a saturated solution of potassium bitartrate (pH 3.56 at 20°C) was used to calibrate the pH meter.

3.6 Yeast Karyotype Analysis

Polymorphic chromosomal banding patterns were used to distinguish between yeasts used in this analysis (EC1118, ACID-, 71B and D432).

The preparation and karyotyping of the microorganisms followed a modified procedure as outlined in the Biorad CHEF-DR™ II Pulsed Field Electrophoresis System Instruction Manual and Applications Guide, 1988. Modifications are as follows; 0.5ml EDTA(0.05M) was used to resuspend cells; NDS buffer contained 0.01M Tris pH 7.5, 0.5M EDTA pH 8.0, 1% Lauroylsarcosine, 1 mg/ml Proteinase K; 1.0% Fastlane agarose was used in gel construction.

Gels were run at approximately 12°C and had a pulse/run time of 60 seconds for 14 hours, followed by 90 seconds for 11 hours.

4.0 RESULTS

Simulated and commercial grape juices were inoculated with different yeast strains. Samples were recovered from the fermenting juice until the completion of each fermentation, as determined by the reducing sugar test. Experimental results from standard solution, laboratory, Simulated Grape Juice, industrial, acid taste and karyotype analyses are presented below.

4.1 Standard Solutions

Statistical analysis of the results of repeated analyses of some of the standard methods used for analyses performed in this research are presented in table 4.1. The results show that the methods tested gave excellent reproducability. However, standard alcohol analyses were performed on only one wine on one day rather than over an extended time period. Therefore, accuracy of the method was not satisfactorily determined. It is commonly accepted that the sensitivity of the test is relatively poor, however, and small differences in alcohol volume cannot be reliably estimated. The level of reliability of titratable acidity analyses decreases, therefore data from this test is only accurate within ±2.31g/L. Data from glucose and fructose analysis is shown to be comparatively unreliable. For this reason this analytical procedure was stopped, and replaced with brix hydrometer measurement. Repeated hydrometer readings maintained a satisfactory level of accuracy.

Table 4.1 Estimation of reproducability of some analytical methods used in this research.

	n	Mean	Standard Deviation	Standard Error	Coefficient of Variation
Malic acid 0.1 g/L	7	0.10	0	0	0
0.2 g/L	28	0.20	0	0	0
T.A. 15 g/L	17	15.42	0.36	0.09	2.31
pH 3.56 (KHT)	5	3.55	0.013	0.006	0.37
Glucose 0.1 g/L	12	1.65	0.21	0.15	12.86
Fructose 0.1 g/L	12	1.40	0.42	0.30	30.31
Alcohol, wine (%v/v)	5	7.01	0.044	0.02	0.63

4.2 Laboratory Grape Juice Fermentations

Commercial grape juice fermentations (18°C) were performed in triplicate in small volumes (2L) in the laboratory with yeast strains EC1118, ACID-, 71B, D432 and M. Analyses (initial and final) of the fermentations were performed in duplicate or triplicate. Repeated test analyses show an excellent degree of reproductability.

The data from each fermentation was statistically compared at the 95% confidence level and results are presented in table 4.2. Individual yeast strain analyses are presented in Appendix A.

Malic Acid

Table 4.2 shows that there was no significant differences found in the malic acid level of wines made from strains EC1118 and ACID- or from wines made from strains D432 and 71B. Significant differences existed between all other wines. Table 4.3 shows that malic acid is degraded by 30% by strain 71B and 28% by strain D432. The smallest percent degradation of malic acid (10.9%) was observed in wines fermented by strain EC1118.

Titratable Acidity

Significant differences in levels of titratable acidity were found amongst all wines except those made from strains ACID - and M (Table 4.2). Table 4.3 shows that strains 71B and D432 reduced titratable acidity by 17% and 13% respectively. Comparitively, strain EC1118 increased titratable acidity by 0.5% (0.06g/L).

pH

Table 4.2 shows significant differences in pH levels were found amongst all wines at the 95% confidence level. Table 4.3 shows that a pH increase of 8.9% was observed in wines fermented by strain 71B. Strain D432 increased pH by 6% whilst the remaining

Table 4.2 Analyses of wines from replicate Laboratory Chardonnay Juice Fermentations*

	EC1118	ACID-	71B	D432	M
Malic acid (g/L)	5.7 ±0.03[a]	5.6 ±0.11[a]	4.5 ±0.13[b]	4.6 ±0.08[b]	5.3 ±0.08[c]
Titratable acidity (g/L)	10.9 ±0.08[c]	10.5 ±0.05[a]	9.0 ±0.03[b]	9.5 ±0.02[d]	10.6 ±0.08[a]
pH	3.15 ±0.00[a]	3.12 ±0.01[b]	3.29 ±0.00[c]	3.20 ±0.01[d]	3.13 ±0.00[e]
Alcohol %(v/v)	9.9 ±0.13[d]	9.5 ±0.05[ab]	9.2 ±0.03[e]	9.4 ±0.01[bc]	9.5 ±0.06[ac]
Brix	-0.7 ±0.05[c]	-0.9 ±0.00[a]	-1.0 ±0.00[ab]	-1.0 ±0.09[b]	-1.2 ±0.05[d]

* Average (3 determinations) initial grape juice analyses: Malic acid, 6.4g/L; Titratable acidity, 10.88g/L; pH, 3.02; Alcohol, 0 % v/v; Brix, 19.1. Mean wine analyses in the table showing the same superscript letter indicate there are no significant differences (95% confidence level) between yeast strains. Final values showing different superscript letters indicate significant differences (95% confidence level).

Table 4.3 Differences between initial juice and final wine component concentrations and (percent change) of replicate Laboratory Chardonnay Juice Fermentations.

	EC1118	ACID-	71B	D432	M
Malic acid change (g/L)	-0.7 (10.9%)	-0.8 (12.5%)	-1.9 (30%)	-1.8 (28%)	-1.1 (17%)
Titratable acidity change (g/L)	+0.06 (+0.5%)	-0.41 (3.7%)	-1.86 (17%)	-1.4 (13%)	-0.3 (2.8%)
pH change	+0.13 (4.3%)	+0.1 (3.3%)	+0.27 (8.9%)	+0.18 (6%)	+0.11 (3.6%)
Alcohol formed %(v/v)	+9.9	+9.5	+9.2	+9.4	+9.5

strains produced a relatively small pH increase (EC1118, 4.3%; M, 3.6%; ACID-, 3.3%).

Alcohol

Final alcohol analyses were onducted when fermentations were complete as determined by reducing sugar test. Table 4.2 shows that there were no significant differences in alcohol levels found amongst wines made from strains ACID - and M, ACID - and D432, or D432 and M. All other wines exhibited significant difference from each other. Table 4.3 shows that 9.9% v/v alcohol was formed by strain EC1118 even when this strain degrades the least malic acid. The strains with most maloethanolic activity (strains 71B and D432) produce notably smaller amounts of alcohol in comparison with strain EC1118. The implications of this are discussed in section 5.1.

Brix

Significant differences in Brix levels were found amongst all wines except those made from strains 71B and D432 and 71B and ACID- (Table 4.2).

4.3 Simulated Grape Juice Fermentations

A series of fermentations were performed with a modified Simulated Grape Juice (Triple M media, see section 3.1.4) in the laboratory. The purpose of these fermentations was to establish the parameters of effective malic acid degradation for each yeast strain.

4.3.1 Standard Fermentations

Standard fermentations were performed at a uniform temperature (18°C) with no alteration of the chemical composition of the modified Simulated Grape Juice. The results from these fermentations serve as a comparison with other trials where medium components were varied.

Some of the results of the standard fermentations are presented below, with a complete set shown in Appendix B.

Malic Acid

Table 4.4 and figure 4.1 clearly show that the yeast strain most capable of degrading malic acid was 442. The strain degrading the least malic acid was EC1118. Most malic acid degradation occurred by the time the amount of sugars (fructose and glucose) in the fermenting juice were reduced by 50% (data not shown). This effect may be due to the yeast strains reaching the stationary growth phase, where greatest biochemical activity occurs.

Figure 4.2 shows an anomaly regarding malic acid degradation. Although overall degradation of malic acid occurs, a slight increase is observed at the end of the fermentation. This effect was rarely observed elsewhere in this research and may be due to yeast cell lysis expelling some malic acid into solution.

Table 4.4 Analyses of fermentations (18°C) of Simulated Grape Juice inoculated with different yeast strains[a].

YEAST	Initial M.A.	Final M.A.	M.A. Change	Initial T.A.	Final T.A.	T.A. Change	Initial pH	Final pH	pH Change	Final Alcohol	Ferm. Duration[b]
EC1118	5.0	4.1	-0.9	10.5	9.9	-0.6	3.34	3.45	+0.11	15.0	16
ACID-	5.0	4.0	-1.0	10.65	11.9	+1.25	3.32	3.37	+0.05	14.0	16
71B	4.9	3.6	-1.3	10.20	9.2	-1.0	3.33	3.57	+0.24	13.0	17
D432	4.7	3.9	-0.8	10.50	9.3	-1.2	3.34	3.50	+0.16	14.0	16
M	5.0	4.3	-0.7	11.40	10.5	-0.9	3.36	3.37	+0.01	14.0	16
442	5.0	2.4	-2.6	10.95	8.8	-2.15	3.30	3.40	+0.10	7.3	10

[a] All results expressed as g/L except alcohol as % v/v.

[b] Fermentations were complete when two consectutive readings of 0% sugar (reducing sugar test) were recorded.

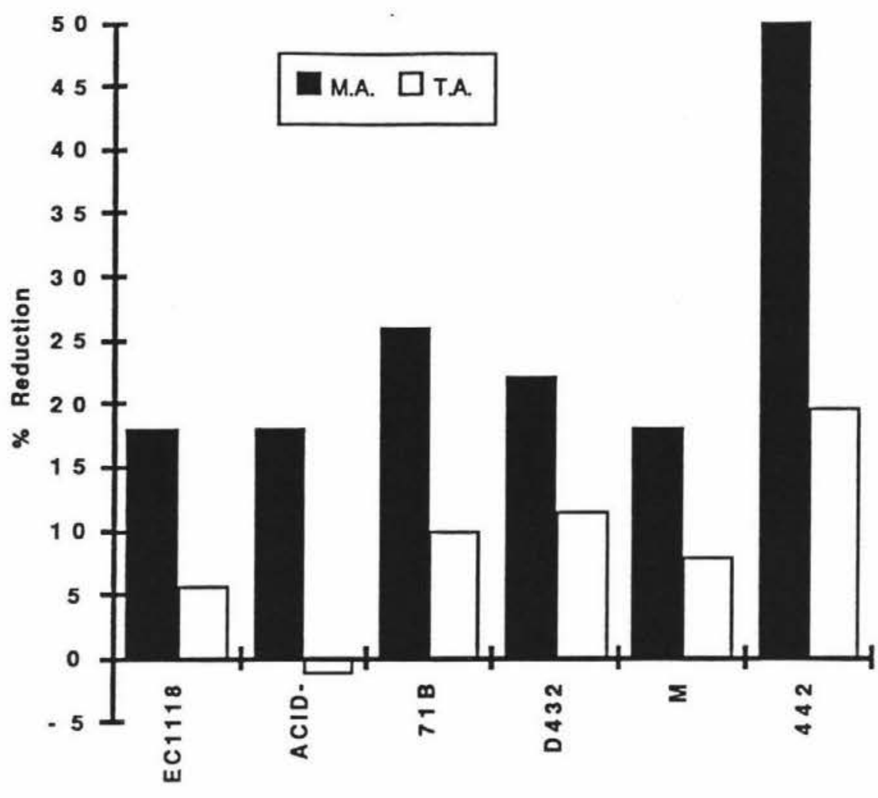


Fig.4.1 Percent reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented (18°C) with different yeast strains.

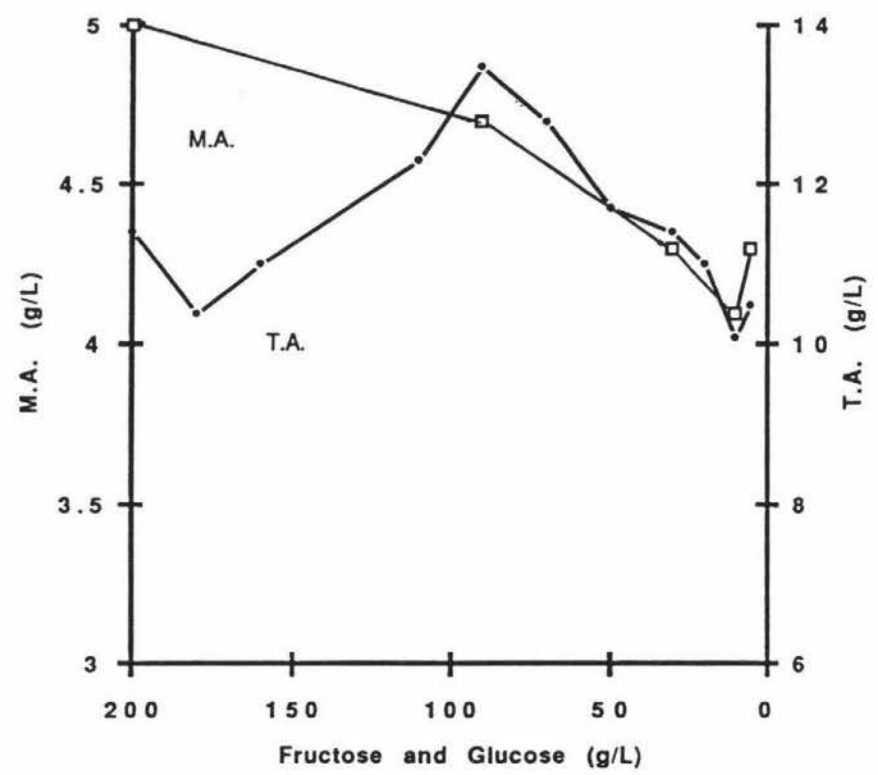


Fig.4.2 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain M. Initial cell count was 2.8×10^6 cells/ml.

Titratable Acidity

Figure 4.1 shows that strains EC1118, 71B, D432, 442 and M all reduced titratable acidity. ACID- increased titratable acidity by 1.25 g/L. This effect is seen again in section 4.1.2 [variable temperature fermentations] and may be due to the formation of acids (eg. succinic acid). Formation of acids will increase titratable acidity.

Figure 4.3 shows a trend which has been observed throughout this research. An initial increase in titratable acidity occurs before subsequent reduction. This initial increase is possibly caused through volatile acid and succinic acid formation. Both acids are formed by maloethanolic yeast strains, generally at the beginning of fermentation (Radler, 1986). In addition, Alves (1995) suggests that yeasts produce succinic acid for antibacterial purposes.

pH

Increases in pH during fermentation are presented in Figure 4.4. The strain producing the greatest increase in pH was 71B (0.24 pH units), followed by D432 (0.16 pH units), EC1118 (0.11 pH units), 442 (0.10 pH units), ACID- (0.05 pH units) and M (0.01 pH units).

Alcohol

Final alcohol analyses were taken on completion of the fermentation (as determined by reducing sugar test) and the results are shown in Table 4.4. Large differences in alcohol concentrations can be detected, and clearly the non-*Saccharomyces* strain 442 was incapable of producing high amounts of alcohol with only half the amount formed compared with the *Saccharomyces* strains.

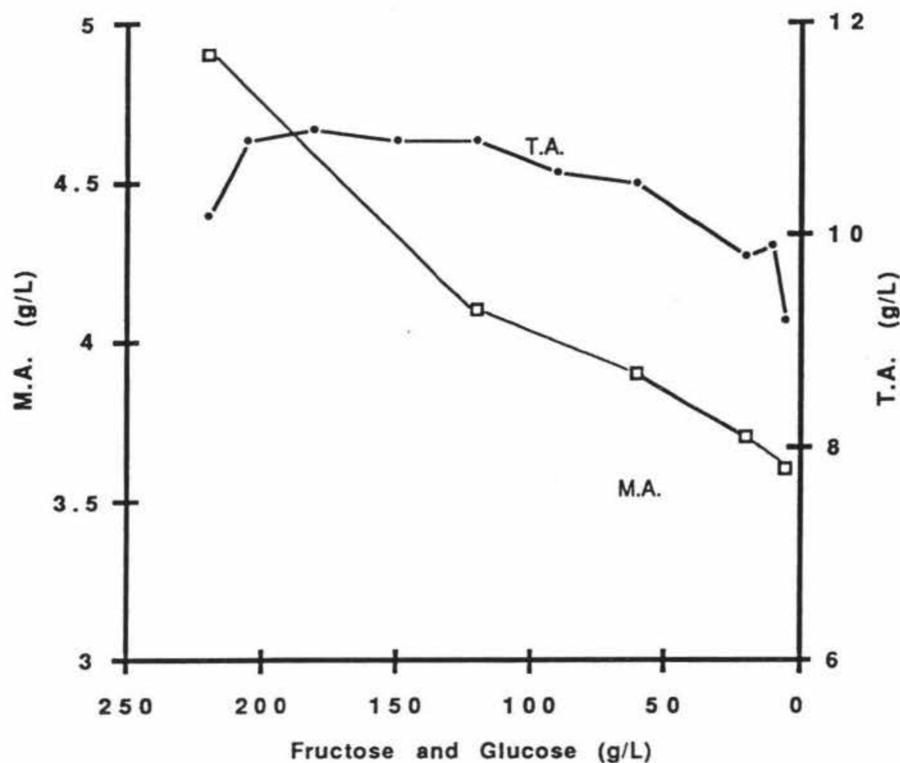


Fig.4.3 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial cell count was 2.6 x 10^6 cells/ml.

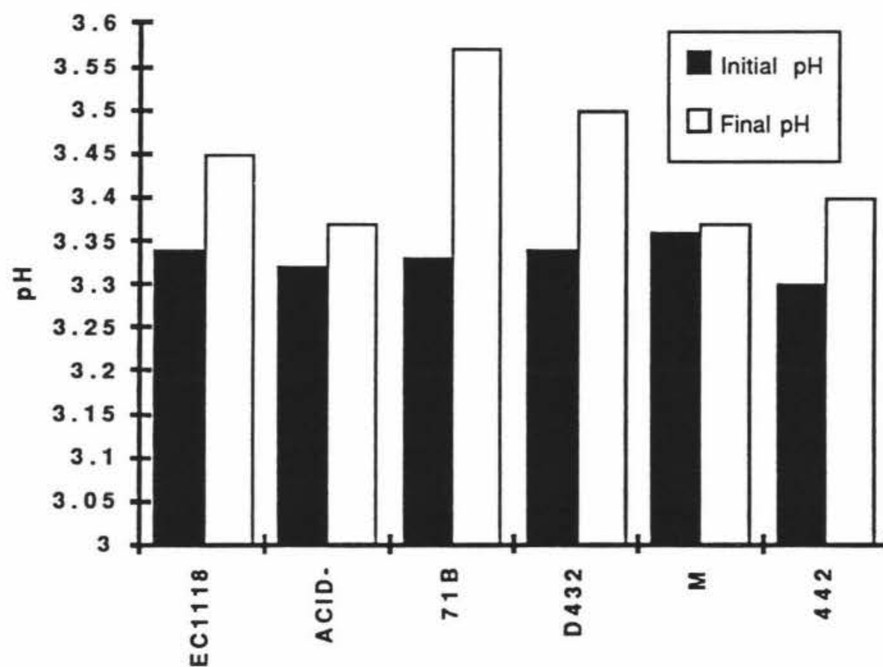


Fig.4.4 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice inoculated with different yeast strains.

4.3.2 Variable Temperature Fermentations

In addition to fermentations conducted at the standard 18°C temperature, fermentations were also conducted at 12°C, 25°C and 37°C to assess the effects of this variable on acid reduction using strains EC1118 and D432.

Some results of the fermentations are presented below and a complete set of results is shown in Appendix C.

Malic Acid

Table 4.5 and figure 4.5 show results of malic acid degradation. The fermentation temperature giving the greatest malic acid reduction is 25°C for yeast strain D432 and 37°C for yeast strain EC1118. Cooler fermentation temperatures such as 12°C may inhibit malic acid degradation due to slower yeast metabolism.

Titratable Acidity

An overall increase in titratable acidity was observed in all fermentations except those conducted at 18°C. Table 4.5 presents these results. The final analyses show that the temperatures influencing greatest increase intitratable acidity were 37°C for strain EC1118 and 25°C for strain D432. At these temperatures both strains also degraded the most malic acid compared with other fermentations with the same strain. This anomaly may be due to other acids being produced (eg. succinic), but at such high levels as the results show this theory seems doubtful. In addition, no determination of succinic acid concentrations at the completion of fermentation were made. It would have been beneficial to the study to do so. The authors analytical techniques were investigated to eliminate any error from this source (see sections 4.1 and 4.2). Therefore, this effect cannot be adequately accounted for. Figure 4.6 is representative of the overall increase in titratable acidity observed in these trials.

Table 4.5 Analyses of fermentations at different temperatures of Simulated Grape Juice inoculated with yeast strains EC1118 and D432[a].

Temp. °C	Yeast	Initial M.A.	Final M.A.	M.A. Change	Initial T.A.	Final T.A.	T.A. Change	Initial pH	Final pH	pH Change	Final Alcohol	Ferm Duration[b]
12	EC1118	4.9	3.7	-1.2	7.4	10.3	+2.9	3.46	3.47	+0.01	11.3	17
18	EC1118	5.0	4.2	-0.8	10.5	9.9	-0.6	3.34	3.45	+0.11	15.0	16
25	EC1118	5.0	4.1	-0.8	7.2	9.5	+2.3	3.47	3.53	+0.04	13.1	17
37	EC1118	5.0	3.7	-1.3	7.5	11.0	+3.5	3.47	3.58	+0.09	11.2	15
12	D432	5.0	3.9	-1.1	8.4	9.0	+0.6	3.44	3.46	+0.02	9.4	17
18	D432	4.7	3.9	-0.8	10.5	9.3	-1.2	3.34	3.50	+0.16	14.0	16
25	D432	4.8	3.3	-1.5	8.9	10.5	+1.6	3.45	3.53	+0.08	12.4	17
37	D432	4.7	3.7	-1.0	8.9	9.2	+0.3	3.47	3.51	+0.04	11.1	15

[a] All results expressed as g/L except alcohol as % v/v.

[b] Fermentations were complete when two consectutive readings of 0% sugar (reducing sugar test) were recorded.

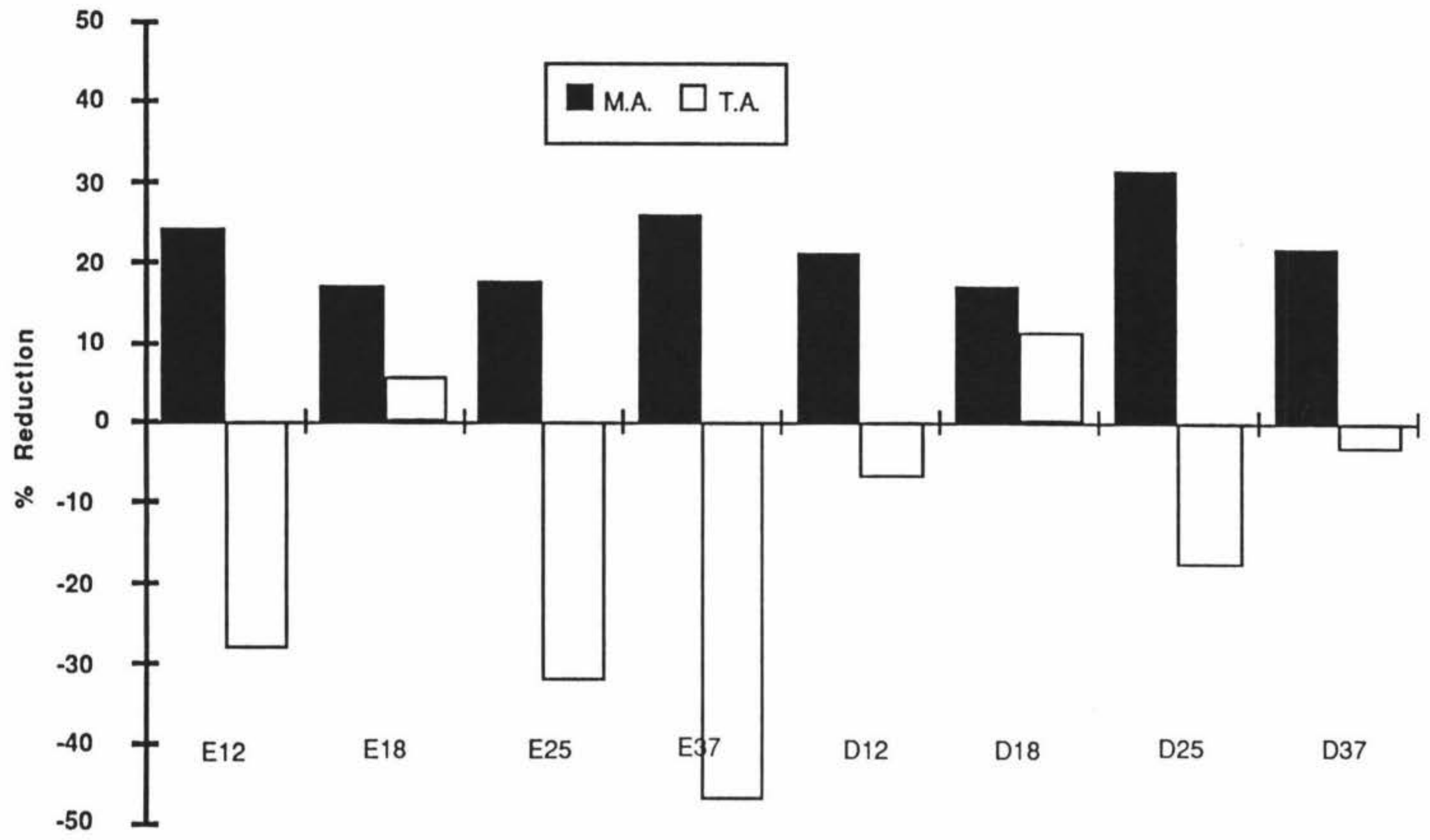


Fig.4.5 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented at different temperatures with yeast strains E (EC1118) and D (D432).

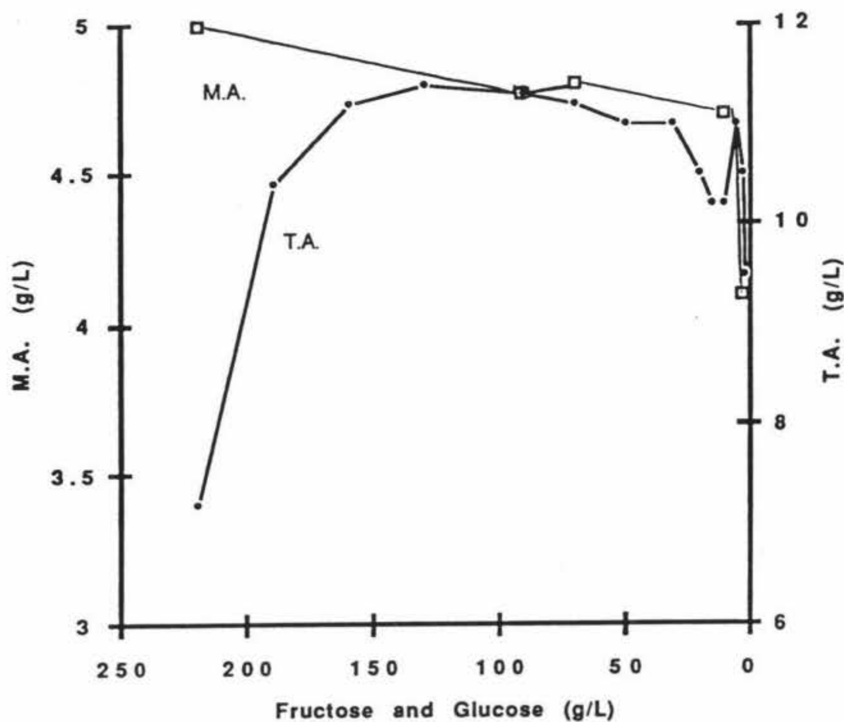


Fig.4.6 Malic acid (M.A.) and titratable acidity concentrations during fermentation (25°C) of Simulated Grape Juice inoculated with strain EC1118. Initial cell count was 6.5 x 10^6 cells/ml.

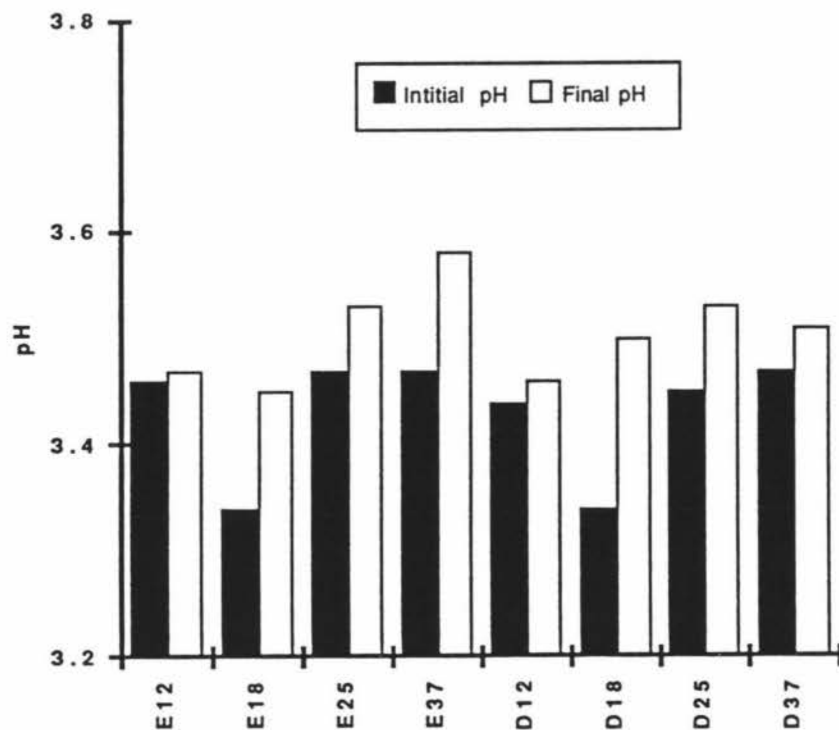


Fig.4.7 Initial and final pH values of fermentations of Simulated Grape Juice at different temperatures inoculated with strains E (EC1118) and D (D432).

pH Increase

Figure 4.7 presents the changes in pH which occurred in these fermentations. In general, pH increases were negligible. The greatest increase was at 37°C by strain EC1118, a mere 0.09 pH units.

Alcohol Formation

Table 4.5 presents the amounts of alcohol formed on completion of the fermentations by the yeast strains. Less alcohol was formed in comparison to that formed in the fermentations described in section 4.3.1. Lower fermentation temperatures may slow the metabolic rate of yeasts, resulting in less malic acid degradation and therefore alcohol formation. Similarly, higher fermentation temperatures may have effected yeast metabolism and caused yeast cell lysis which generates lower alcohol yields.

4.3.3 Variable Malic Acid Concentration Fermentations

In these trials, the initial amounts of malic acid added to Simulated Grape Juice were varied from 5.0 g/L to 2.5 g/L and 7.5 g/L to determine the effect of malic acid concentration on acid reduction.

Some results of the fermentations are presented below and a complete set of results is shown in Appendix D.

Malic Acid

The amount of malic acid degraded is presented in Table 4.6 and Figure 4.8. A general trend emerged. Greatest amounts of malic acid initially added resulted in the most malic acid degraded on completion of the fermentation. Therefore, to compare effective malic acid degradation by each yeast strain, the percentage malic acid must be examined. All strains degraded the greatest percentage of malic acid when 7.5g/L was initially added to the Simulated Grape Juice. Strains EC1118 and D432 degraded similar percentages at all initial concentrations, wheras strain 71B was ineffective at degrading malic acid at 2.5g/L initial malate.

Titratable Acidity

All yeast strains reduced the titratable acidity and results are presented in Table 4.6 and figure 4.8. When 5.0 g/L and 7.5 g/L malic acid was initially added strain 71B reduced the titratable acidity the most. Comparitively, strain D432 reduced the titratable acidity the most at 2.5 g/L initial malic acid.

pH

Table 4.6 and figure 4.9 shows pH increase during the course of the fermentations. All strains at each initial level of malic acid increased the pH, except strain D432 (2.5 g/L initial malic acid) where no change in pH resulted.

Table 4.6 Analyses of fermentations (18°C) of Simulated Grape juice with varying concentrations of malic acid[a].

M.A. g/L	Yeast	Initial M.A.	Final M.A.	M.A. Change	Initial T.A.	Final T.A.	T.A. Change	Initial pH	Final pH	pH Change	Final Alcohol	Ferm. Duration[b]
2.5	EC1118	2.60	2.10	-0.50	7.43	7.20	-0.23	3.36	3.41	+0.05	13.9	19
5.0	EC1118	5.00	4.17	-0.83	7.00	6.80	-0.20	3.42	3.55	+0.13	13.9	21
7.5	EC1118	7.50	6.09	-1.41	10.80	9.90	-0.90	3.45	3.57	+0.12	13.9	19
2.5	71B	2.6	2.6	0.0	7.20	6.60	-0.60	3.42	3.63	+0.19	12.7	25
5.0	71B	5.0	3.9	-1.1	7.00	6.30	-0.70	3.44	3.60	+0.16	13.9	24
7.5	71B	7.7	5.9	-1.8	11.55	9.75	-1.80	3.46	3.64	+0.18	13.0	25
2.5	D432	2.4	2.0	-0.4	6.75	5.85	-0.90	3.44	3.44	0.00	12.4	23
5.0	D432	5.0	4.1	-0.9	9.90	9.75	-0.15	3.46	3.57	+0.11	13.4	21
7.5	D432	7.2	5.5	-1.7	10.05	9.15	-0.90	3.41	3.51	+0.10	12.8	24

[a] All results expressed as g/L except alcohol as % v/v.

[b] Fermentations were complete when two consecutive readings of 0% sugar (reducing sugar test) were recorded.

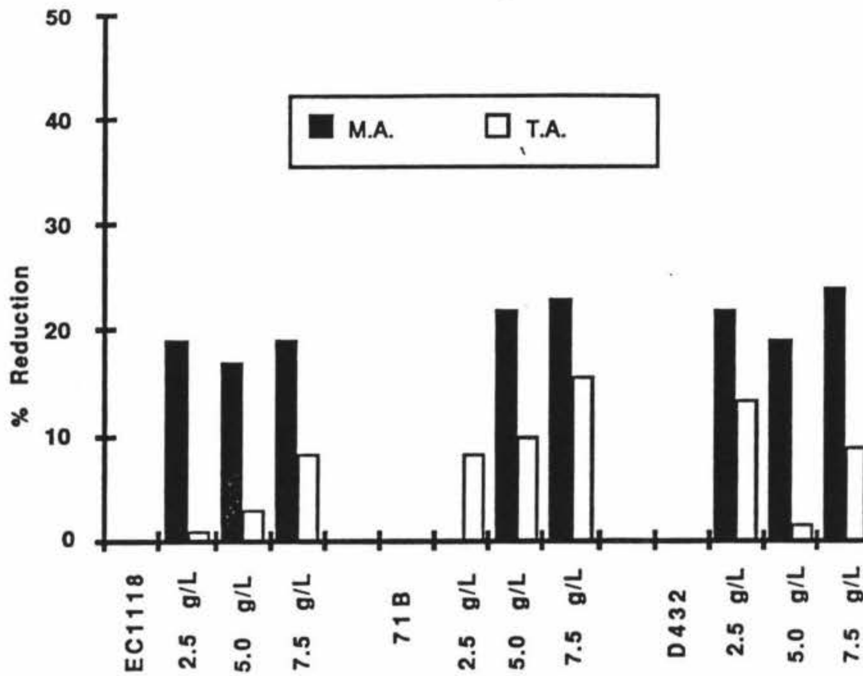


Fig.4.8 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice with varying initial concentrations of malic acid (g/L) fermented (18°C) with yeast strains EC1118, 71B and D432.

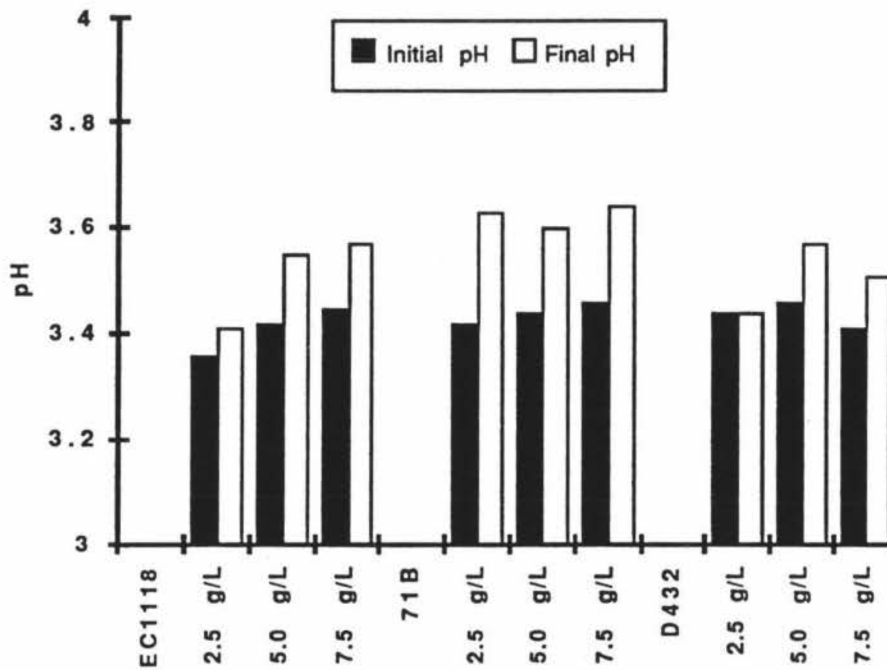


Fig.4.9 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice with different initial concentrations of malic acid (g/L) inoculated with yeast strains EC1118, 71B and D432.

4.3.4 Variable Nitrogen Concentration Fermentations

The initial nitrogen concentrations in the Simulated Grape Juice medium were varied from 1152mg/L to 578mg/L and 463mg/L to determine the effect of nitrogen level on acid reduction. The nitrogen concentration of proline (172mg/L) was included in these figures, although this amino acid is not utilised under anaerobic conditions.

Some results of the fermentations are presented below and a complete set of results is shown in Appendix E.

Malic Acid

The amounts of malic acid degraded are presented in table 4.7 and figure 4.10. At each initial nitrogen concentration, strain 71B degraded the most malic acid. Strain D432 degraded a consistent amount of malic acid at each initial nitrogen concentration. In comparison, strain EC1118 degraded the least (percentage) malic acid at 463mg/L initial nitrogen but the same percentage at 578mg/L and 1152mg/L initial nitrogen.

Titratable Acidity

At an initial concentration of 578mg/L nitrogen, a trend emerged where the greatest reduction in titratable acidity was observed (Fig. 4.10). As with malic acid degradation, strain 71B was most effective in reducing the titratable acidity at all initial levels of nitrogen.

pH Increase

Figure 4.11 shows that generally the least pH increase was observed at the standard (1152 mg/L) initial level of nitrogen. Greater increases in pH were observed at 462mg/L and 578mg/L initial nitrogen levels.

Table 4.7 Analyses of fermentations (18°C) of Simulated Grape Juice with varying initial concentrations of nitrogen[a].

Nitrogen mg/L	Yeast	Initial M.A.	Final M.A.	M.A. Change	Initial T.A.	Final T.A.	T.A. Change	Initial pH	Final pH	pH Change	Final Alcohol	Ferm. Duration[b]
463	EC1118	5.0	4.1	-0.9	8.60	8.33	-0.27	3.50	3.70	+0.20	14.1	22
578	EC1118	4.8	4.3	-0.5	8.70	8.25	-0.45	3.45	3.65	+0.20	12.3	22
1152	EC1118	5.0	4.2	-0.8	7.00	6.80	-0.20	3.42	3.55	+0.13	13.9	21
463	71B	5.0	3.4	-1.6	9.98	9.30	-0.68	3.25	3.54	+0.29	12.9	27
578	71B	4.8	3.8	-1.0	8.70	7.95	-0.75	3.45	3.73	+0.28	12.5	22
1152	71B	5.0	3.8	-1.2	7.00	6.30	-0.70	3.44	3.60	+0.16	13.9	24
463	D432	5.0	4.0	-1.0	9.0	8.4	-0.6	3.45	3.65	+0.20	12.7	23
578	D432	5.0	4.0	-1.0	9.0	8.4	-0.6	3.46	3.65	+0.19	13.2	23
1152	D432	5.0	4..1	-0.9	9.9	9.8	-0.1	3.46	3.57	+0.11	13.4	21

[a] All results expressed as g/L except alcohol as % v/v.

[b] Fermentations were complete when two consecutive readings of 0% sugar (reducing sugar test) were recorded.

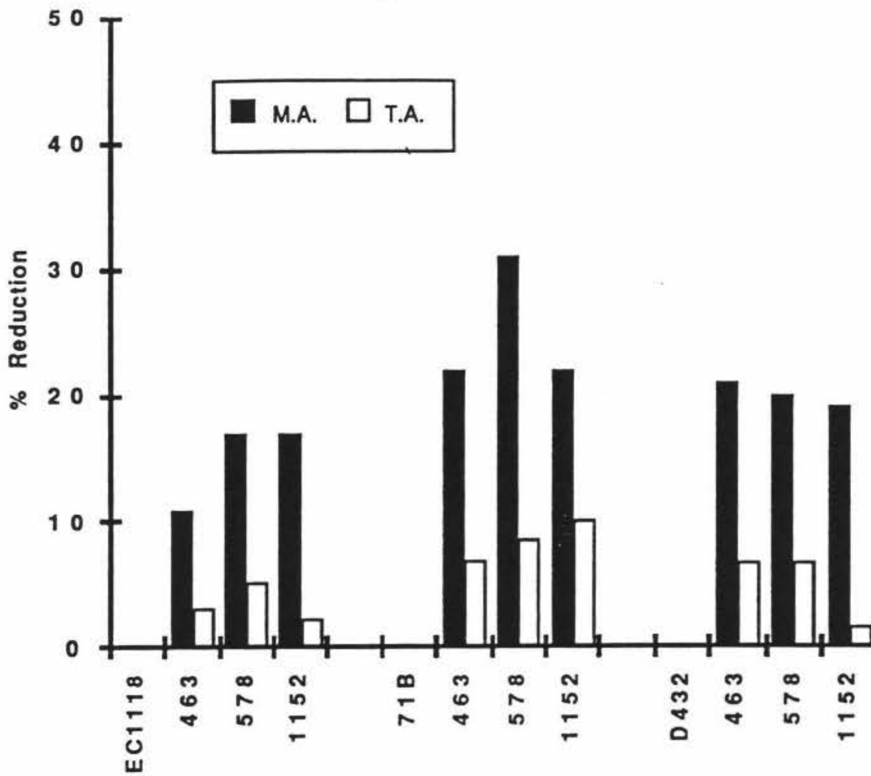


Fig.4.10 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice with varying initial concentrations of nitrogen (mg/L) fermented (18°C) with yeast strains EC1118, 71B and D432.

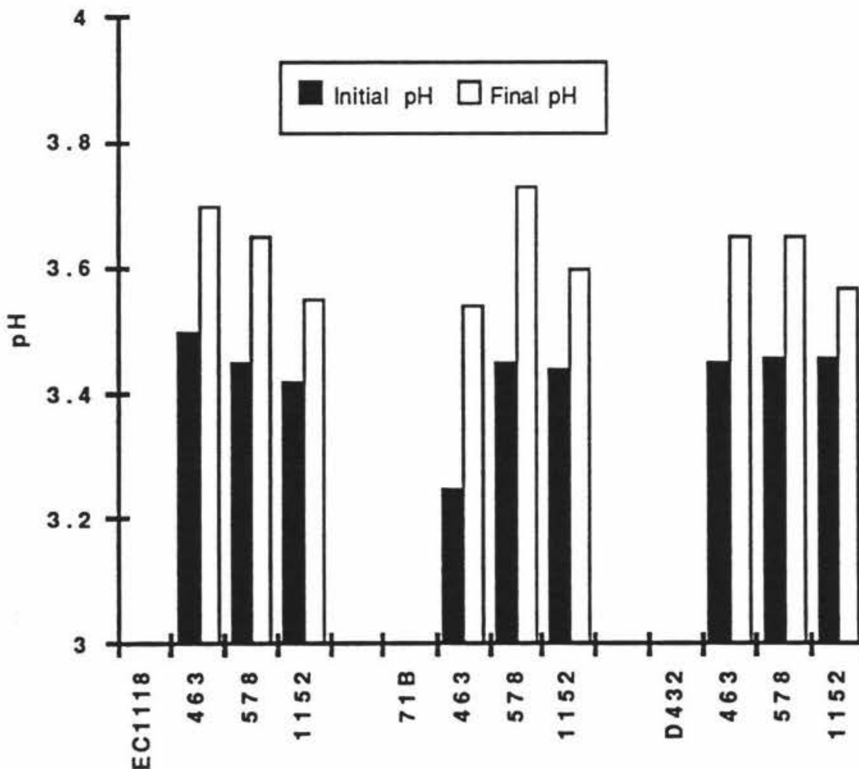


Fig.4.11 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice with different initial concentrations of nitrogen (mg/L) inoculated with yeast strains EC1118, 71B and D432.

4.3.5 Variable pH Level Fermentations

Initial pH levels of Simulated Grape Juice medium were adjusted (before autoclaving) to three pH levels, 3.0, 3.5 and 4.0 to determine the effect of pH on acid reduction.

Some results from the fermentations are presented below and a complete set of results is shown in Appendix F.

Malic Acid

Table 4.8 and figure 4.12 presents results of malic acid degradation. At all initial pH levels, strain D432 consistently degraded high amounts of malic acid. Strains 71B and EC1118 degraded malic acid at pH 3.5 but at pH 3.0 and 4.0, comparatively little malic acid was degraded by these yeasts. This is probably because the pH of wine is approximately 3.5, and lower pH levels are inhospitable to many wine and non wine yeasts. At higher pH levels the uptake of malic acid may be inhibited in some yeast strains.

Titratable Acidity

Titratable acidity was reduced at all initial pH levels (Fig. 4.12). The greatest reduction occurred at pH 3.0 by strain 71B but this fermentation became stuck and was stopped on day 30. Therefore this result may be invalid due to possible spoilage organisms causing reduction rather than 71B. Strain D432 was effective at reducing titratable acidity at this pH, as was strain EC1118 at pH 4.0.

pH Increase

As figure 4.13 shows, the smallest pH increase was observed at pH 4.0. A proportional relationship was formed whereby the lower the initial pH level the greater the observed pH increase.

Table 4.8 Analyses of fermentations (18°C) of Simulated Grape Juice at different pH levels[a].

pH	Yeast	Initial M.A.	Final M.A.	M.A. Change	Initial T.A.	Final T.A.	T.A. Change	Initial pH	Final pH	pH Change	Final Alcohol	Ferm. Duration[b]
3.0	EC1118	5.1	4.5	-0.6	10.70	10.65	-0.05	2.99	3.13	+0.14	13.1	21
3.5	EC1118	5.0	4.2	-0.8	7.00	6.80	-0.20	3.42	3.55	+0.13	13.9	21
4.0	EC1118	5.2	4.7	-0.5	9.00	7.80	-1.20	3.90	3.86	-0.04	13.8	21
3.0	71B	4.7	4.1	-0.6	12.00	8.40	-4.60	2.98	3.23	+0.25	12.9	(30)[c]
3.5	71B	5.0	3.9	-1.1	7.00	6.30	-0.70	3.44	3.60	+0.16	13.9	24
4.0	71B	5.3	4.9	-0.4	7.20	6.30	-1.10	3.95	3.95	0.00	14.3	21
3.0	D432	5.3	4.5	-0.8	11.25	10.05	-1.20	3.03	3.20	+0.17	13.8	24
3.5	D432	5.0	4.1	-0.9	9.90	9.75	-0.15	3.46	3.57	+0.11	13.4	21
4.0	D432	5.1	3.9	-1.2	6.75	6.60	-0.15	3.90	3.86	-0.04	13.3	24

[a] All results expressed as g/L except alcohol as % v/v.

[b] Fermentations were complete when two consectutive readings of 0% sugar (reducing sugar test) were recorded.

[c] Fermentation became stuck and was stopped at Day 30.

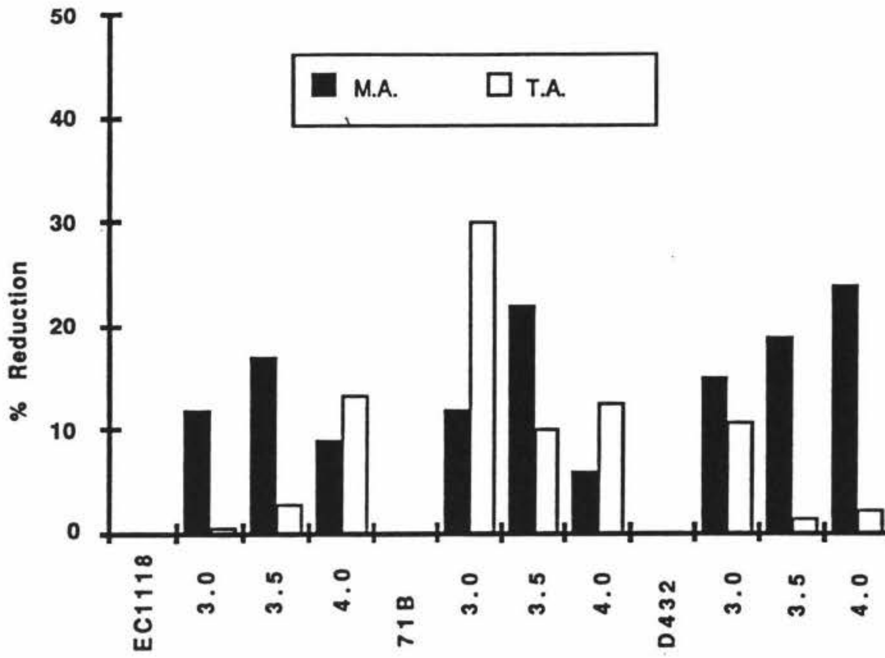


Fig.4.12 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice at different initial pH levels fermented (18°C) with yeast strains EC1118, 71B and D432.

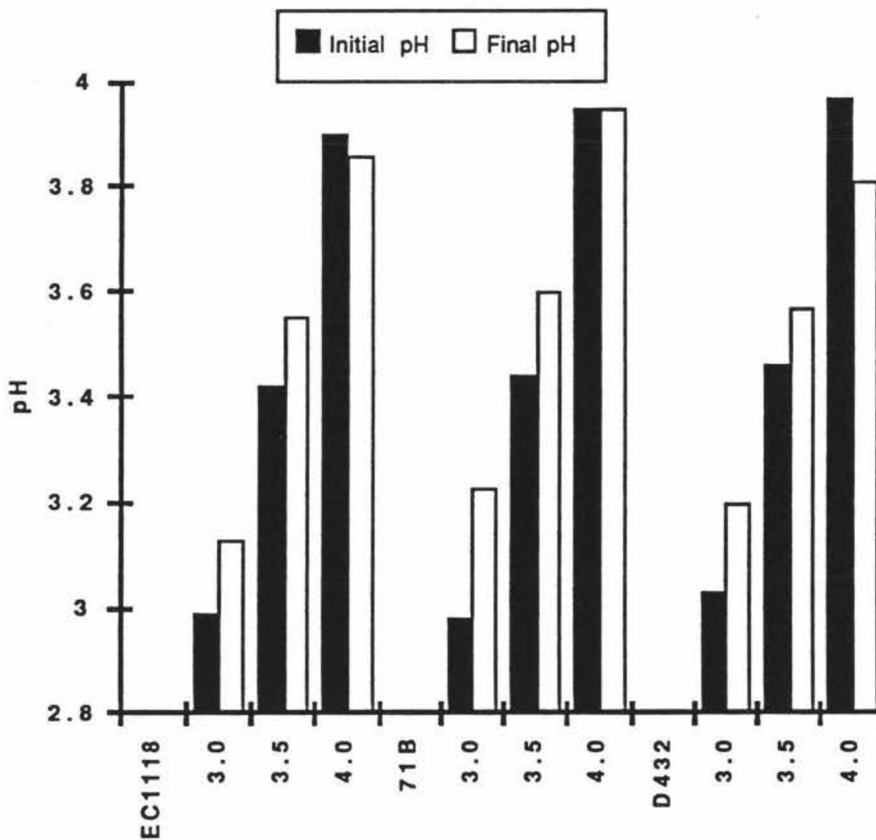


Fig.4.13 pH values of fermentations (18°C) of Simulated Grape Juice with different initial pH levels inoculated with yeast strains EC1118, 71B and D432.

4.4 Industrial Fermetations

Strains EC1118, ACID-, 71B and D432 were inoculated into commercial Chardonnay and Sauvignon Blanc juices in two commercial wineries in the Hawkes Bay, New Zealand. The purpose of these fermentations was to assess the activity of these yeasts in an industrial situation for possible future application and to compare results with those from Simulated Grape Juice fermentations.

4.4.1 Chardonnay Juice Fermentations

Some results of the fermentations are presented below and a complete sets of results for sections 4.4.1 and 4.4.2 are shown in Appendix G.

Malic Acid

Table 4.9 and figure 4.14 show that strain 71B was most effective at degrading malic acid (42%,34%), followed by D432 (23%,22%), ACID- (19%,14%) and finally EC1118 (6%,5%).

Titratable Acidity

Table 4.9 and figure 4.14 show that strain 71B reduced the titratable acidity to the greatest extent (17%, 15% reduction) while strain D432 reduced it the least (4%,3% reduction).

Reinoculation of the juice with strains 71B, ACID - and EC1118 was required, possibly due to copper residue from vineyard sprays. Such spray residues can inhibit yeast viability and growth. Figure 4.15 is representative of the effect caused by the first inoculation failing. It shows the titratable acidity rapidly declining until reinoculation occurs. This may have been caused by the precipitation of potassium bitartrate crystals.

Table 4.9 Analyses of fermentations of commercial Sauvignon Blanc and Chardonnay juices with different yeast strains[a].

	Initial	Final	M.A.	Initial	Final	T.A.	Initial	Final	pH	Final	Ferm.
	M.A.	M.A.	Change	T.A.	T.A.	Change	pH	pH	Change	Alcohol	Duration[b]
Sauvignon Blanc											
EC1118	3.6	2.8	-0.8	8.25	7.20	-1.05	3.30	3.36	+0.06	11.4	19
ACID-	3.9	3.0	-0.9	8.25	7.35	-0.90	3.29	3.37	+0.08	11.5	22
71B	4.4	2.3	-2.1	7.95	6.20	-1.75	3.45	3.62	+0.17	11.5	20
D432	3.6	2.1	-1.5	8.80	6.60	-2.20	3.30	3.42	+0.12	11.3	15
Chardonnay											
EC1118 (A)	6.4	6.0	-0.4	12.08	11.30	-0.78	3.11	3.29	+0.18	10.8	14
EC1118 (B)	6.4	6.0	-0.4	11.60	11.20	-0.40	3.10	3.30	+0.18		13
ACID- (A)	6.7	5.7	-1.0	10.80	10.50	-0.30	3.15	3.30	+0.15	11.0	18
ACID- (B)	6.7	5.4	-1.3	10.95	10.50	-0.45	3.18	3.29	+0.11		18
71B (A)	7.3	4.8	-2.5	12.20	10.40	-1.80	3.11	3.31	+0.20	10.9	24
71B (B)	7.3	4.2	-3.1	12.15	10.05	-2.10	3.14	3.36	+0.22		24
D432 (A)	6.4	5.0	-1.4	10.35	10.05	-0.30	3.17	3.37	+0.20	10.9	9
D432 (B)	6.4	5.1	-1.3	10.40	9.98	-0.42	3.18	3.38	+0.20		9

[a] All results expressed as g/L except alcohol as % v/v.

[b] Fermentations were complete when two consectutive readings of 0% sugar (reducing sugar test) were recorded.

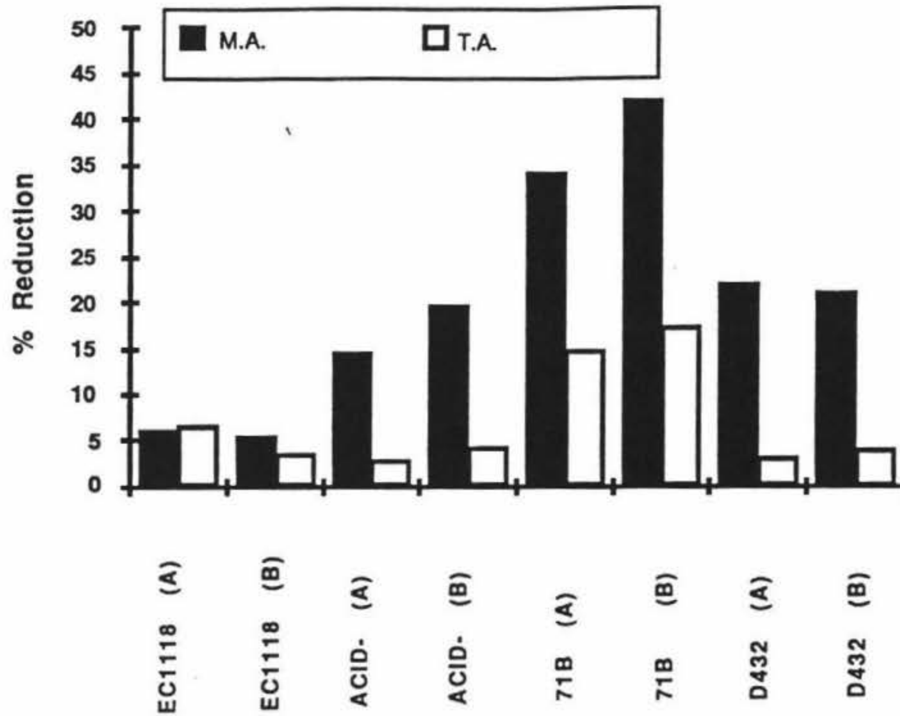


Fig.4.14 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of wines vinified in duplicate from commercial Chardonnay juice with strains EC1118, ACID-, 71B and D432.

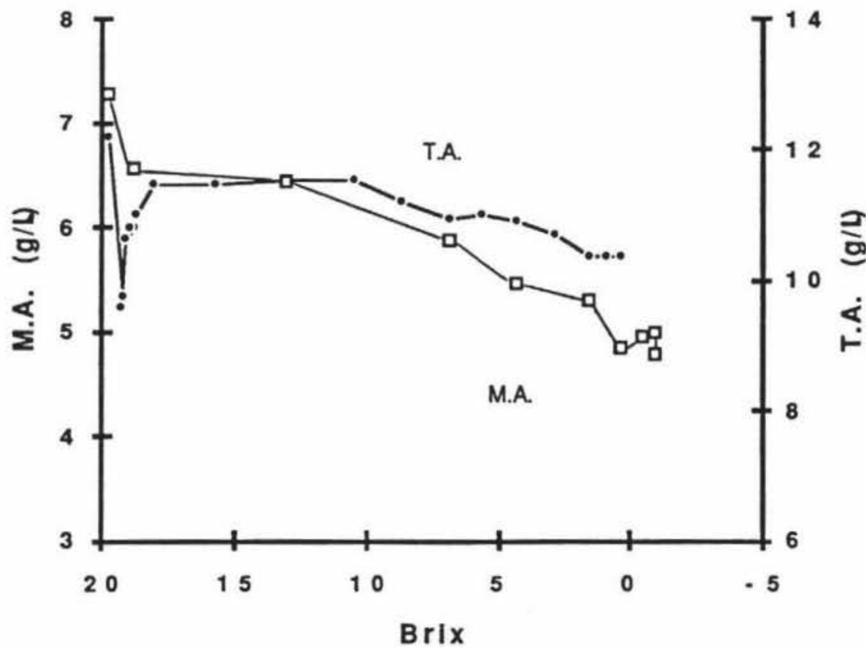


Fig.4.15 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain 71B (a). Initial cell count was 5.6 x 10^6 cells/ml.

pH

The greatest pH increases were observed in fermentations from strains D432 (0.2,0.2 pH units) and 71B (0.2, 0.22 pH units) as shown in Figure 4.16. The smallest increase in pH was observed with strain ACID- (0.15, 0.11 pH units).

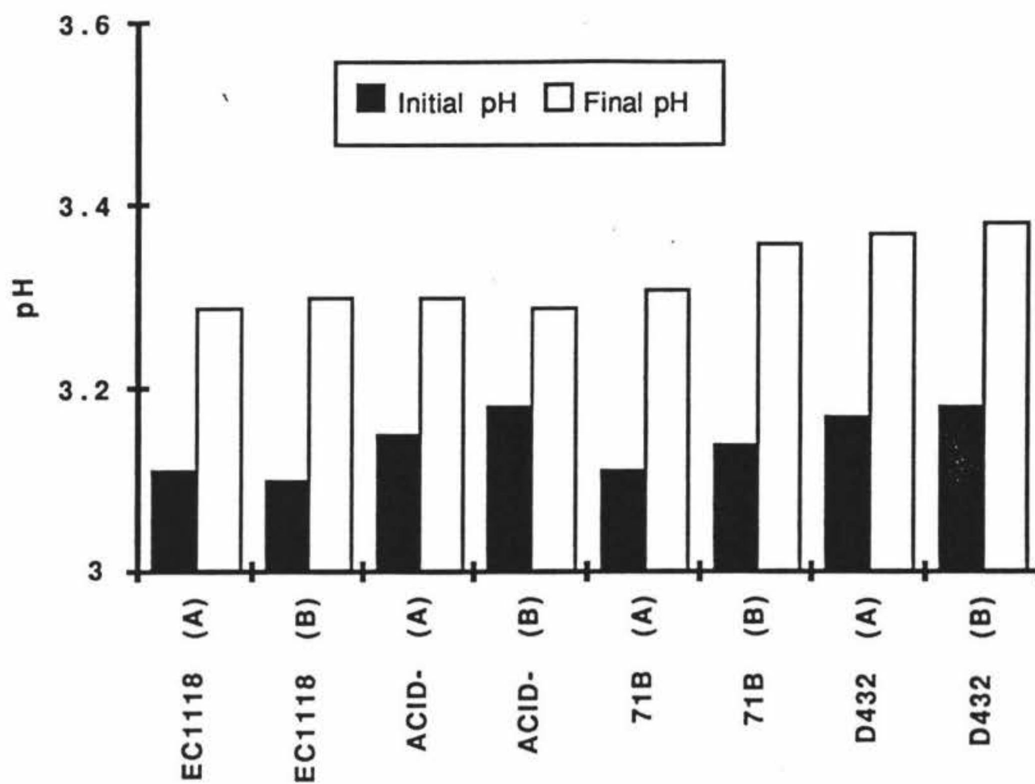


Fig.4.16 Initial and final pH values of commercial Chardonnay juice vinified in duplicate with yeast strains EC1118, ACID-, 71B and D432.

4.4.2 Sauvignon Blanc Juice Fermentations

Malic Acid

As occurred in section 4.4.1, strain 71B degraded the most malic acid in these fermentations (49%) followed by D432 (43%), ACID- (23%) and EC1118 (22%). Results are presented in table 4.9 and figure 4.17.

Titratable Acidity

Table 4.9 and figure 4.17 show that strain D432 is most effective in reducing titratable acidity (24% reduction), whilst strain ACID- is least effective (11% reduction).

pH

The greatest increase in pH was observed in the fermentation conducted by strain 71B (0.17 pH units) and this is shown in figure 4.18. Strain EC1118 produces the smallest change in pH (0.06 pH units).

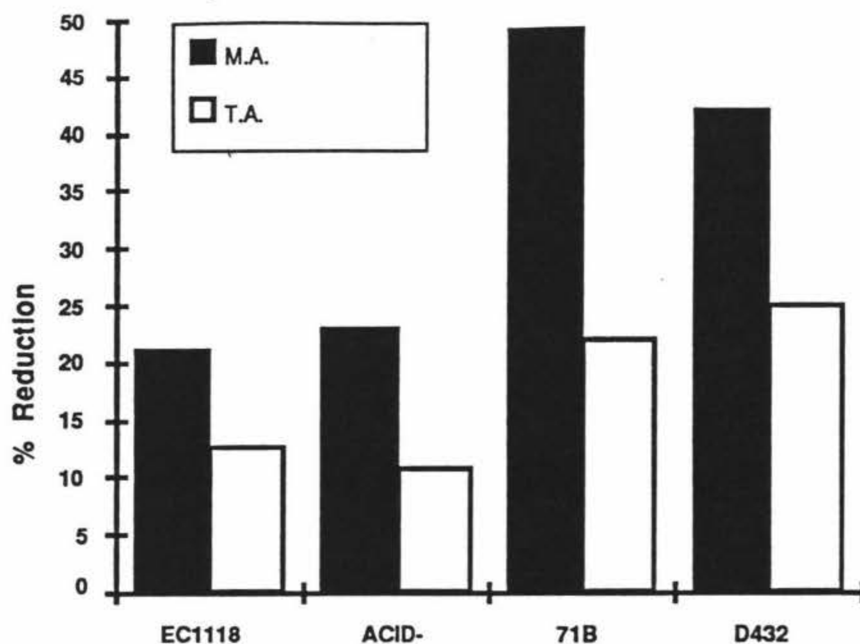


Fig.4.17 Percentage reduction in malic acid (M.A.) and titratable acidity (T.A.) of wines vinified from commercial Sauvignon Blanc juice with strains EC1118, ACID-, 71B and D432.

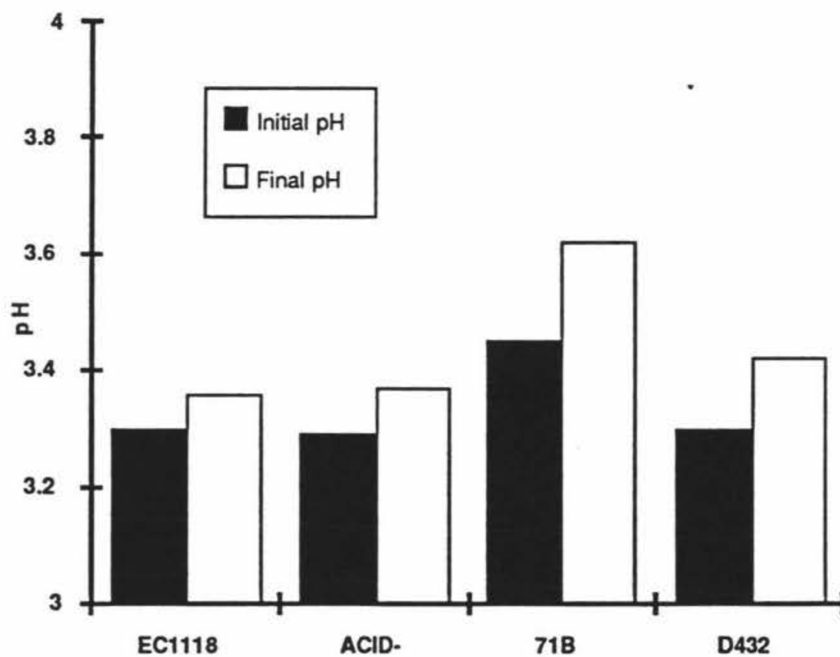


Fig.4.18 Initial and final pH values of commercial Sauvignon Blanc juice vinified with yeast strains EC1118, ACID-, 71B and D432.

4.4.3 Acid-Ranking Taste Tests

Wines from the industrial trials were bottled and aged for three months. Taste trials were performed to determine if differences in acid taste could be detected between the wines. Judges (four winemakers) were asked to rank the wines in order from highest to lowest acid taste. The results of the judges scores have been analysed following statistical methods outlined by Amerine and Roessler (1976).

In the Chardonnay juice taste tests (Table 4.10), wine made from strain D432 was found to be significantly different in acid taste to all other wines (95% confidence level). Judges consistently ranked this wine as having the least acid taste.

In the Sauvignon Blanc juice taste tests (Table 4.11), wine made from strain 71B was found to be significantly different in acid taste to all other wines (95% confidence level). This wine was constantly ranked as having the least acid taste.

Table 4.10 Taste tests of Chardonnay wine produced by different yeast strains.

Ranking Scores for acid taste (1 = highest acid)*

Wines	EC1118	ACID-	71B	D432
Judges				
J1 (A)	3	1	2	4
J1 (B)	3	2	1	4
J2 (A)	1	3	2	4
J2 (B)	2	3	1	4
J3 (A)	1	2	4	3
J3 (B)	1	3	2	4
J4 (A)	1	2	3	4
J4 (B)	1	2	3	4
Total	13	18	18	31
Mean	1.6[a]	2.3[a]	2.3[a]	3.9[b]

* Statistical analyses of acid taste ranking scores. Mean values showing the same superscript letter indicate there are no significant differences (95% confidence level) in acid taste. Mean values showing different superscript letters indicate significant differences (95% confidence level).

Table 4.11 Taste tests of Sauvignon Blanc wine produced by different yeast strains

Ranking scores for acid taste (1 = highest acid)*

Wines	EC1118	ACID-	71B	D432
Judges				
J1 (A)	4	1	3	2
J1 (B)	2	3	4	1
J2 (A)	1	4	3	2
J2 (B)	3	1	4	2
J3 (A)	2	3	4	1
J3 (B)	1	2	3	4
J4 (A)	1	2	3	4
J4 (B)	1	3	4	2
Total	15	19	28	18
Mean	1.9[a]	2.4[a]	3.5[b]	2.3[a]

* See Table 4.10

4.4.4 Karyotype Analysis of Yeasts

Industrial trial fermentations were examined by molecular genetic techniques to determine yeast strains present at the most vigourous stage of the fermentation (a fall in six degrees Brix). The purpose of this was to prove that the yeast strains inoculated into the juice actually conducted the fermentations.

Figures 4.19 and 4.20 show the ability to differentiate the four yeast strains by chromosomal banding patterns. The yeast strains used in these gels were commercially produced strains. The banding patterns of these yeasts serve as references for the isolates to be identified (Figs. 4.21 to 4.26). Figures 4.19 to 4.20 show polymorphisms in the banding pattern of each yeast. For example, band 2 of each strain is located in a slightly different position on the gel. More convincingly, the final two bands show definite differences in gel position.

Figure 4.19 has a 'wave' appearance due to the gel lifting off the platform. This problem was amended with the setting of the gel onto the platform with agar. Figure 4.20 has bands which appear 'fuzzy'. This happened to many of the gels due to the automatic temperature control being raised for unknown reasons. This effect could not be prevented. It appears from results of this research that temperature is critical to the outcome of any karyotype analysis.

Figures 4.21 to 4.26 present banding patterns of isolates to be identified. These isolates were cultured from juice samples which were taken during the most vigorous part of the fermentation (initial fall in six degrees brix). YM plates were streaked with the sample juice and three individual colonies, after incubation, were chosen at random from each plate and subcultured onto YM slopes. Each different colony was given the identification code of isolate A, B or C.

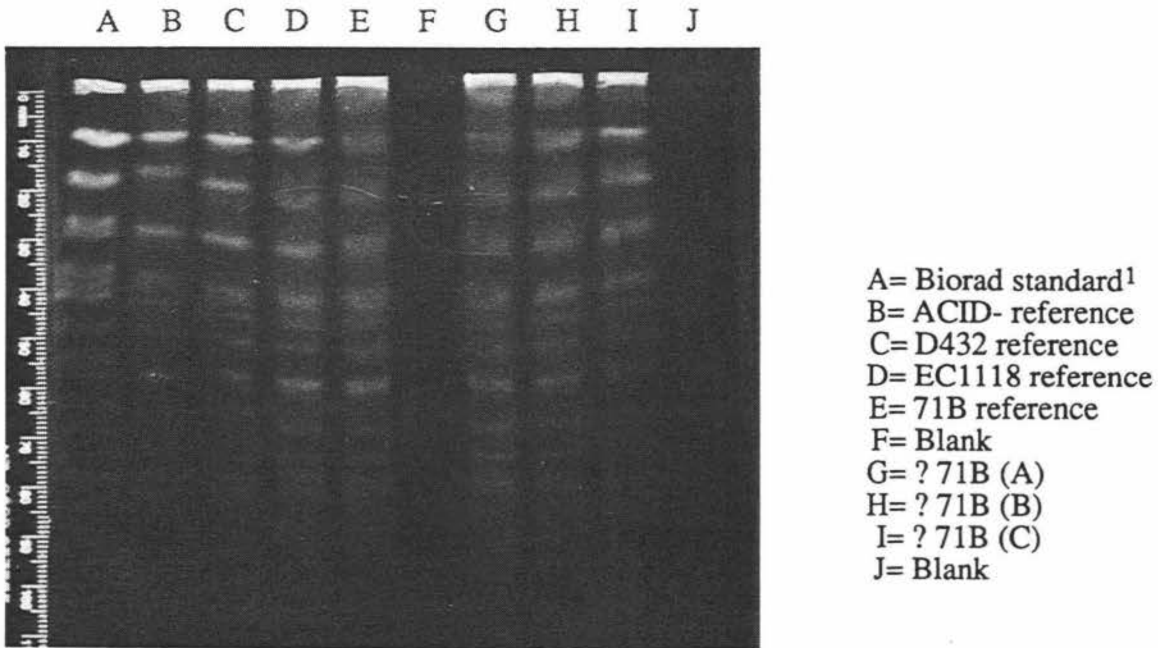


Fig. 4.19 CHEF electrophoresis of reference strains (columns B-E) and isolates removed from fermenting Sauvignon Blanc juice (column G-I). Biorad *S. cerevisiae* standard[1] 170-3605 was used.

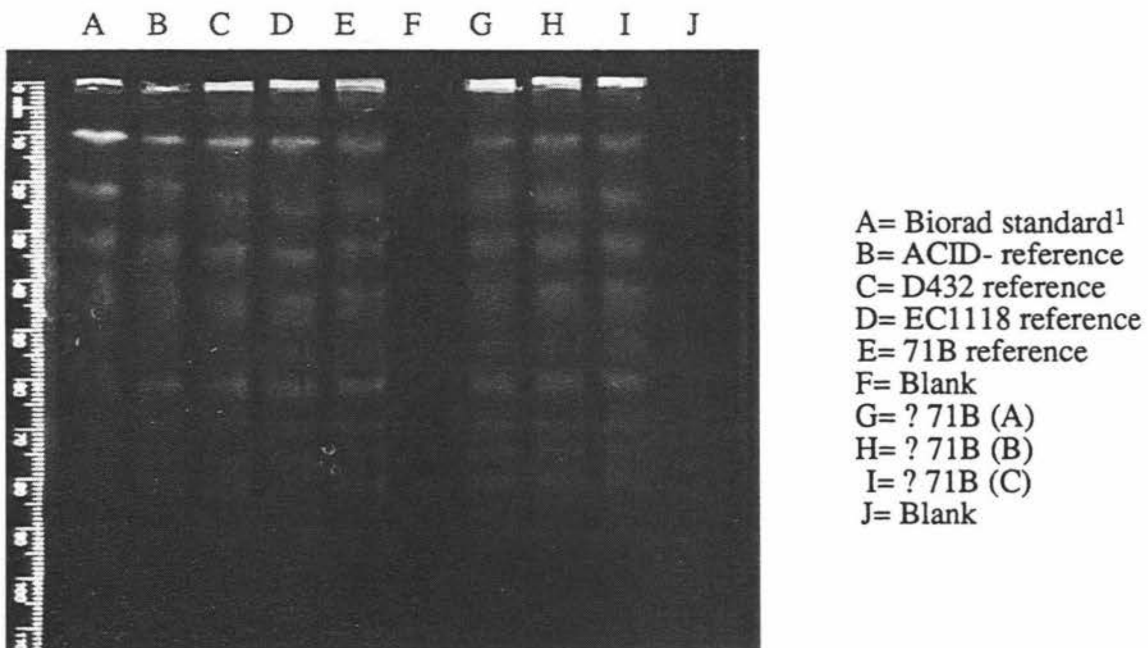
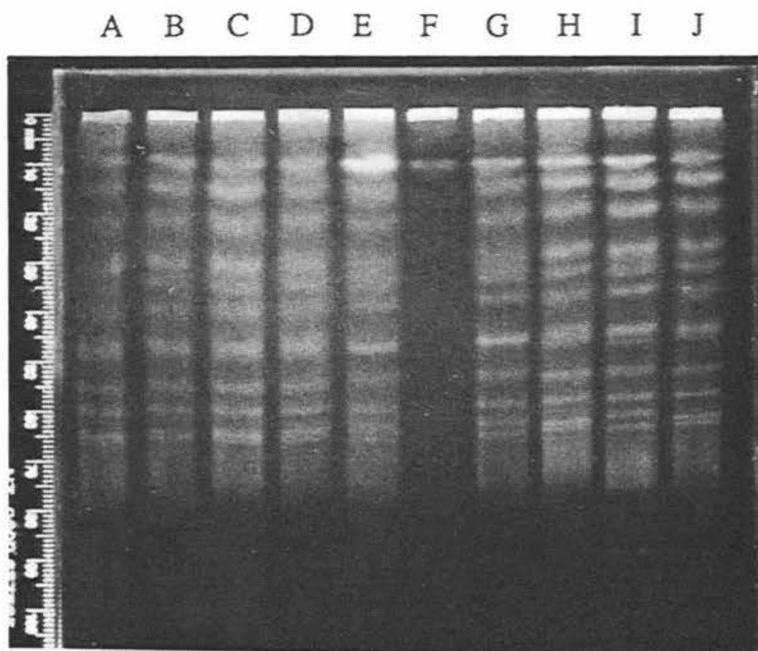


Fig. 4.20 CHEF electrophoresis of reference strains (columns B-E) and isolates removed from fermenting Sauvignon Blanc juice (column G-I). Biorad *S. cerevisiae* standard[1] 170-3605 was used.

Figures 4.21 and 4.22 show very definite and clear chromosomal bands. Two gels were run to confirm the banding patterns. In figure 4.21, columns A, B and C show identical banding patterns. On comparison with the reference strain these isolates are assumed to be strain 71B. Column F however is blank. This same result occurred in column C (Fig. 4.22). Both columns represent the same isolate. It is possible that no banding patterns occurred because a contaminant grew on the plate which was subcultured rather than the desired yeast strain, indicating a level of contamination in the wine. In this case, DNA would not have been obtained due to inadequate cell breakage conditions, resulting in no bands. This seems the most logical explanation because isolates A and B both produced clear bands indicating strong presence of 71B in the fermenting wine.

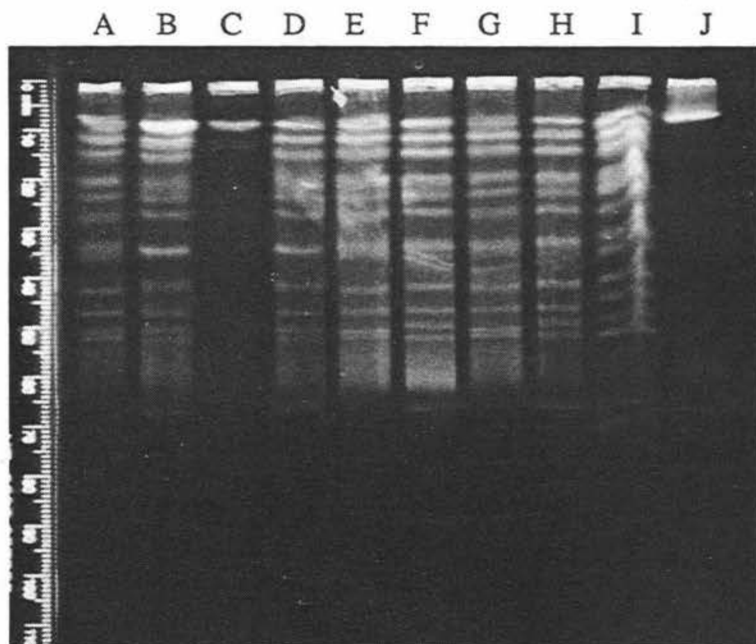
Columns D to I (Fig. 4.21) show polymorphisms in the banding patterns of the last two chromosomes. Columns A to F (Fig. 4.22) show the same result. Column G (Fig. 4.22) contains a reference strain of 71B and Column H (Fig. 4.22) contains a reference strain of EC1118. On comparison with these two columns, it appears that Chardonnay juice fermentations which were inoculated with strain 71B also contained strain EC1118. This may have been due to existing EC1118 colonies in winery equipment (although measures were taken to prevent this) or contamination of the wines through the air or sampling equipment. In addition, the period before reinoculation occurred (see section 4.4.1) may have allowed some EC1118 to multiply in the juice.

Figure 4.23 clearly shows the banding pattern of only one yeast strain. On comparison with the reference strain, it was confirmed that at the most vigorous part of fermentation, EC1118 was the predominant yeast strain in the fermenting vessel. Banding patterns of this strain have not travelled as far down the gel due to temperature complications (see above).



A= ? 71B (A)
B= ? 71B (B)
C= ? 71B (C)
D= 71B reference
E= ? a71B (A)
F= ? a71B (B)
G= ? a71B (C)
H= ? b71B (A)
I= ? b71B (B)
J= ? b71B (C)

Fig. 4.21 CHEF electrophoresis of isolates removed from fermenting Sauvignon Blanc juice (column A-C) and fermenting Chardonnay juice (columns E-J). a and b represent duplicate fermentations. Column D contains reference strain 71B.



A= 71B reference
B= ? a71B (A)
C= ? a71B (B)
D= ? a71B (C)
E= ? b71B (A)
F= ? b71B (B)
G= ? b71B (C)
H= EC1118 reference
I= Biorad standard[1]
J= Biorad standard[2]

Fig. 4.22 CHEF electrophoresis of reference strains (columns A and H) and isolates removed from fermenting Chardonnay juice (column B-G). a and b represent duplicate fermentations. Biorad *S. cerevisiae* standard[1] 170-3605 and *S. pombe* standard[2] 170-3633 were used.

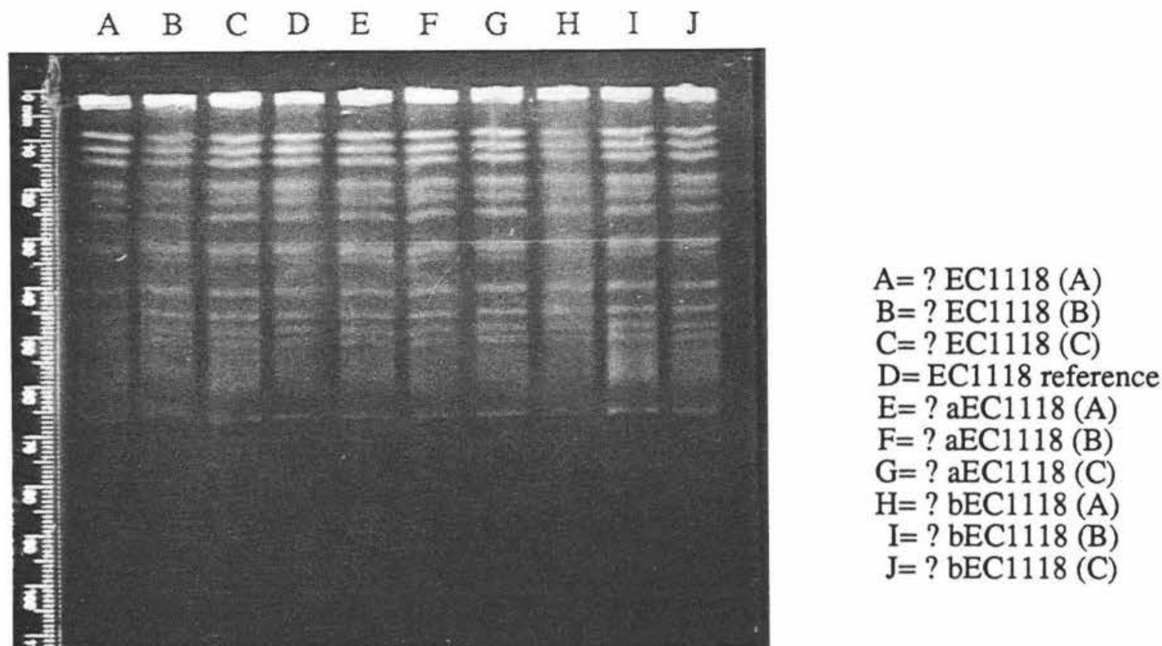


Fig. 4. 23 CHEF electrophoresis of isolates removed from fermenting Sauvignon Blanc juice (column A-C) and fermenting Chardonnay juice (columns E-J). a and b represent duplicate fermentations. Column D contains reference strain EC1118.

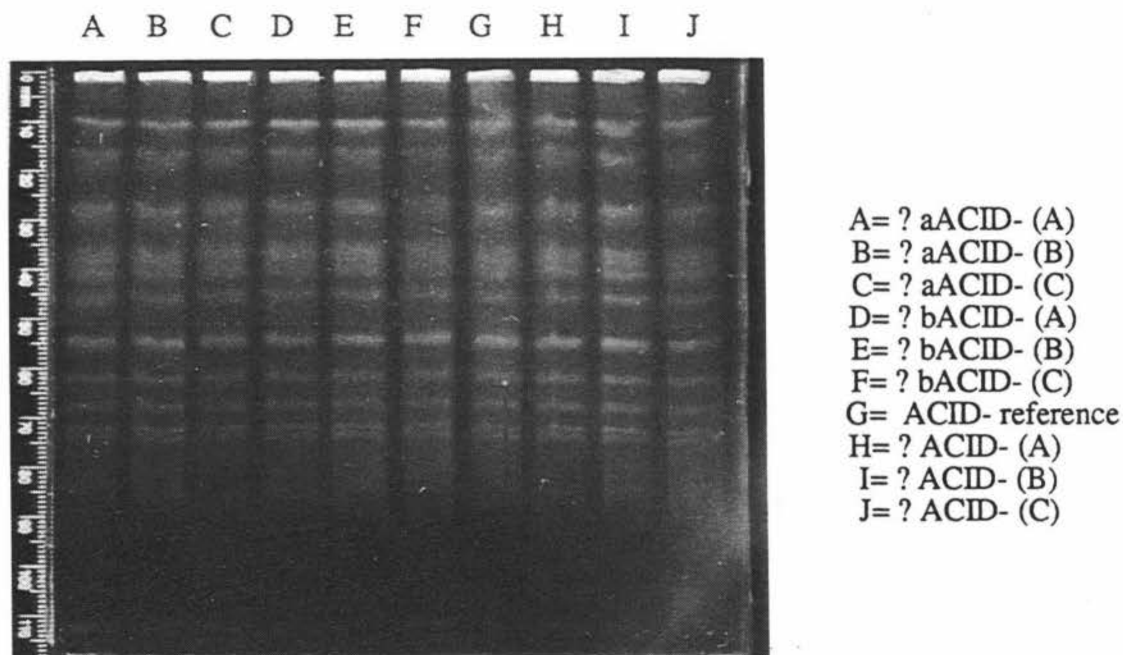
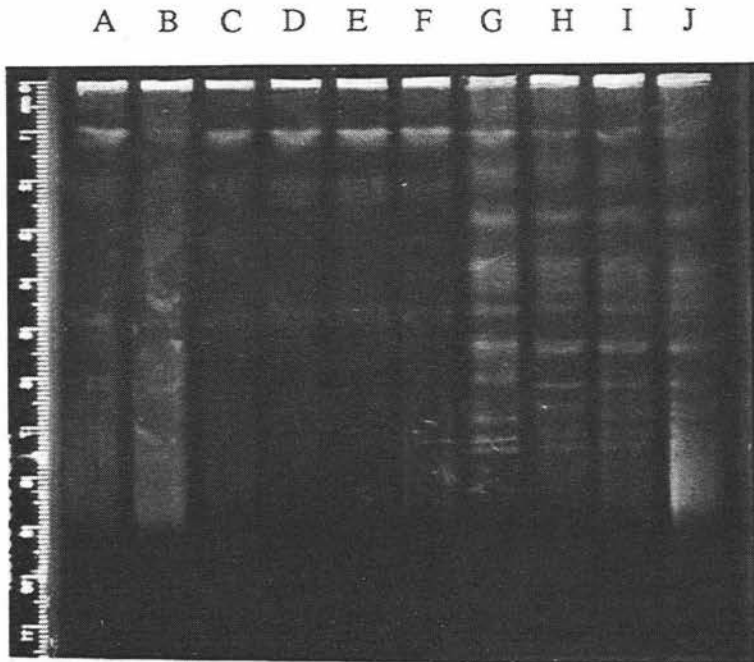


Fig. 4.24 CHEF electrophoresis of isolates removed from fermenting Chardonnay juice (columns A to F) and fermenting Sauvignon Blanc juice (columns H-J). a and b represent duplicate fermentations. Reference strain ACID- is positioned in column G.

Figure 4.24 shows one definite banding pattern, indicating the presence of only one yeast strain. On comparison with the reference strain, it was concluded that ACID- was the predominant yeast strain at the most vigorous part of the fermentation.

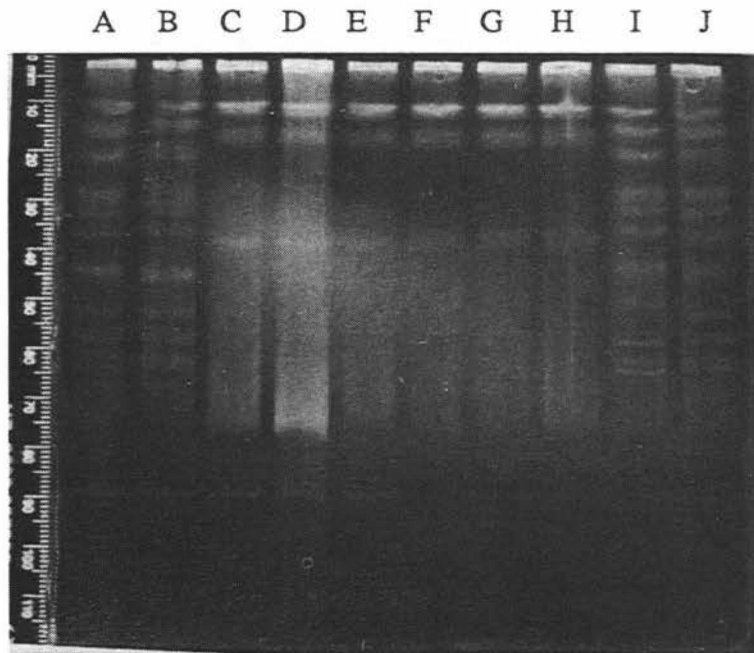
Columns G to I (Fig. 4.25) show the same banding pattern indicating that strain D432 was the predominant strain in the Sauvignon Blanc fermentations. However an anomaly occurs in the Chardonnay fermentations as columns A to F appear blank. To confirm this result, a second gel was run (Fig. 4.26) with reference strains loaded strategically on either side of the isolates. However, the same result was produced. Microscopic examination of the YM slope confirmed that a contaminant had grown. The contaminant was apiculate in shape and budding occurred from both ends when cultured. This indicated that a strain of *Kloeckera* or *Hanseniaspora* had contaminated the original plates and was reisolated rather than strain D432. It was assumed that strain D432 did in fact control the fermentation in the Chardonnay juice trials due to the following reasons:

- A large inoculum of strain D432 was added to the juice as is required by the manufacturers specifications. Therefore little competition from other yeast strains would have occurred.
- Wine yeasts require temperatures of approximately 30°C to grow on YM plates. There was no incubation room at this temperature in either winery, so plates had to be left out on the lab bench. In addition, fridges for the storage of YM plates were overfilled (increasing temperature of the interior) and the plates were often examined by unauthorised personnel.
- Strain D432 grows slowly on YM plates. Spoilage yeasts such as *Kloeckera* are ubiquitous in the winery and could have easily out competed strain D432 due to the ability to grow rapidly on YM plates.
- The fermentations went rapidly to completion. If a spoilage yeast such as *Kloeckera* dominated the fermentation high amounts of acetic acid would have been produced causing the fermentation to become 'stuck'.



A= ? aD432 (A)
B= ? aD432 (B)
C= ? aD432 (C)
D= ? bD432 (A)
E= ? bD432 (B)
F= ? bD432 (C)
G= D432 reference
H= ? D432 (A)
I= ? D432 (B)
J= ? D432 (C)

Fig. 4.25 CHEF electrophoresis of isolates removed from fermenting Chardonnay juice (column A-F) and fermenting Sauvignon Blanc juice (columns H-J). a and b represent duplicate fermentations. Column G contains reference strain D432.



A= EC1118 reference
B= ACID- reference
C= ? aD432 (A)
D= ? aD432 (B)
E= ? aD432 (C)
F= ? bD432 (A)
G= ? bD432 (B)
H= ? bD432 (C)
I= D432 reference
J= 71B reference

Fig. 4.26 CHEF electrophoresis of reference strains (columns A, B, I, J) and isolates removed from fermenting Chardonnay juice (column C-H). a and b represent duplicate fermentations.

Possible ways to prevent such contamination in future experiments include the use of adequate culturing conditions and microscopic examination of the isolate before karyotypic analysis begins. In addition, karyotyping more yeast isolates (10 isolates compared to 3) would have provided results with greater statistical validity. Time factors and limited equipment availability prevented this from occuring.

5.0 DISCUSSION

This research has entailed the investigation of six yeast strains in Simulated Grape Juice and commercial grape juice fermentations. Analytical data was collected during the fermentations and from this, conclusions have been made regarding the acid reducing capabilities of each yeast strain.

The result of any single analysis performed in this research has been shown by statistical methods to be reliable (Table 4.1). The coefficients of variation for standard solutions of Brix, pH, malic acid and alcohol analyses are extremely low which denotes an excellent level of reproducability. However, standard alcohol analyses were not performed over an extended time period, therefore the sensitivity of the method was not satisfactorily determined. As aforementioned, sensitivity of the test is such that small differences in alcohol cannot be reliably estimated. In addition, titratable acidity analyses were found to only be accurate within ±2.31g/L of the data presented. This indicates a margin of error in results from any single analysis of titratable acidity presented in this research.

Replicate laboratory grape juice fermentations were performed to observe significant differences in malic acid degradation, titratable acidity, pH and alcohol amongst yeast strains EC1118, ACID-, 71B and D432 (Table 4.2, 4.3). It was shown that strains 71B and D432 degraded significantly greater amounts of malic acid and reduced a greater proportion of the titratable acidity than strains EC1118, ACID- and M. In addition, results show that strain 71B significantly differed from all other strains in the amount of alcohol formed (9.2% v/v). The smallest amount of alcohol was formed in wines made from this strain, although the greatest malic acid degradation occurred. This anomaly may be attributed to ester formation by 71B from secondary products of pyruvate metabolism. However, only small amounts of ethanol are formed (0.34g/L) from maloethanolic fermentation in relation to the large amounts formed from the metabolism

of grape sugars. In addition, significant differences observed from chemical analysis may not be detected organoleptically.

Furthermore, the level of reproducability shown from these fermentations was excellent which indicates that under the same trial conditions, similar results should be obtained.

5.1 Comparison of Simulated Grape Juice and Commercial Grape Juice Fermentations

Industrial, laboratory and chemically Simulated Grape Juice fermentations were conducted to determine yeast activity in defined and non-defined environments. Simulated grape juice was a controlled environment which allowed relatively easy comparisons to be made about yeast activity between fermentations, due to standard chemical composition (unless deliberately altered). Commercial juices however, can exhibit considerable variability in chemical composition, which makes comparisons more difficult.

Laboratory and industrial fermentations of commercial grape juices were performed to compare yeast activity in grape juices to that in Simulated Grape Juice. Results of these fermentations show that wines produced by strains D432 and 71B, a greater percentage of malic acid is degraded compared with Simulated Grape Juice (Tables 4.2 to 4.9). Approximately the same percentage degradation was observed for strain ACID- in both commercial and Simulated Grape Juice fermentations. However, strain EC1118 degraded less malic acid (5%) in Chardonnay juice trials than in any Simulated Grape Juice fermentation (Fig. 4.14). This was possibly due to interactions between EC1118 and the juice constituents. The high malic acid content of the juice is not a factor because Simulated Grape Juice fermentations conducted at a similar initial malic acid

concentration (7.5 g/L) resulted in four times the amount of degradation as that observed in the industrial fermentations (Fig. 4.8).

Correspondingly greater increases in pH and reductions in titratable acidity were also observed in commercial grape juice fermentations compared with Simulated Grape Juice fermentations. Furthermore, the activation step (see section 2.2.1) of D432 was utilised in industrial fermentations causing a much more rapid fermentation. This step was not employed in Simulated Grape Juice fermentations because the juice was first sterilised, eliminating competition from other yeast strains and because standard procedures for all yeast strains were used. The fermentations inoculated with strain D432 took a similar duration to other yeast strains.

Although Simulated Grape Juice closely mimics grape juice composition, winemakers must be aware that this juice does not accurately mimic the outcome of a fermentation. Each yeast strain will behave differently in juices due to variation in juice composition. However, repeated Simulated Grape Juice fermentations (18°C) inoculated with the same yeast strain show that generally a consistent percentage of malic acid was degraded (data not shown). This indicates that Simulated Grape Juice can reliabily reproduce results from one fermentation to the next.

Simulated Grape Juice fermentations will never replace commercial grape juice fermentations. The benefit of using this medium however is in the ability to alter the chemical composition in order to study different variables. Therefore, Simulated Grape Juice can be seen as a beneficial tool in the research of yeast activity in wine. It can be recommended for use in future research so long as the researcher is aware of its limitations.

5.2 Comparison of Variables

Alteration of temperature and the chemical composition of Simulated Grape Juice allowed different variables to be studied. Variables were investigated to observe the ranges at which the yeast strains were able to effectively conduct fermentation and degrade malic acid. These ranges are presented in section 5.3.

The variables investigated were a selection of factors which influenced malic acid degradation (Tortia *et al*, 1993) which include: fermentation temperature, malic acid concentration, nitrogen composition, hydrogen ion concentration, oxygen level, sugar concentration, thiamine and biotin. Due to constraints on time, only the first four factors were investigated in this research.

Fermentation at 30°C is cited by Tortia *et al* (1993) to promote malic acid degradation. Temperatures above this level (30-35°C) are suggested by Reed and Nagodawithana (1991) to cause yeast cell lysis which will harm the wine. In this research, results on temperature variation (Table 4.5) were not conclusive due to anomalies in titratable acidity and pH results. It would have been appropriate therefore to repeat these trials. However, it appears from variable temperature fermentations, that strains D432 and EC1118 degraded more malic acid at the higher fermentation temperatures (25°C and 37°C respectively).

Tortia *et al* (1993) also investigated the influence of initial malic acid concentration ion the total malic acid degraded. They found that the total amount degraded was influenced by initial malic acid concentration but the percentage degraded remained constant. They also found that, on average, between 10-35% malic acid was degraded. In this research, strain EC1118 gave constant percentage malic acid degradation while strains 71B and D432 were variable (Table 4.6, Fig. 4.8). These latter two strains both degraded a greater percentage malic acid when a greater amount of malic acid was initially present.

In addition, more than 35% malic acid was degraded by strains 71B and D432 in industrial fermentations (Figs. 4.14, 4.17) but in laboratory grape juce fermentations malic acid was only degraded by a maximum of 30% (Table 4.3).

Tortia *et al* (1993) also claim that in juices where the sole nitrogen source was in the form of ammonium salts (as compared with amino acids), greater malic acid degradation occurred. Vezinhet and Barre (1982) report that the minimum required level of nitrogen in a juice for satisfactory yeast growth is between 120-140 mg N/L. Even lower figures (70-140 mg N/L) have been cited by Reed and Nagodawithana (1991). In this research, it was impossible to lower the initial nitrogen concentrations to these levels. However, fermentations with the lowest concentrations of nitrogen (463mg/L including proline) showed high levels of malic acid degradation by all yeast strains except EC1118 (Table 4.7, Fig. 4.10).

Tortia *et al* (1993) claim that between pH 2.0 and 3.5, the hydrogen ion concentration of the substrate did not effect malic acid degradation. Higher pH levels were not investigated by the authors. Results of laboratory grape juice fermentations (Table 4.2, 4.3) indicate that greatest malic acid degradation occured at pH 3.5. From results of Simulated Grape Juce fermentations (Table 4.8), pH 3.5 was observed to increase malic acid degradation by strains EC1118 and 71B and pH 4.0 by strain D432. Therefore, if deacidification is not required by the winemaker in a juice of pH 4.0, the utilisation of yeast strains other than D432 is recommended.

Oxygen levels were reported by the authors as influencing malic acid degradation due to aerobic conditions stimulating development of biomass. However, oxygen concentration levels were not given. In this research all fermentations were conducted anaerobically in order to mimic standard winery practices.

Initial high sugar concentrations are also reported to encourage greater malic acid degradation (Tortia *et al* , 1993). The authors state most malic acid degradation occurs within the first 5-6 days of the fermentation, when low oxygen and high carbohydrate sources are available. The results of this research generally support these findings.

Finally, thiamine is claimed by Tortia *et al* (1993) to promote maloethanolic fermentation by aiding activation of the thiamine-pyrophosphate complex (TPP) which catalyses (in the presence of alcohol dehydrogenases) the reaction of pyruvic acid to ethanol. Experiments performed with thiamine additions have shown mixed results, however. As reported by the authors an increase in malic acid degradation due to thiamine additions can also correspond to increases in volatile acidity. However, this has been disputed by experiments where no or less volatile acidity, in comparison to the controls, has been reported. Therefore it would be beneficial to research thiamine additions in New Zealand juices and musts as the findings presented above are not conclusive.

5.3 Maloethanolic Performance of Yeast Strains

Maloethanolic performance was observed in laboratory, Simulated Grape Juice and industrial fermentations. From these results, ranges of effective malic acid degradation for each variable and yeast strain are presented below.

5.3.1 Strain EC1118

Strain EC1118 was used as a reference strain in this research due to wide usage in New Zealand wineries. Although strain EC1118 is widely known, effective degradation of malic acid has not been defined for New Zealand conditions.

Results from this research have shown that EC1118 degraded malic acid within the range of 2.5-7.5 g/L initial malate (Tables 4.3, 4.4, 4.6), with most degradation occuring at 7.5 g/L. Higher fermentation temperatures (37°C) increased the level of degradation (Table 4.5). Higher (578mg/L) nitrogen concentrations (Table 4.7) and pH 3.5 (Table 4.8) encouraged greater levels of malic acid degradation.

The manufacturer of strain EC1118 claim that this strain is able to conduct fermentations within a temperature range of 8°C to 30°C. However, Simulated Grape Juice fermentations show that strain EC1118 degraded more malic acid at the higher (37°C) fermentation temperatures (Table 4.5).

As expected, results from industrial fermentations (Table 4.9) show that strain EC1118 is degrades little malic acid compared to strains 71B and D432. Significantly smaller amounts of malic acid degradation were shown in results from the statistical analysis of laboratory grape juice fermentations (Table 4.2).

Therefore, strain EC1118 does not degrade malic acid to the same extent as strains 71B and D432. However, EC1118 was used in this research for reference purposes only and manufacturers of EC1118 do not imply that this strain has high maloethanolic activity. Strain EC1118 is widely used due to proven and reliable performance even when mishandled in wineries. In addition, strain EC1118 rapidly initiates fermentation and produces wines of good organoleptic properties. Furthermore, many wineries use Malolactic Fermentation to deacidify wines, in which case the yeast strain conducting alcoholic fermentation is used primarily for alcohol production and organoleptic characteristics.

The findings of this research however, promote the use of strains 71B and D432 over lower malic acid degrading strains such as EC1118 in high acid juices requiring deacidification.

5.3.2 Strain ACID-

Simulated Grape Juice and industrial fermentations with ACID- were conducted. Relatively little malic acid degradation occured by this yeast strain in these fermentations (Tables 4.3, 4.4, 4.9). In addition, acid taste tests of the wines made in the industrial fermentations (Tables 4.10, 4.11) did not distinguish wines produced by ACID- from any other wines fermented by other yeast strains. The judges also commented on the undesirable organoleptic properties of the wines. The manufacturer of this strain however, claim that this strain of ACID- was selected in preference over other strains which produced poor taste. The manufacturer also claims rapid fermentation by this strain (5-6 days at 30^{0}C) which was not observed in this research. Generally, the length of ACID- fermentations were similar to other strains.

Because little malic acid degradation was observed in Simulated Grape Juice and industrial fermentations, ACID- was omitted from the final stages of research and effort was focused on strains with higher maloethanolic activity

It is proposed that ACID- may be beneficial in blending situations (to counter poor organoleptic properties) and in wines requiring MLF (due to poor amounts of malic acid being degraded).

5.3.3 Strain 71B

On the basis of results from this research it was observed that high amounts of malic acid (up to 50%) were degraded by strain 71B within the range of 4.4-7.5 g/L initial malate (Tables 4.3, 4.4, 4.6, 4.9). The greatest percentage malic acid degradation occured when the initial malic acid level was 7.5 g/L (Table 4.6). However, no degradation was observed at 2.5 g/L initial malate. In addition, the initial nitrogen level of the juice does not effect the degradative capabilities of this strain (Table 4.7). Also, optimum malic acid

degradation occured at pH 3.5 with fermentations at lower initial pH levels becoming stuck (Table 4.8).

The manufacturer of 71B claims that between 25-30% of the initial malic acid can be degraded by strain 71B. As observed in industrial fermentations however, up to 50% degradation can occur (Table 4.9). Strain 71B degraded more malic acid than any other strain in the industrial fermentations, even when the pH of the juice was as low as pH 3.1. However, in Simulated Grape Juice fermentations strain 71B did not degrade malic acid at pH 3.0 (Table 4.8). Therefore, strain 71B is effective at degrading malic acid at almost all initial malate concentrations and almost every initial pH level typical of a New Zealand grape juice. In addition, it was observed by statistical analysis of laboratory grape juice fermentations (Table 4.2) that strain 71B significantly degrades greater amounts of malic acid than strains EC1118, M and ACID-.

This research has shown strain 71B to better than strains EC1118 and ACID- in the amount of malic acid degraded. It is assumed that this strain is not of wider usage, due to manufacturers claims of ester and (higher) alcohol formation giving fruity aromas to the wine, which for many New Zealand wines would be uncharacteristic, hence undesirable. Further research into organoleptic properties of wines produced by strain 71B may help to qualify this claim, although no undesirable aromas were reported by judges at acid ranking taste tests.

5.3.4 Strain D432

It was found from this research that strain D432 degraded malic acid within the range of 2.5-7.5 g/L initial malate (Tables 4.3, 4.4, 4.6, 4.9), with no observed optimum (consistent degradation at all levels). Higher fermentation temperatures favoured malic acid degradation (Table 4.5). However, industrial Sauvignon Blanc juice fermentations (Table 4.9) conducted at 13°C degraded approximately twice the amount of malic acid in comparison with levels degraded at 18°C and 25°C. The initial nitrogen concentration of

the juice did not influence the amount of degradation (Table 4.7) but pH 3.5 favoured acid reduction although high levels of malic acid were also degraded at pH 4.0.

After strain 71B, industrial fermentations show that D432 degraded the highest levels of malic acid (Table 4.9). Due to the poor viability of this strain an activation step is required. The activation step involves a 2-3 day growth period of strain D432 in 10% of the juice. After this time, yeast viability is approximately the same as other yeasts requiring pied de cuvee inoculation (see section 2.2.1).

Tortia *et al* (1993) trialed strain D432 in 1964, 1966, 1987 and 1988 vintages. The authors observed greater than 50% degradation by this strain in 1964 and 1966 vintages, but not in the other years. They postulate that the high initial sugar concentration of the juices may have produced this effect. In the 1987 and 1988 vintages, strain D432 consistently degraded more than twice the amount than spontaneous controls. In addition, the authors claim that differences in taste could be detected between wines made from strain D432 and the spontaneous control. These taste differences could not be detected after MLF.

This research has shown D432 to be more effective at degrading malic acid than strains EC1118, ACID- and M. Statistical analysis of laboratory grape juice fermentations have confirmed this (Table 4.2). Further research to ratify organoleptic properties produced in wines by strain D432 would be beneficial to the winemaker. However, Gianni (personal communication) claims that advantages of using strain D432 include: rapid fermentation which gives low amounts of hydrogen sulphide and sulphur dioxide; selected in areas of Italy over other yeast strains due to the production of high quality ranked wines (comments such as typical, varietal and clean described these wines); and wines undergo MLF easily.

Through this research the potential for strain D432 in industry has been established.

5.3.5 Strain M

Strain M was used as a reference strain in this research. Simulated Grape Juice and laboratory grape juice fermentations with strain M were conducted. It was omitted from further Simulated Grape Juice and industrial fermentations due to space availability and the assumption that strains 71B, D432 and ACID- were more worthwhile to investigate. This assumption was based on little malic acid being degraded in Simulated Grape Juice and laboratory grape juice fermentations (Tables 4.2, 4.3, 4.4). It is noted however, that the manufacturer of strain M does not claim high maloethanolic activity.

5.3.6 Strain 442

Strain 442 is not a wine yeast: it is a *Schizosaccharomyces* strain with high malic acid degrading capabilities (Rodriguez and Thornton, 1990). High amounts of malic acid degradation were observed in results of Simulated Grape Juice fermentations (Table 4.4). Strain 442 also produced the greatest reduction in titratable acidity and conducted an extremely rapid fermentation in these trials. Satisfactory pH increase was observed but comparatively little alcohol was formed.

Further study with strain 442 was not undertaken as *Schizosaccharomyces* are still not a viable option for use in industry (Auriol *et al*, 1990). This is due to off flavours and aromas being produced by this genera (Kunkee and Bisson, 1993). In addition, large amounts of hydrogen sulphide are formed in wines produced by this strain (van Rooyen and Tracey, 1987).

Continued genetic research with strain 442 and other *Schizosaccharomyces* strains (see section 2.5) may prove beneficial in future to the wine industry.

5.3.7 Acid Ranking Taste Tests

Blind tastings of wines produced from industrial grape juice fermentations with strains EC1118, ACID-, 71B and D432 were conducted. The objective of these trials was to determine if any judge could distinguish by taste, any differences in acid in wines produced by these yeast strains.

Strain D432 was significantly different in acid taste than wines produced by strains EC1118, ACID- and 71B in the Chardonnay juice fermentations (Table 4.10). Strain 71B was shown to be significantly different in acid taste than wines produced by strains EC1118, ACID- and D432 in the Sauvignon Blanc juice fermentations (Table 4.11). These findings reinforce the potential benefits of strains 71B and D432 in industry. They also meet the objectives of this research.

However, although strain 71B caused the greatest chemical degradation in the Chardonnay juice fermentations, taste tests indicated that this reduction in acidity could not be detected organoleptically. Possible reasons for this effect may be the interactions between strain 71B and the high acid Chardonnay juice producing flavours and aromas which masked the effect of deacidification. This anomaly highlights the need for further organoleptic research to be conducted on wines produced by strains 71B and D432.

5.3.8 Karyotype Analysis

The objective of this analysis was to confirm that the yeast strains inoculated into the commercial Sauvignon Blanc and Chardonnay juices were dominant at the most vigorous stage of fermentation. Using CHEF electrophoresis (refer to section 2.6), the

chromosomal banding patterns of the isolates were examined and compared to reference strains to prove homogeny.

From analysis of the karyotypes, all strains inoculated into Sauvignon Blanc juice were dominant at the most vigorous stage of fermentation Figs. 4.21 to 4.25).

In the Chardonnay fermentations (Figs. 4.21 to 4.26), all inoculated strains were dominant, but problems existed with the analysis of strains 71B and D432. It was concluded that strains 71B and EC1118 were both present in the fermentations initially inoculated with strain 71B. The levels of malic acid degradation observed in these fermentations, however, suggest that strain 71B played a dominant role. Such high levels of malic acid degradation would not have been observed if strain EC1118 was dominant. In addition, it was concluded that strain D432 dominated the Chardonnay fermentations in which it was inoculated, although this cannot be proven.

Domination by strains 71B and D432 in both types of juice serve to reinforce the potential benefits of these strains in industry. In addition, karyotypic analysis supports the theory that the yeast strain inoculated into the juice will almost certainly conduct the fermentation. Research by Schutz and Gafner (1993) supports this theory.

The major flaw in karyotype analysis performed in this research, was the small number of isolates karyotyped. A greater number of isolates would have given greater statistical validity to results. Time factors and limited equipment availability did not allow for this.

Vaughan-Martini *et al* (1993) claim that further problems with karyotype analysis exist. These problems include; identical electrophoretic karyotypes when DNA sequence homology between two strains is over 85%, and when similar, but not identical, karyotypes are examined differences in the chromosomal banding pattern are difficult to

distinguish. As the strains used in this research are so closely related, reliability of this test is put into question.

Karyotypic analysis is not yet a viable tool for use in most wineries. Many New Zealand wineries are small, and the cost of the equipment, trained personnel and the limited (seasonal) use of this equipment does not justify its use. In addition, the methodology is labour intensive and time consuming. A faster, more reliable method of identification that can separate strains would be desirable. For research purposes however, karyotype analysis is generally a more accurate method of identification when compared to traditional methods of identification.

6.0 GENERAL CONCLUSIONS

New Zealand's climate generally induces the production of grapes high in acid. There is a need to reduce this acidity through the vinification process. At present, MLF is widely used as a biological deacidifying tool in New Zealand wineries. The widespread use of MLF may be through lack of a better option, but may also be because many winemakers favour the flavour characteristics produced through the MLF pathway. Although the actual pathway only reduces 0.1-0.3% of the acidity, the flavour characteristics play a key sensory role which appears to reduce perceived acid levels in the wine.

However, degradation of malic acid by maloethanolic fermentation has been shown by this research to be a viable and beneficial option for use in industry.

Preliminary trials with maloethanolic yeast strains have indicated that;

1. The use of Simulated Grape Juice in the investigation of yeast activity does not accurately mimic a grape juice, but has a number of benefits including rapid and direct trial assessment. In addition, the composition of Simulated Grape Juice is constant, but individual components can be varied for study.

2. Strains D432, 442 and 71B degrade high levels of malic acid, producing wines of lower acidity.

3. The amount of malic acid degraded depended mainly upon the yeast strain. Other contributing factors included initial malic acid and nitrogen concentration, fermentation temperature, and initial pH.

4. The greatest percentage of malic acid was degraded by strains 71B and D432 when the initial malic acid concentration was high (7.5 g/L). However, the initial amount of malic acid did not influence the percent reduction by strain EC1118.

5. The greatest amount of malic acid degradation occured at pH 3.5 for all strains although strain D432 also degrades high amounts of malic acid at pH 4.0.

6. Low levels of nitrogen (463mg N/L including proline) favoured greatest malic acid degrdation by strains EC1118, D432 and 71B.

7. In Simulated Grape Juice, laboratory grape juice and industrial fermentations, strains 71B and D432 were the most efficient strains to degrade malic acid. Up to 50% of the initial malate in a juice can be degraded by these strains.

8. A lower acid taste can be perceived in wines made from strains 71B and D432 compared with ACID- and the reference strain EC1118.

9. Industrial yeast strains currently used in New Zealand (eg. EC1118) degrade little malic acid in comparison with strains 71B and D432.

10. MEF using strains 71B and D432 has enormous potential benefit to the New Zealand Wine Industry, especially in high acid juices requiring deacidification.

Further research is required regarding the organoleptic properties strains 71B and D432 impart on New Zealand juices and wines. Also, future trials with all strains may involve the investigation of more variables, such as initial sugar concentration, presence of thiamine and oxygen level. It would also be interesting to observe the effect(s) of MLF on wines produced by these strains.

Future use of MEF using yeast strains 71B and D432 to replace or use with MLF has potential in the New Zealand wine industry. Observed benefits would include savings in time, labour and expense. In addition, a relatively easier induction and controlled fermentation would take place compared to methods now in use.

APPENDIX A

Table A1 Analyses of replicate wines from Laboratory Grape Juice fermentations inoculated with yeast strain EC1118.

TEST	EC (A)	EC (B)	EC (C)	COMBINED TOTAL
M.A.(g/L)	5.69± 0.078 (2)	5.75± 0.014 (2)	5.72± 0.057 (2)	5.72± 0.027 (6)
T.A.(g/L)	11.05± 0.043 (3)	10.90± 0.087 (3)	10.88± 0.075 (3)	10.94± 0.077 (9)
pH	3.16 (2)	3.15 (2)	3.15 (2)	3.15± 0.004 (6)
Alcohol(v/v)	9.89± 0.042 (2)	9.79± 0.191 (2)	10.10 (2)	9.93± 0.131 (6)
Brix	-0.79 (2)	-0.69 (2)	-0.69 (2)	-0.72± 0.047 (6)

Table A2 Analyses of replicate wines from Laboratory Grape Juice fermentations inoculated with strain ACID-.

TEST	AC (A)	AC (B)	AC (C)	COMBINED TOTAL
M.A.(g/L)	5.46± 0.085 (2)	5.65± 0.092 (2)	5.72± 0.156 (2)	5.61± 0.109 (6)
T.A.(g/L)	10.43± 0.075 (3)	10.55± 0.040 (3)	10.45± 0.111 (3)	10.47± 0.054 (9)
pH	3.13 (2)	3.11 (2)	3.11 (2)	3.12± 0.009 (6)
Alcohol(v/v)	9.51± 0.035 (2)	9.46± 0.106 (2)	9.58± 0.141 (2)	9.51± 0.051 (6)
Brix	-0.89 (2)	-0.89 (2)	-0.89 (2)	-0.89 (6)

Table A3 Analyses of replicate wines from Laboratory Grape Juice fermentations inoculated with strain 71B.

TEST	71B (A)	71B (B)	71B (C)	COMBINED TOTAL
M.A.(g/L)	4.38± 0.163 (2)	4.69± 0.021 (2)	4.47± 0.205 (2)	4.51± 0.130 (6)
T.A.(g/L)	8.98± 0.04 (3)	9.03± 0.046 (3)	9.05± 0.046 (3)	9.02± 0.032 (9)
pH	3.29 (2)	3.29 (2)	3.29 (2)	3.29 (6)
Alcohol(v/v)	9.18± 0.035 (2)	9.16± 0.057 (2)	9.18± 0.035 (2)	9.17± 0.007 (6)
Brix	-0.99 (2)	-0.99 (2)	-0.99 (2)	-0.99 (6)

Table A4 Analyses of replicate wines from Laboratory Grape Juice fermentations inoculated wuth strain D432.

TEST	D4 (A)	D4 (B)	D4 (C)	COMBINED TOTAL
M.A.(g/L)	4.69± 0.099 (2)	4.51± 0.028 (2)	4.69± 0.156 (2)	4.63± 0.084 (6)
T.A.(g/L)	9.50± 0.046 (3)	9.45 (3)	9.48± 0.046 (3)	9.48± 0.022 (9)
pH	3.20 (2)	3.20 (2)	3.19 (2)	3.20± 0.005 (6)
Alcohol(v/v)	9.29± 0.014 (2)	9.38 (2)	9.48 (2)	9.38± 0.077 (6)
Brix	-0.89 (2)	-1.09 (2)	-1.09 (2)	-1.02± 0.094 (6)

Table A5 Analyses of replicate wines from Laboratory Grape Juice fermentations inoculated with strain M.

TEST	M (A)	M (B)	M (C)	COMBINED TOTAL
M.A. (g/L)	5.25± 0.085 (2)	5.44± 0.007 (2)	5.33± 0.007 (2)	5.34± 0.075 (6)
T.A. (g/L)	10.65 (3)	10.63± 0.04 (3)	10.48± 0.040 (3)	10.58± 0.076 (9)
pH	3.13 (2)	3.13 (2)	3.14 (2)	3.13± 0.004 (6)
Alcohol(v/v)	9.53± 0.078 (2)	9.52± 0.148 (2)	9.40± 0.106 (2)	9.48± 0.061 (6)
Brix	-1.19 (2)	-1.09 (2)	-1.19 (2)	-1.16± 0.047 (6)

APPENDIX B

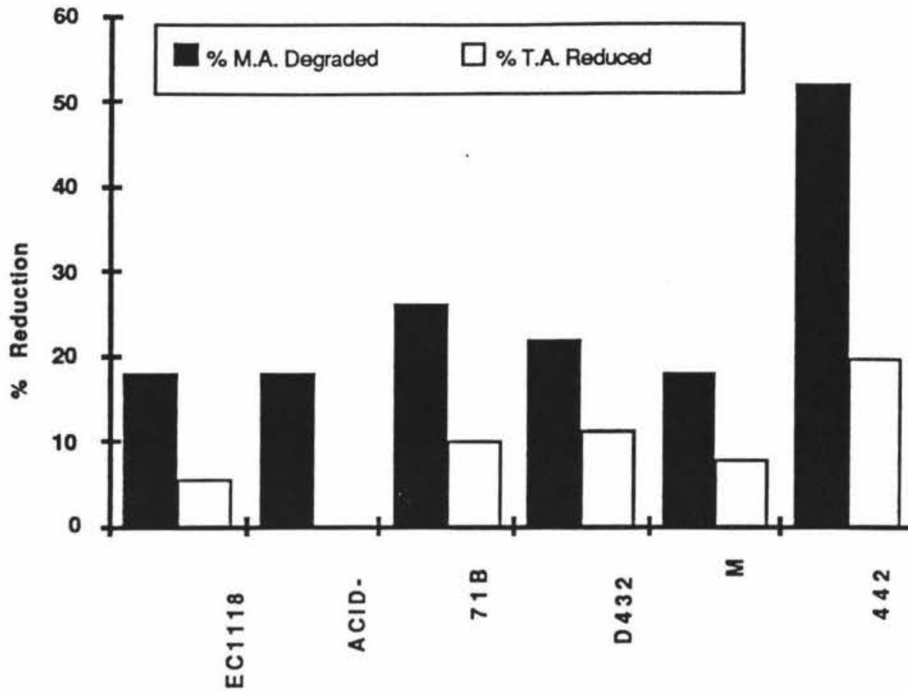


Fig.B1 Percent reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented (18°C) with different yeast strains.

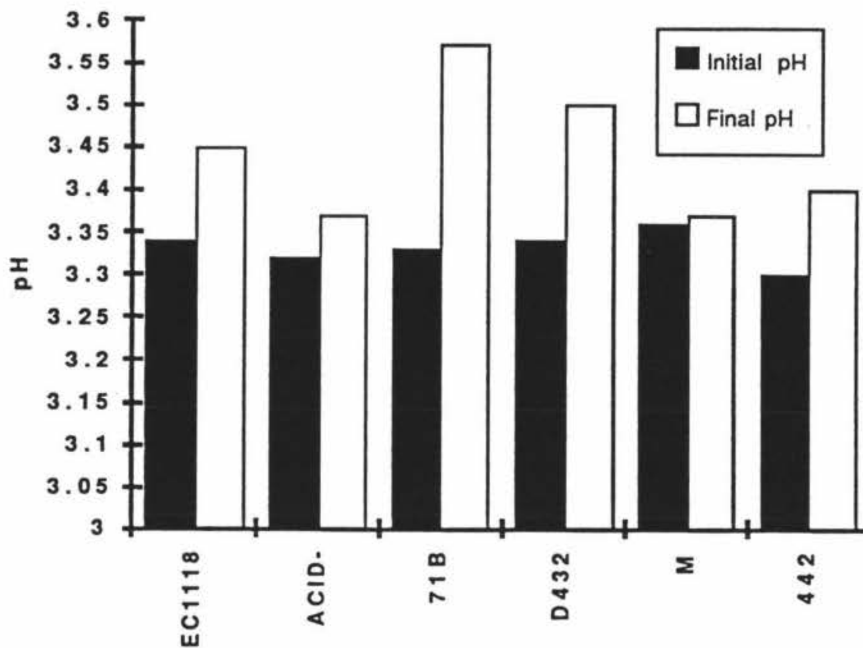


Fig.B2 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice inoculated with different yeast strains.

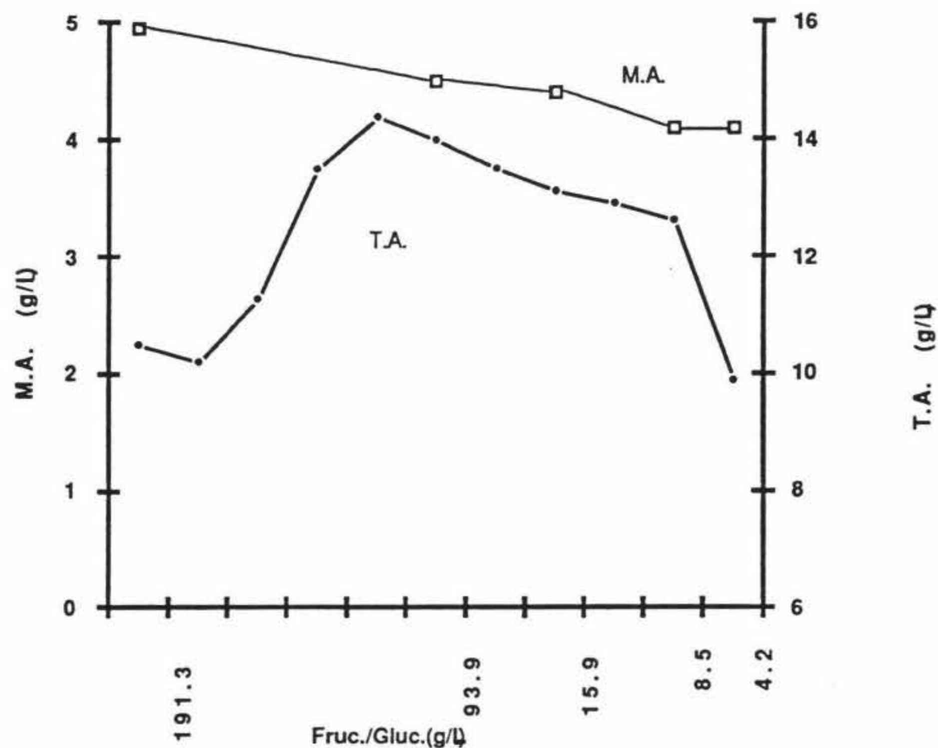


Fig.B3 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial cell count was 1.2 x 10^6 cells/ml.

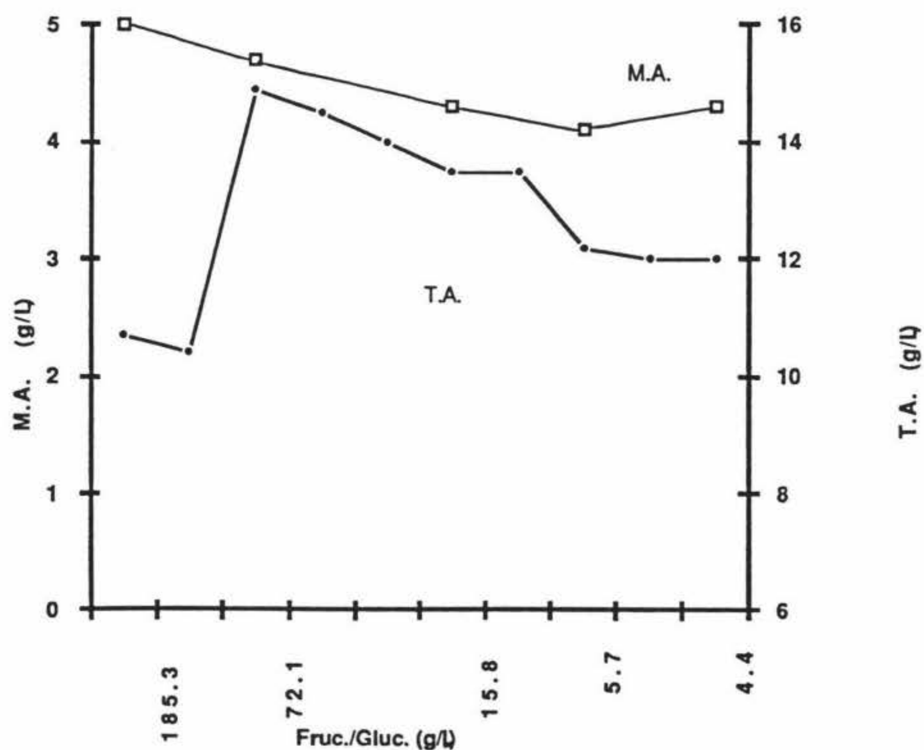


Fig.B4 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain ACID-. Initial cell count was 1.9 x 10^6 cells/ml.

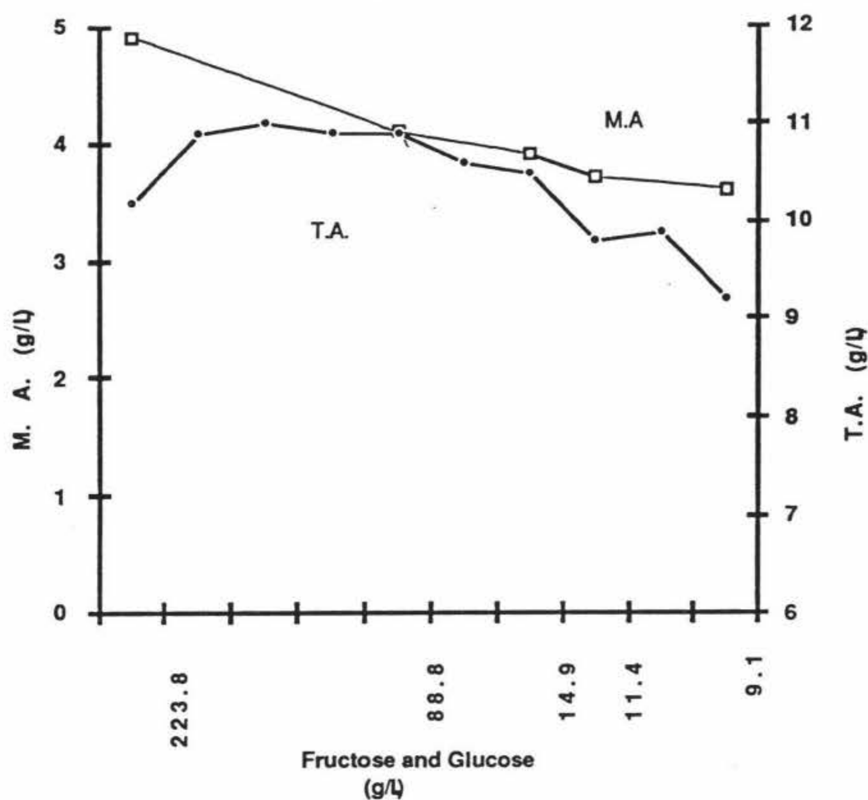


Fig.B5 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial cell count was 2.6 x 10^6 cells/ml.

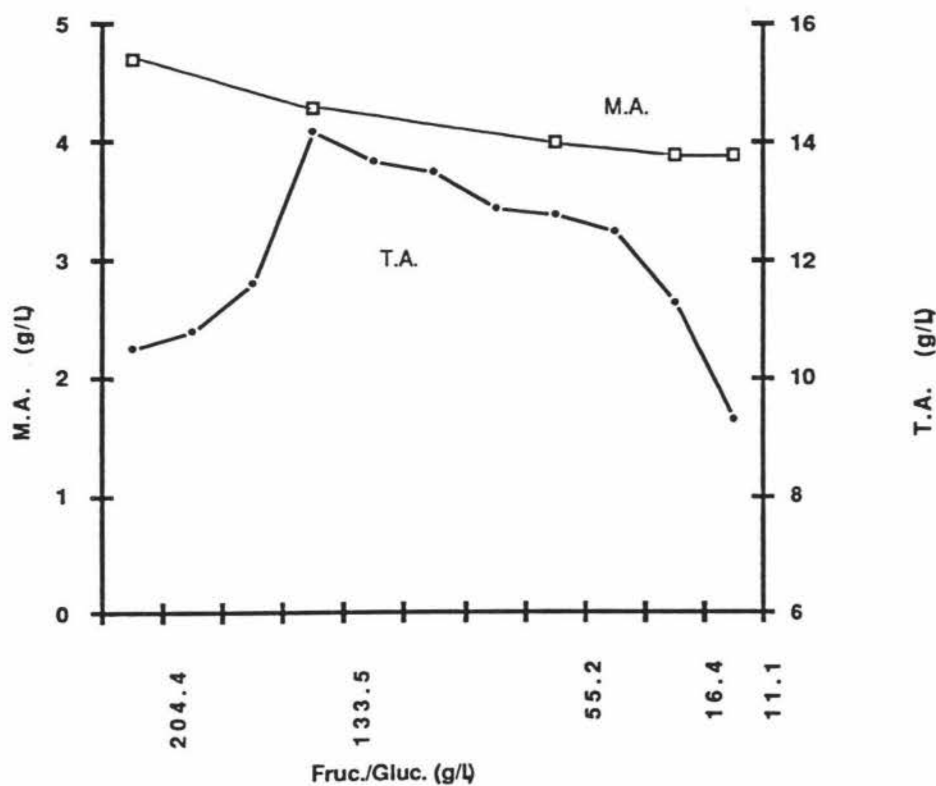


Fig.B6 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial cell count was 6.2 x 10^6 cells/ml.

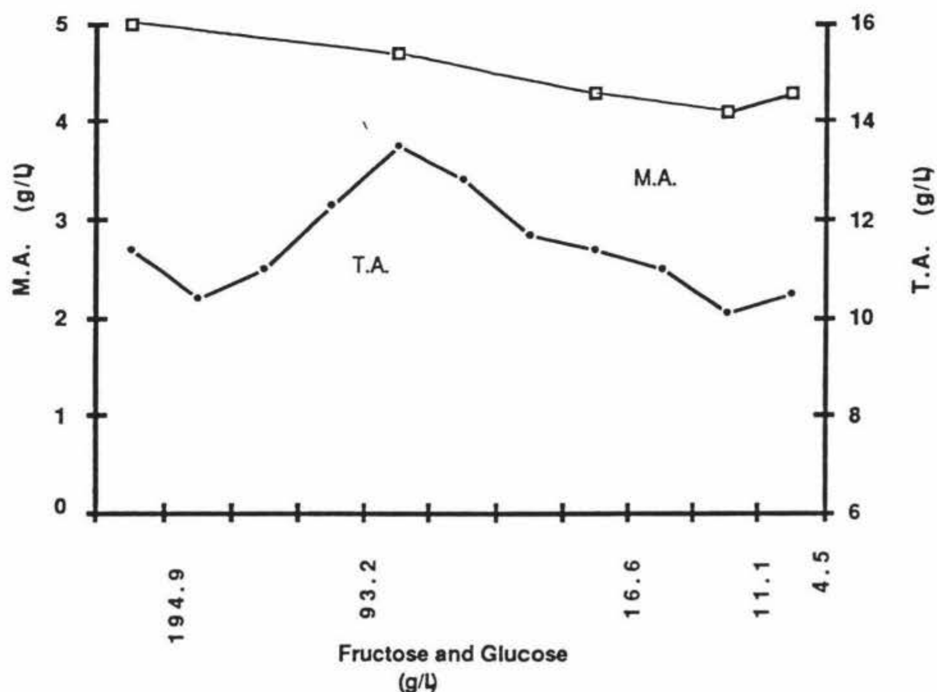


Fig.B7 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain M. Initial cell count was 2.8 x 10^6 cells/ml.

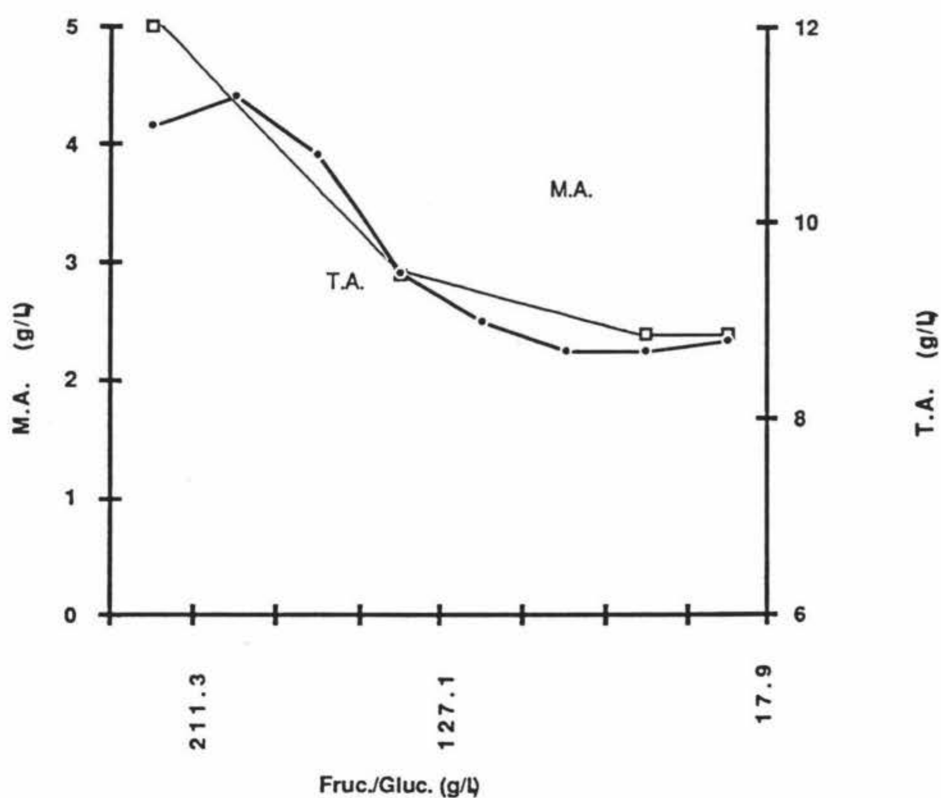


Fig.B8 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 442. Initial cell count was 5.0 x 10^5 cells/ml.

APPENDIX C

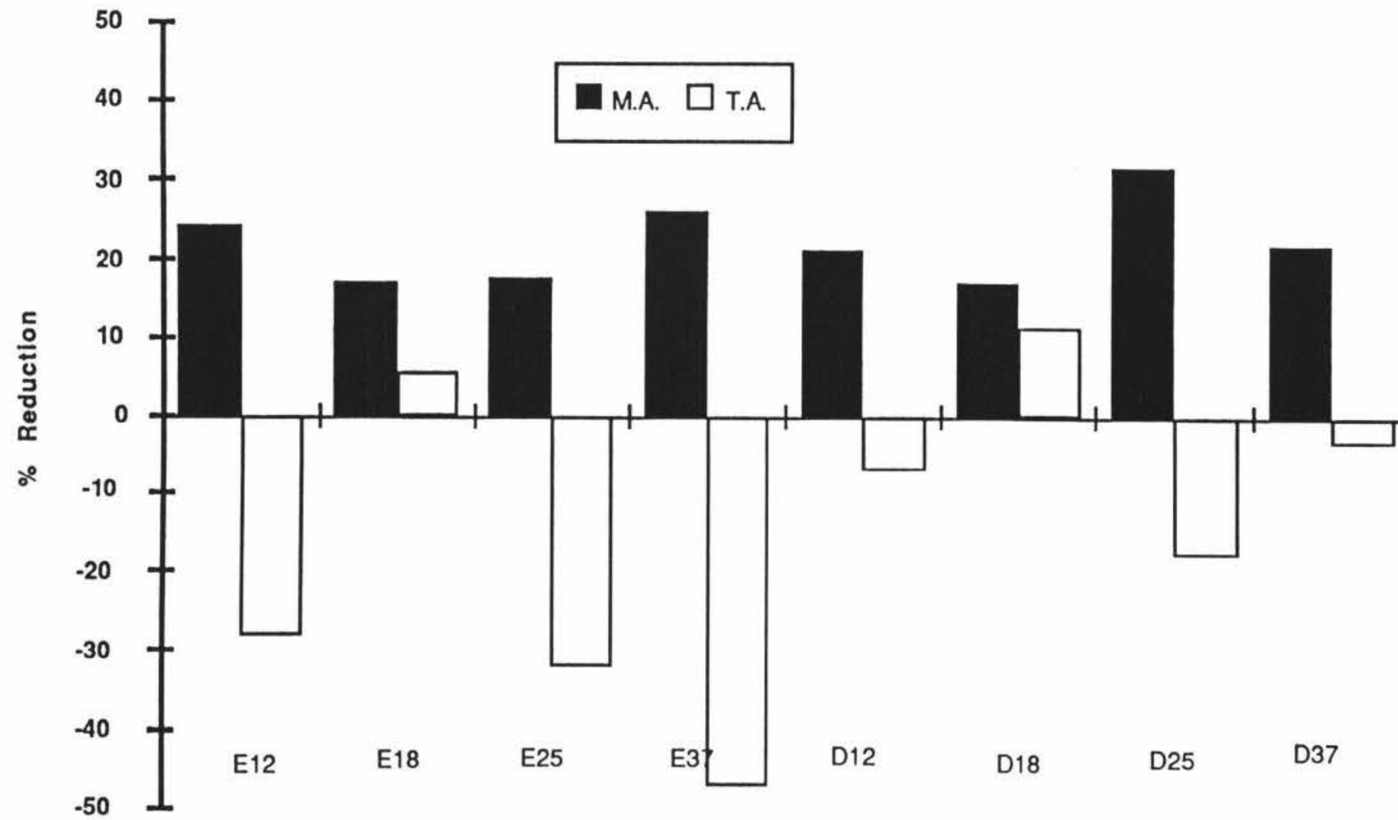


Fig.C1 Reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice fermented at different temperatures with yeast strains E (EC1118) and D (D432).

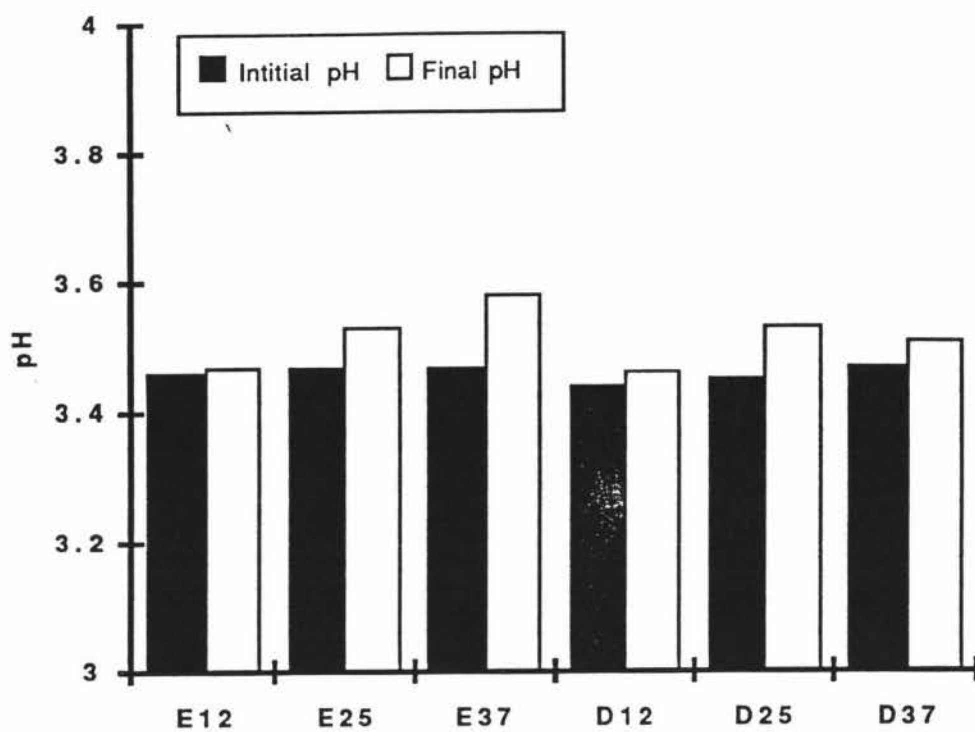


Fig.C2 Initial and final pH of maloethanolic wines (non uniform temperature trials).

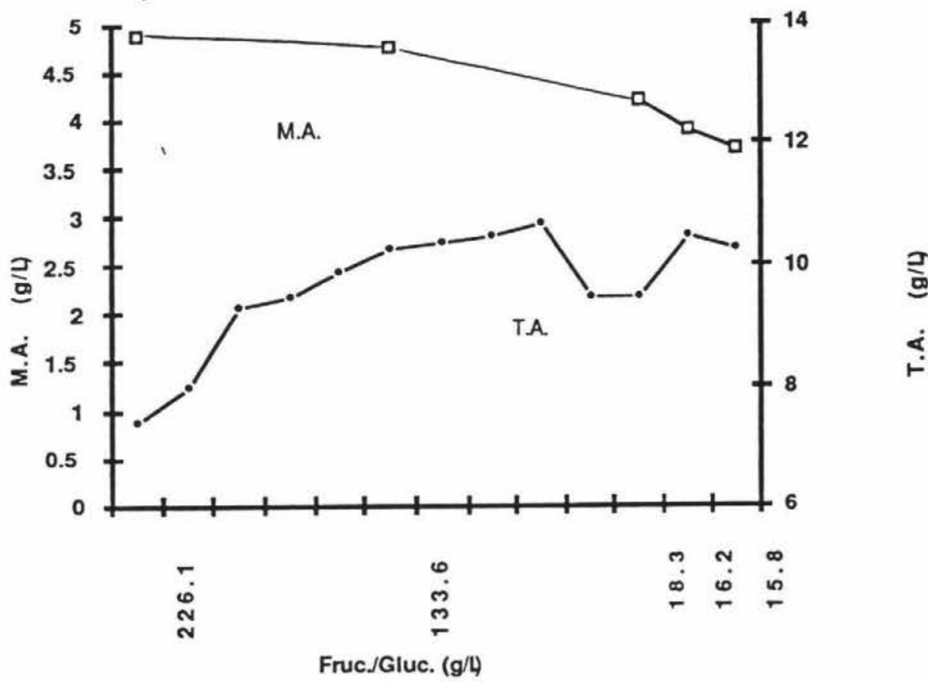


Fig.C3 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (12°C) of Simulated Grape Juice inoculated with strain EC1118. Initial cell count was 6.5 x 10^5 cells/ml.

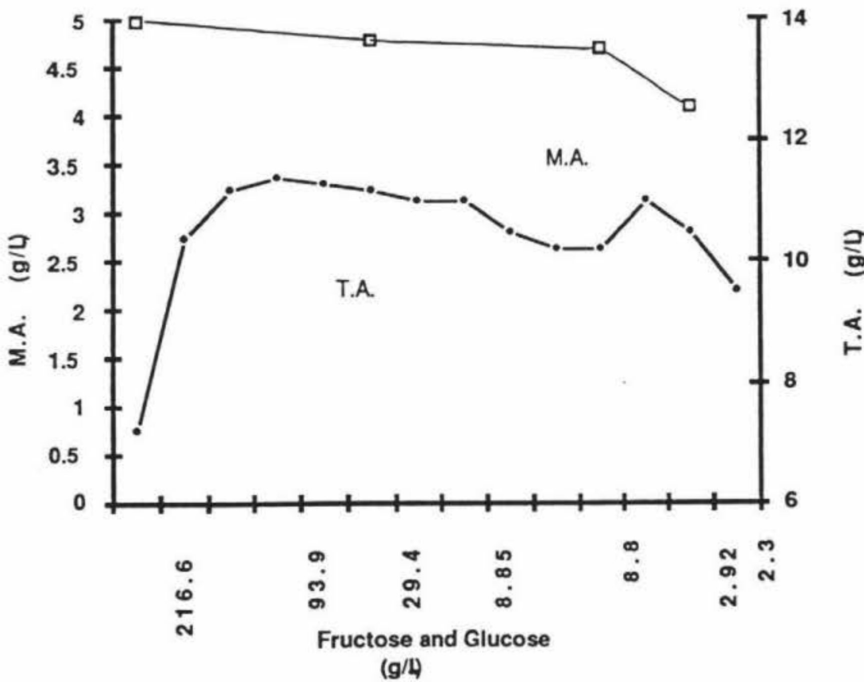


Fig.C4 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (25°C) of Simulated Grape Juice inoculated with strain EC1118. Initial cell count was 6.5 x 10^6 cells/ml.

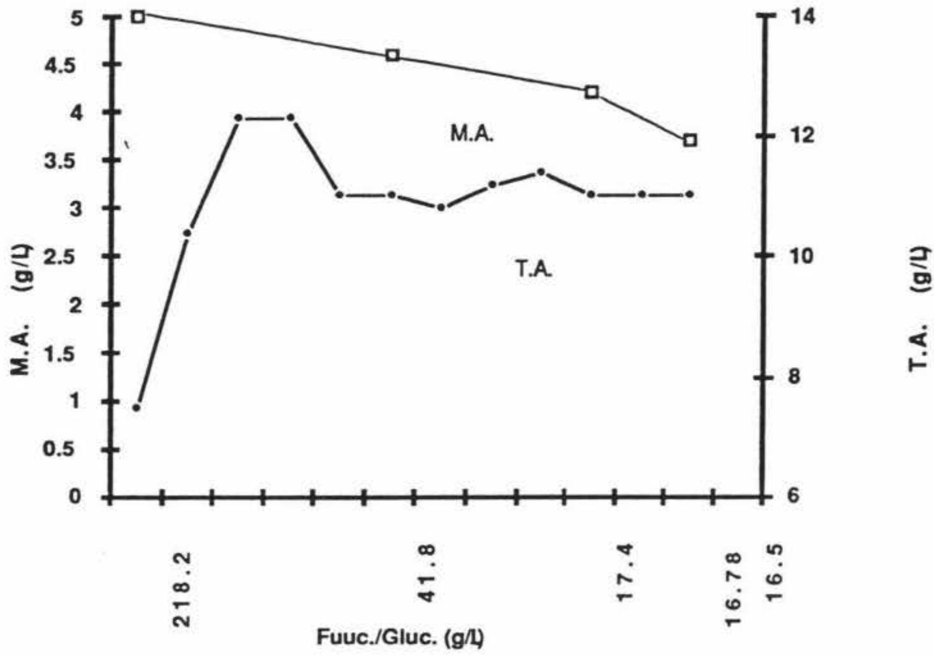


Fig.C5 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (37°C) of Simulated Grape Juice inoculated with strain EC1118. Initial cell count was 7.0×10^5 cells/ml.

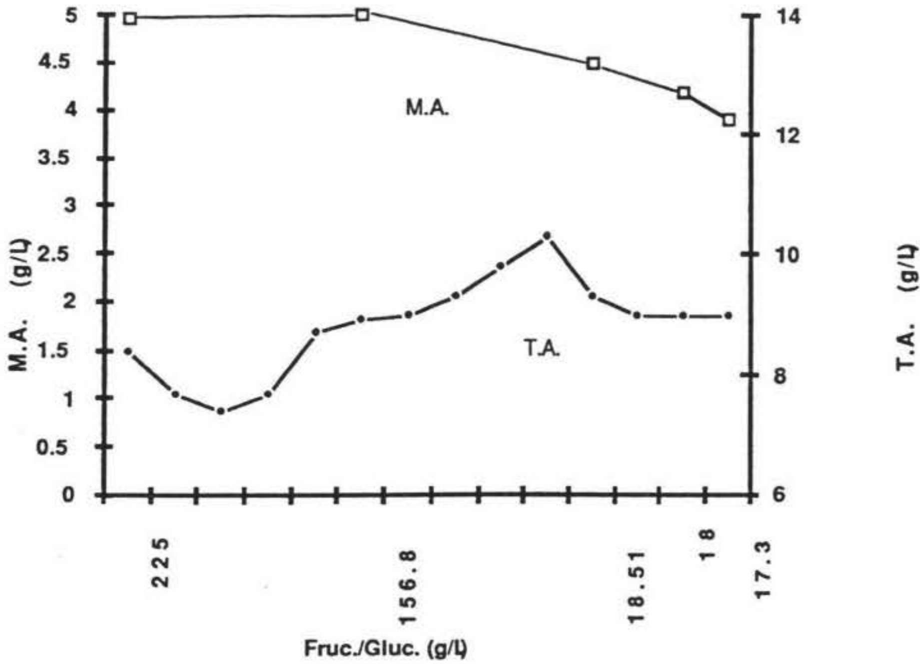


Fig.C6 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (12°C) of Simulated Grape Juice inoculated with strain D432. Initial cell count was 4.2×10^6 cells/ml.

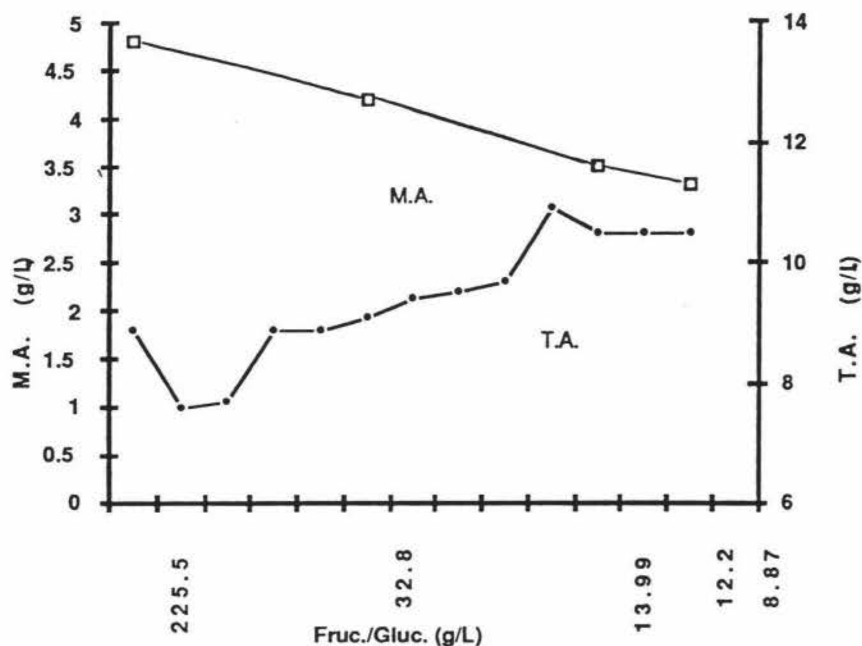


Fig.C7 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (25°C) of Simulated Grape Juice inoculated with strain D432. Initial cell count was 4.8 x 10^6 cells/ml.

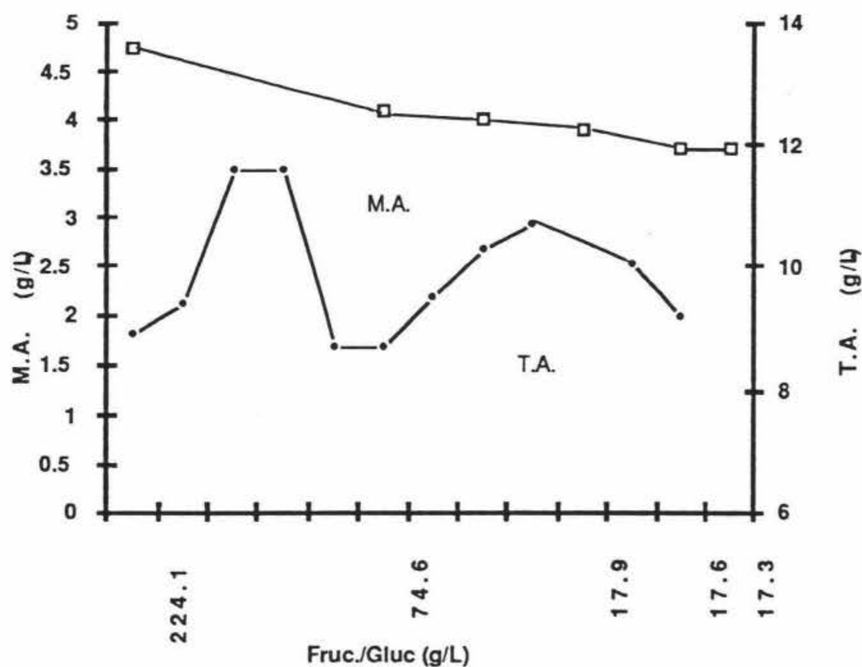


Fig.C8 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (37°C) of Simulated Grape Juice inoculated with strain D432. Initial cell count was 5 x 10^5 cells/ml.

APPENDIX D

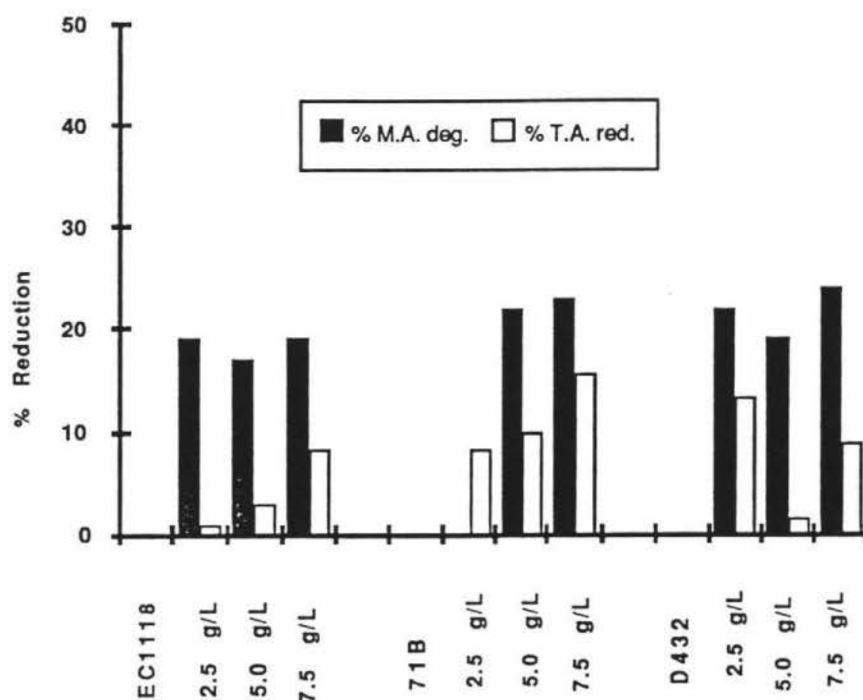


Fig.D1 Percentage reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice with varying initial concentrations of malic acid (g/l) fermented (18°C) with yeast strains EC1118, ACID-, 71B and D432.

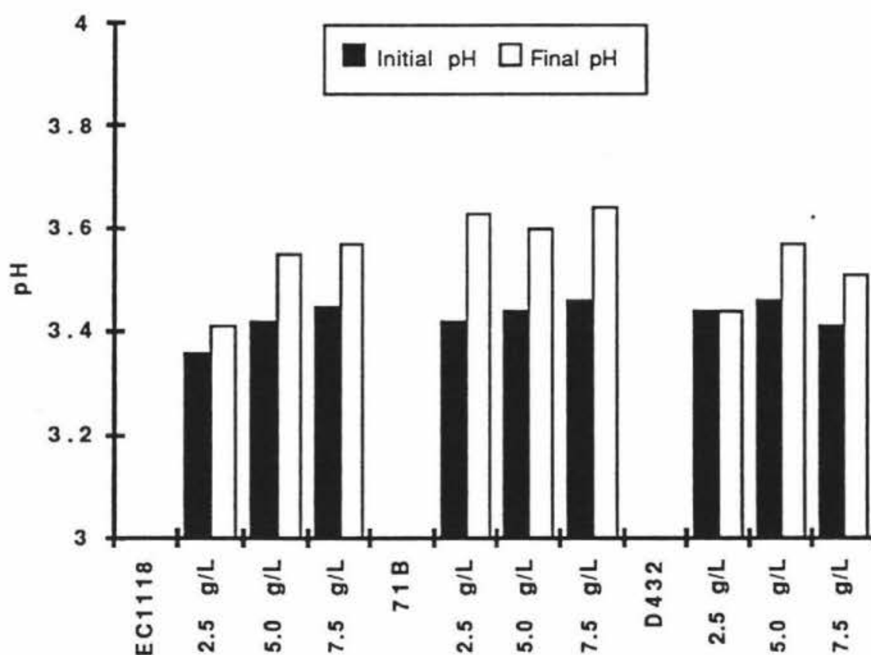


Fig.D2 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice wuth different initial concentrations of malic acid (g/L) inoculated wuth strains EC1118, 71B and D432.

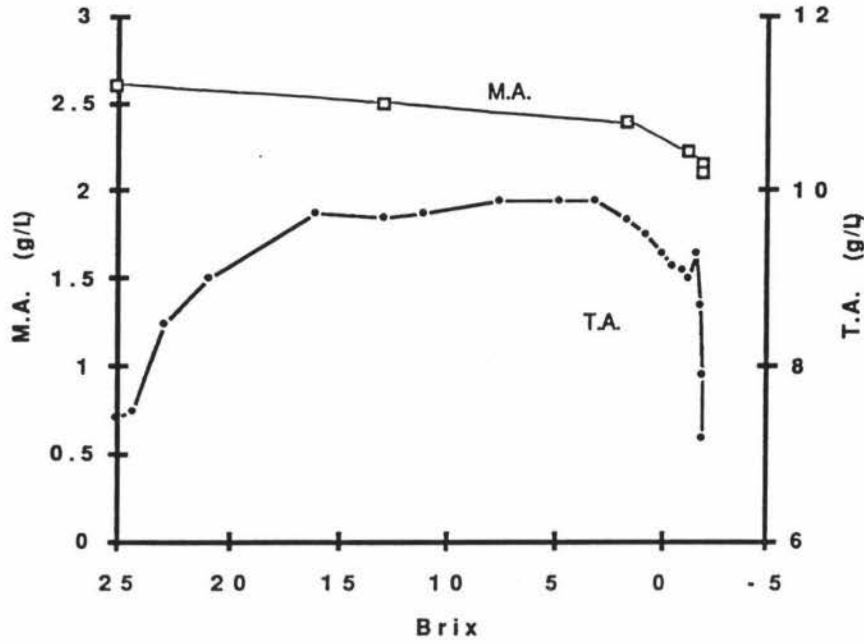


Fig.D3 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial malic acid level was 2.5g/L and initial cell count was 1.3 x 10^6 cells/ml.

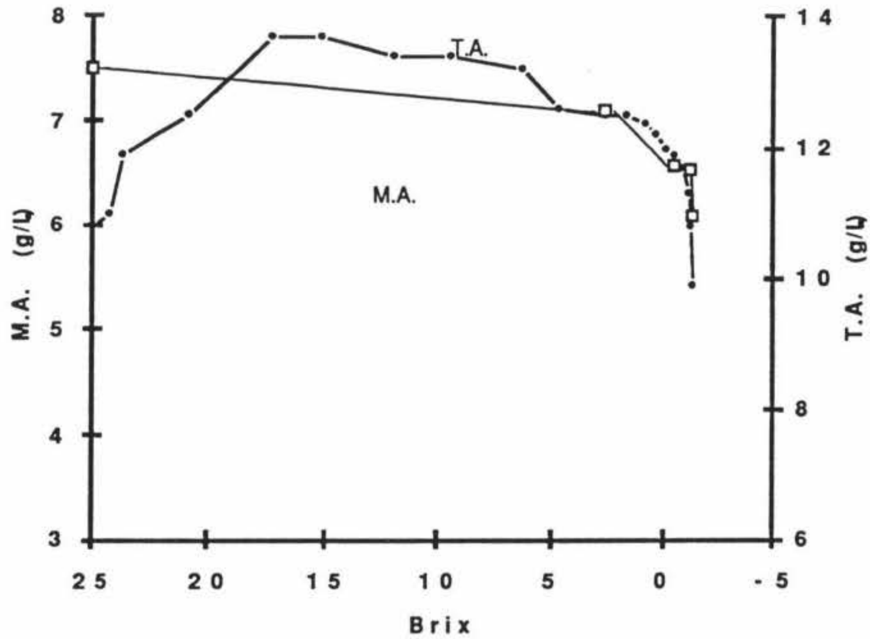


Fig.D4 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial malic acid level was 7.5g/L and initial cell count was 4.2 x 10^6 cells/ml.

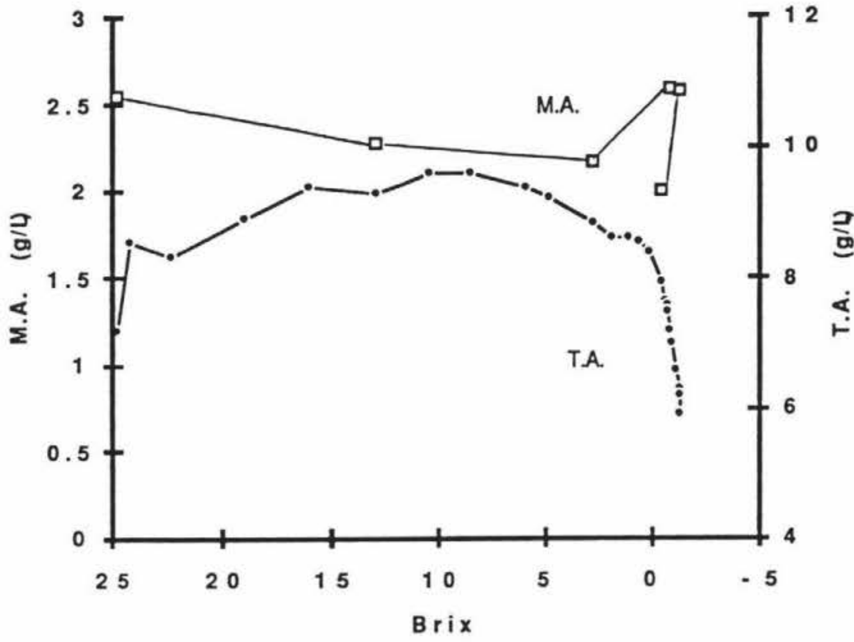


Fig.D5 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial malic acid level was 2.5g/L and initial cell count was 1.8 x 10^6 cells/ml.

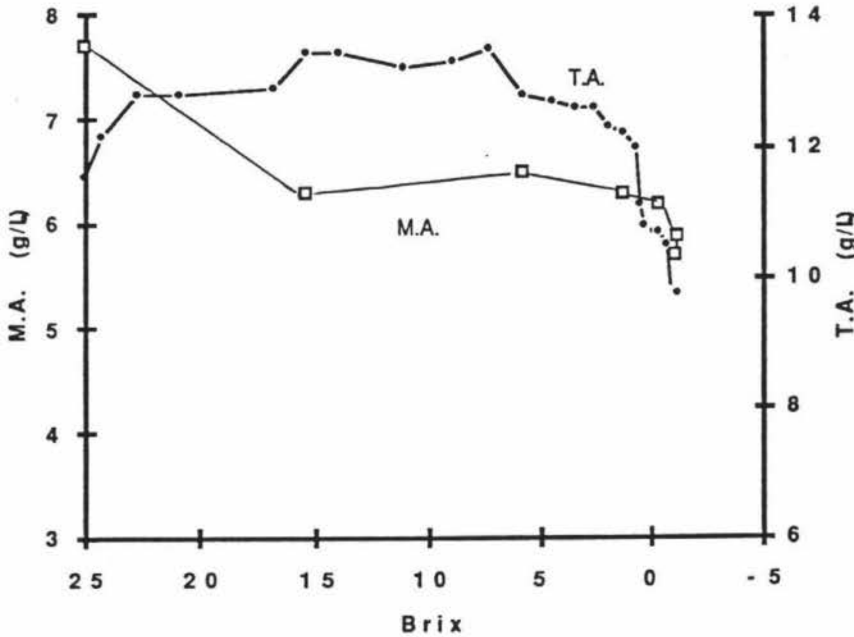


Fig.D6 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial malic acid level was 7.5g/L and initial cell count was 1.6 x 10^6 cells/ml.

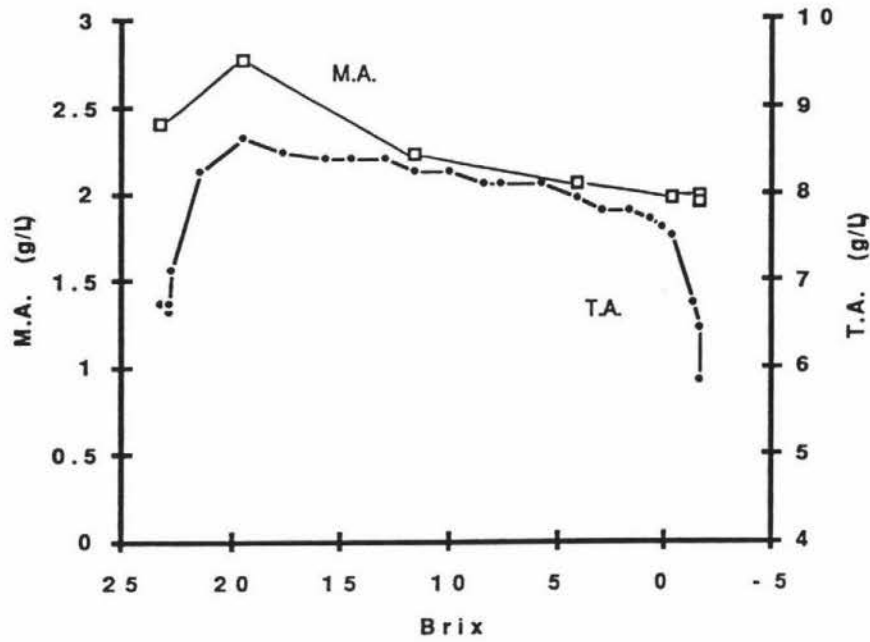


Fig.D7 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial malic acid level was 2.5g/L and initial cell count was 6.4 x 10^6 cells/ml.

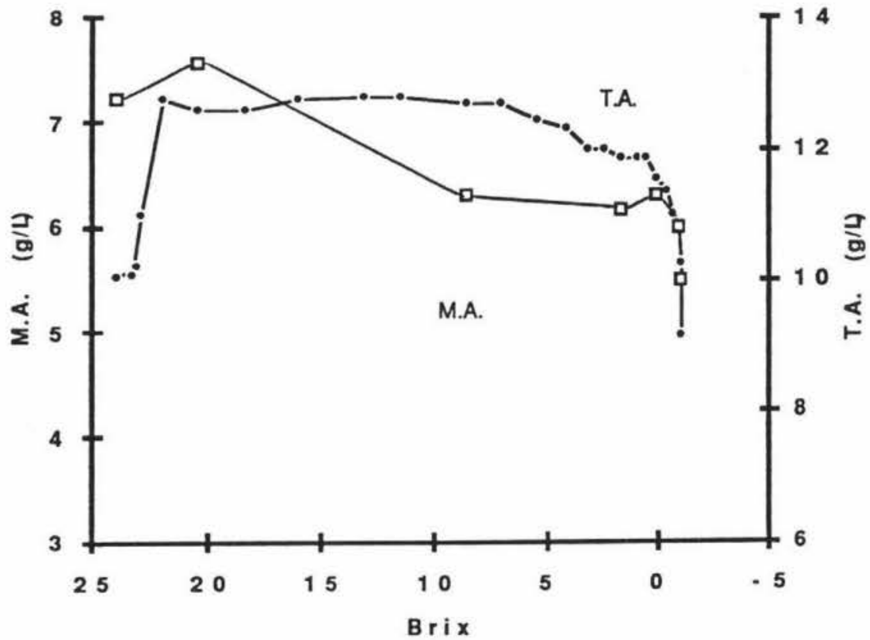


Fig.D8 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial malic acid level was 7.5g/L and initial cell count was 6 x 10^6 cells/ml.

APPENDIX E

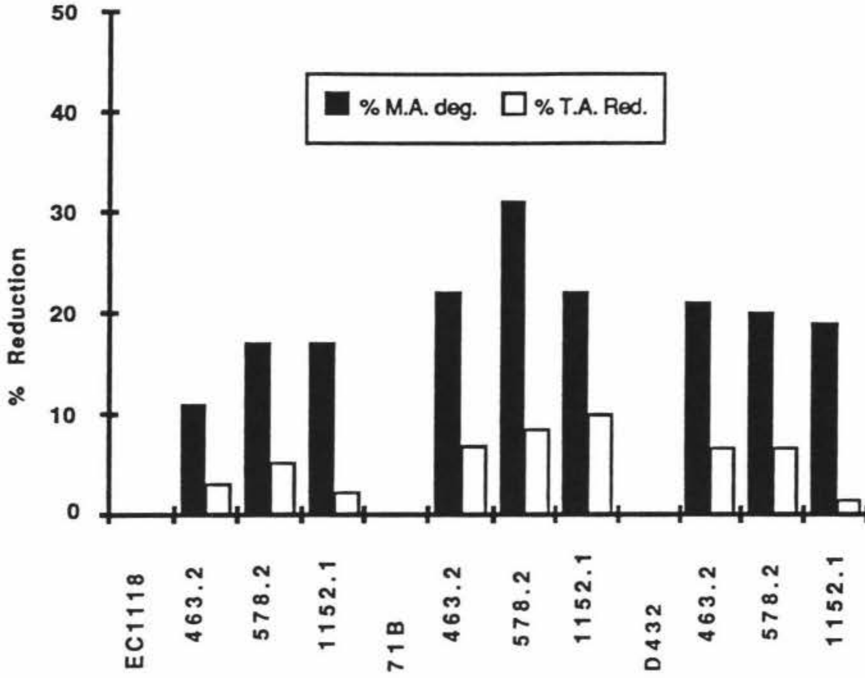


Fig.E1 Percent reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice with varying initial concentrations of nitrogen (mg/L) fermented (18°C) with yeast strains EC1118, 71B and D432.

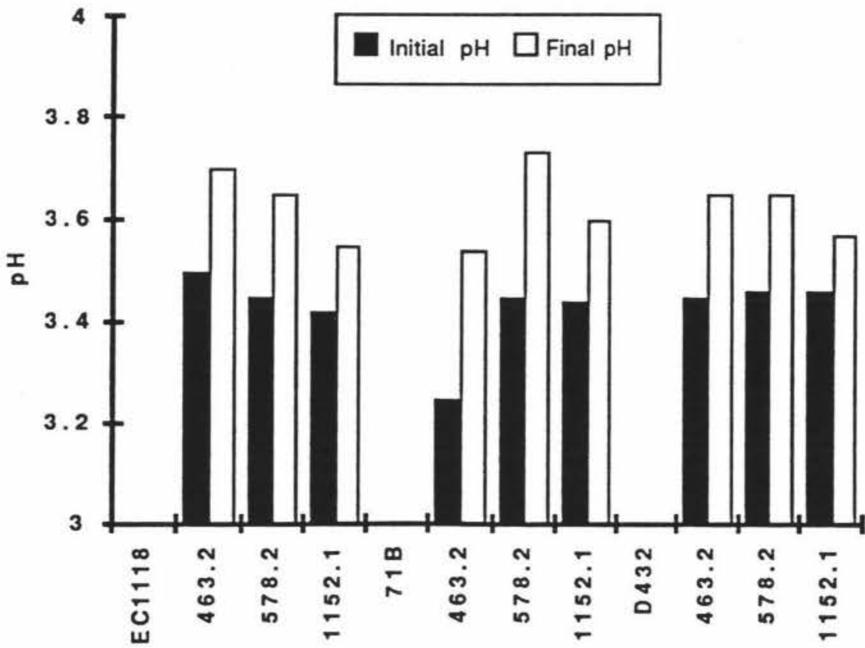


Fig.E2 Initial and final pH values of fermentations (18°C) of Simulated Grape Juice with different initial concentraions of initial nitrogen (mg/L) inoculated with yeast strains EC1118, 71B and D432.

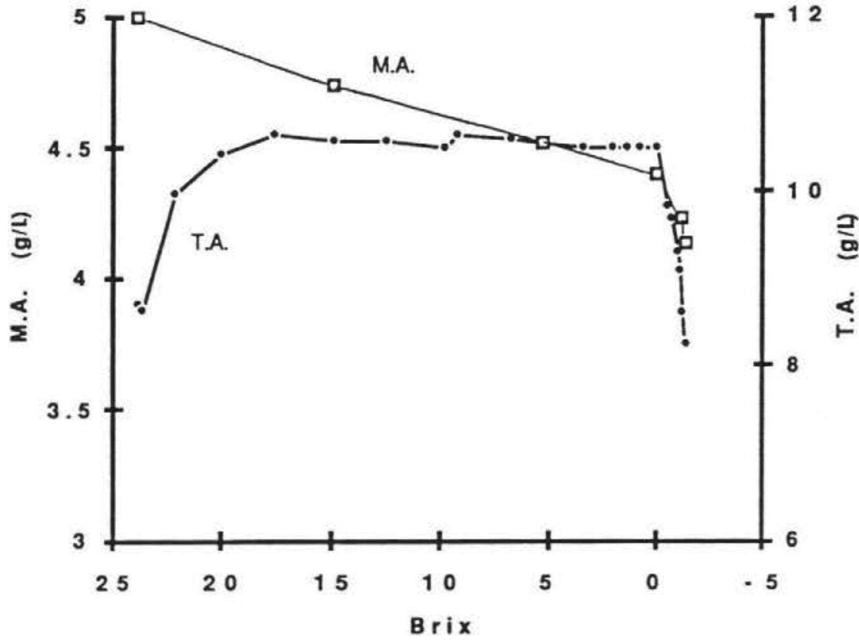


Fig.E3 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial nitrogen level was 463mg/L and initial cell count was 1.7 x 10^6 cells/ml.

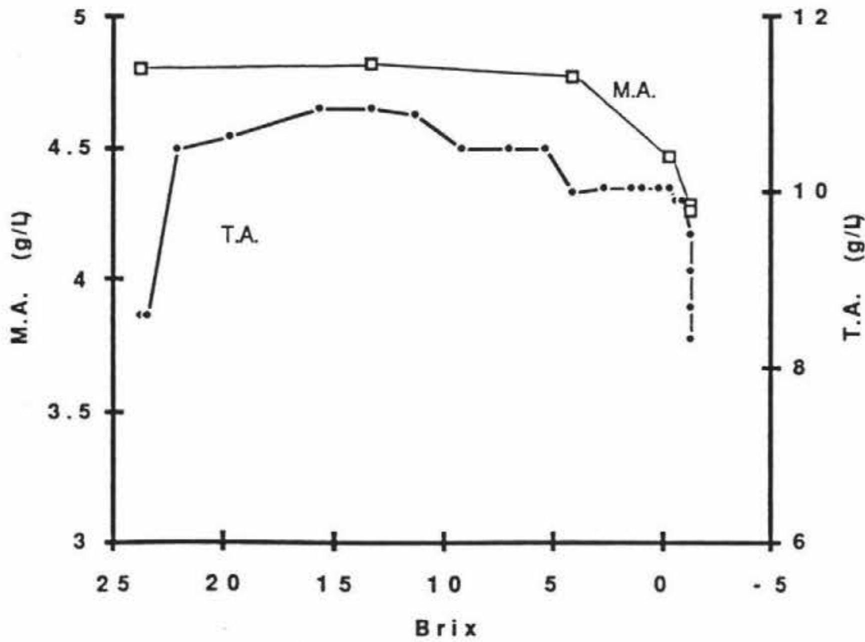


Fig.E4 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial nitrogen level was 578mg/L and initial cell count was 2.0 x 10^6 cells/ml.

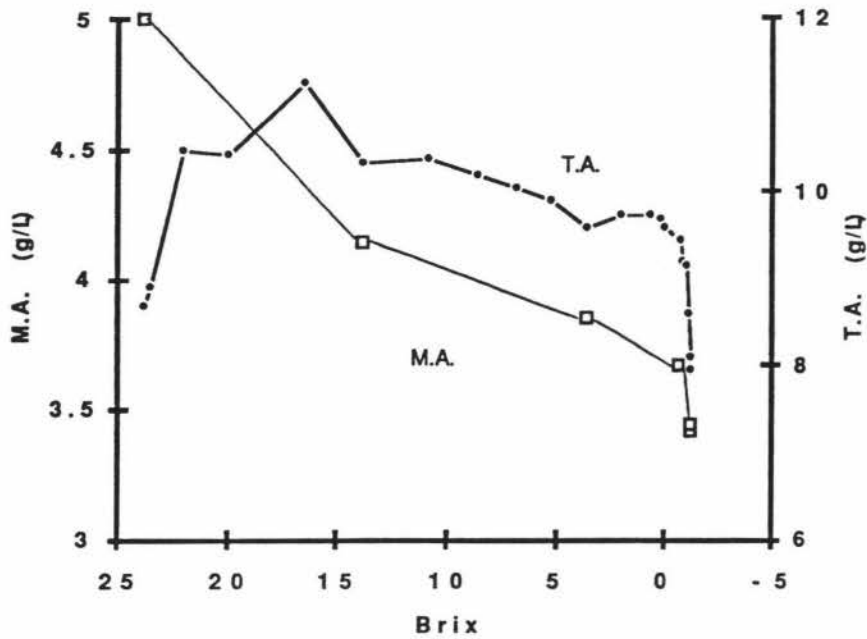


Fig.E5 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial nitrogen level was 463mg/L and initial cell count was 1.8 x 10^6 cells/ml.

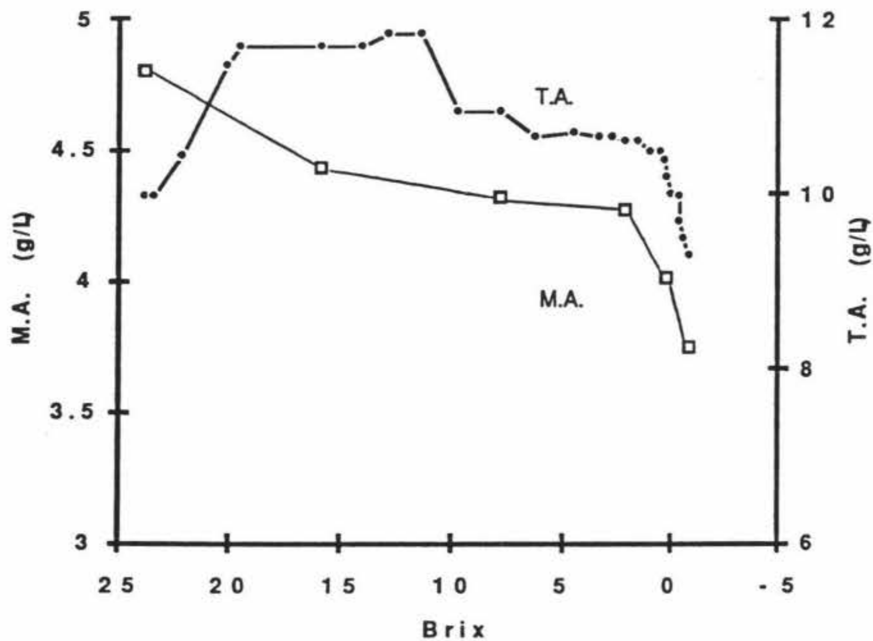


Fig.E6 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial nitrogen level was 578mg/L and initial cell count was 2.0 x 10^6 cells/ml.

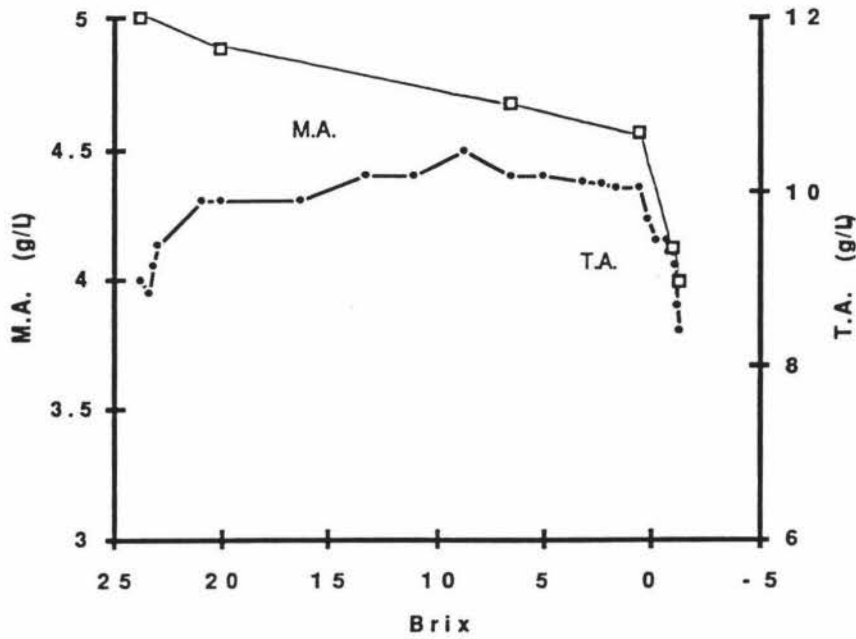


Fig.E7 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial nitrogen level was 463mg/L and initial cell count was 6.1 x 10^6 cells/ml.

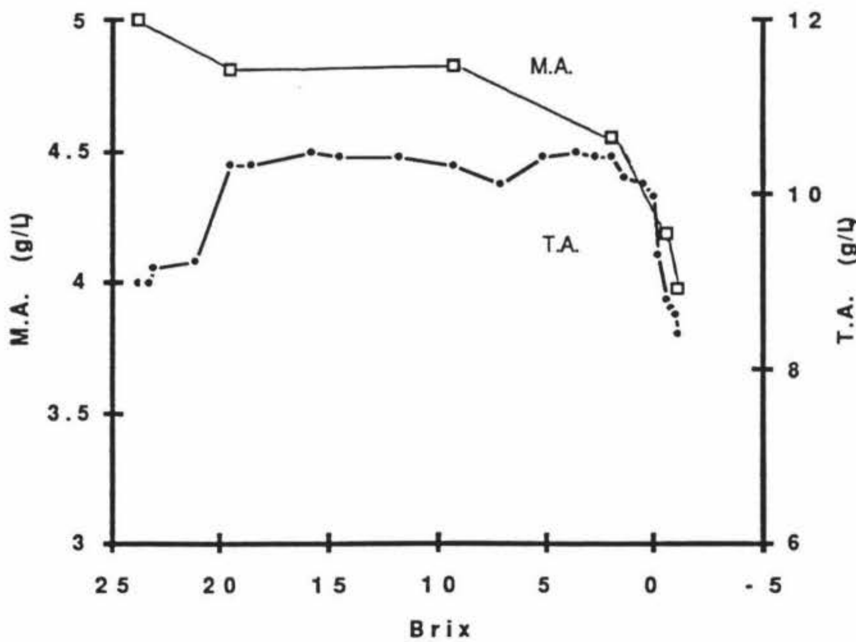


Fig.E8 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial nitrogen level was 578mg/L and initial cell count was 5.7 x 10^6 cells/ml.

APPENDIX F

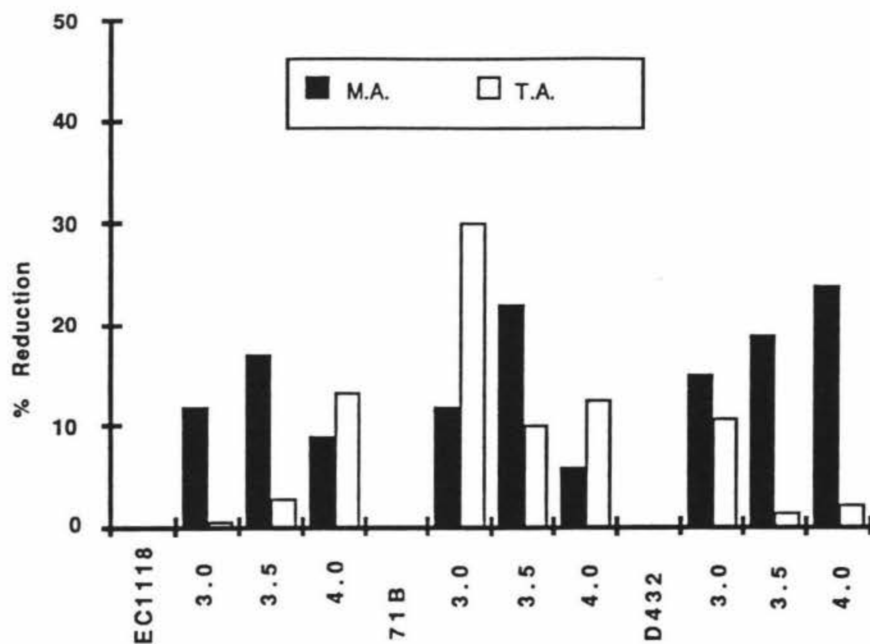


Fig.F1 Percent reduction in malic acid (M.A.) and titratable acidity (T.A.) of Simulated Grape Juice at different initial pH levels fermented (18°C) with yeast strains EC1118, 71B and D432.

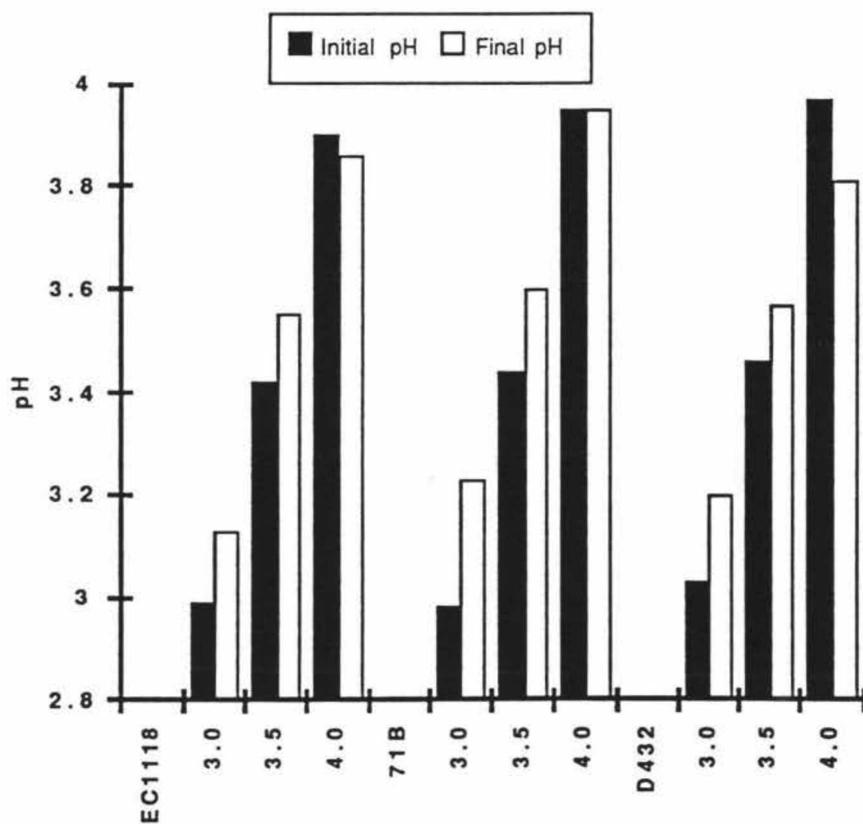


Fig.F2 pH values of fermentations (18°C) of Simulated Grape Juice with different initial pH levels inoculated with yeast strains EC1118, 71B and D432.

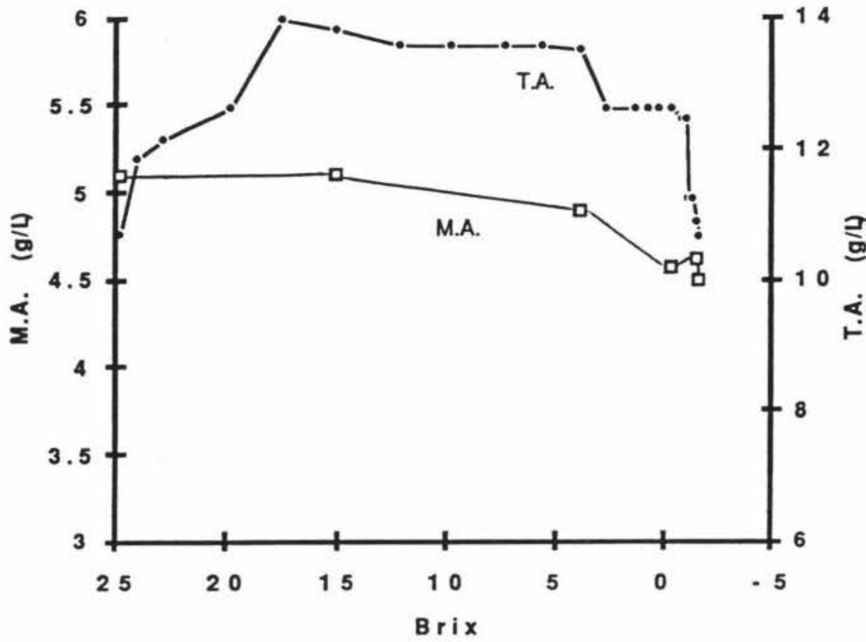


Fig.F3 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial pH was 3.0 and initial cell count was 2.3 x 10^6 cells/ml.

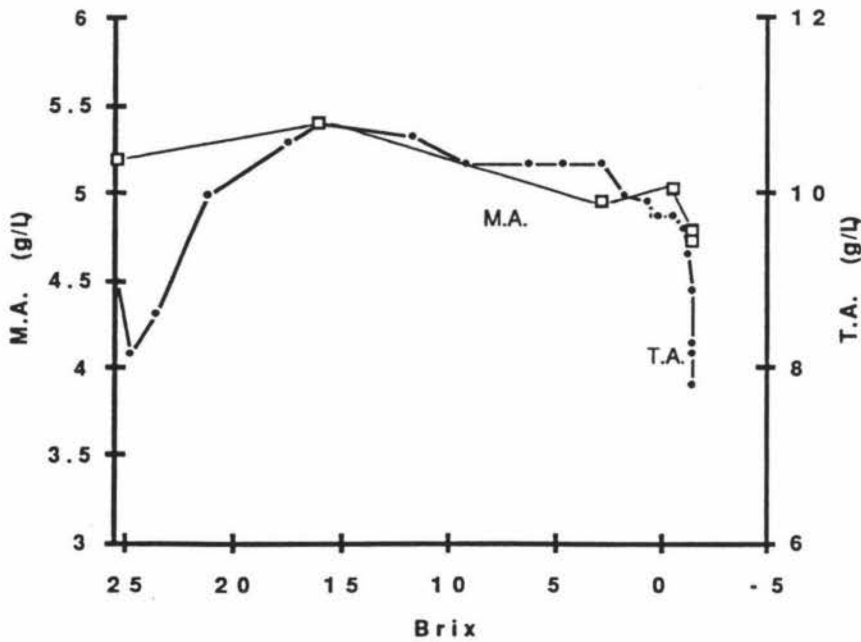


Fig.F4 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain EC1118. Initial pH was 4.0 and initial cell count was 3.2 x 10^6 cells/ml.

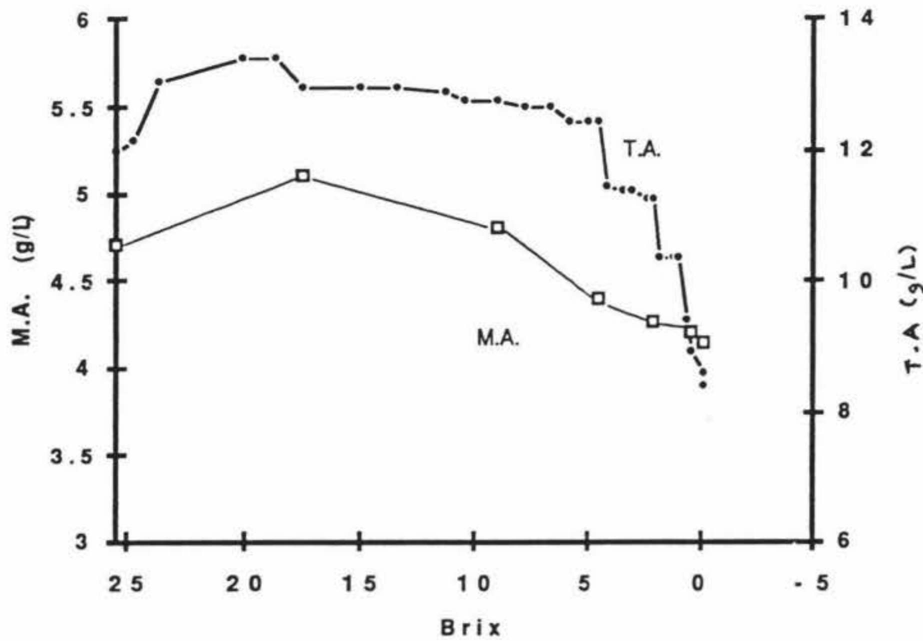


Fig.F5 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial pH was 3.0 and initial cell count was 6.0 x 10^6 cells/ml.

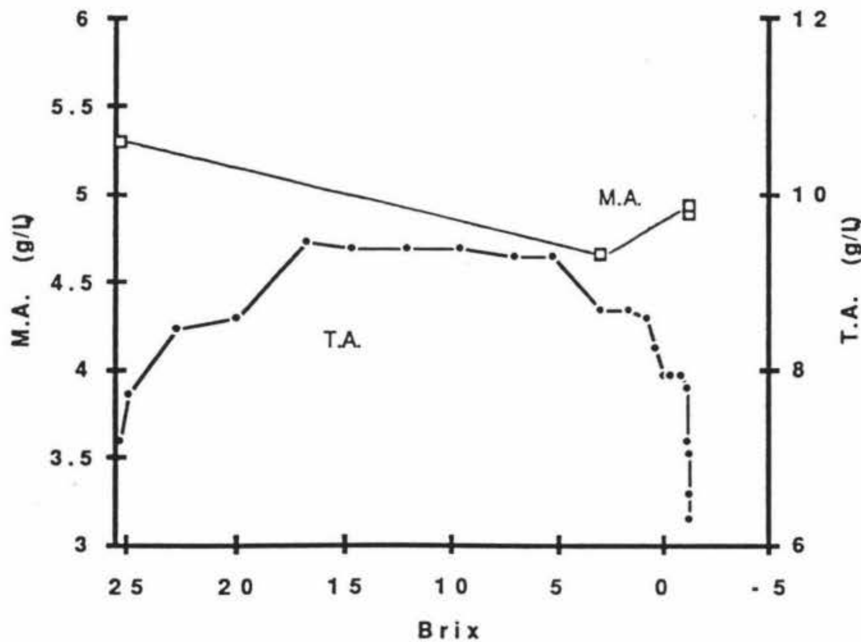


Fig.F6 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain 71B. Initial pH was 4.0 and initial cell count was 4.0 x 10^6 cells/ml.

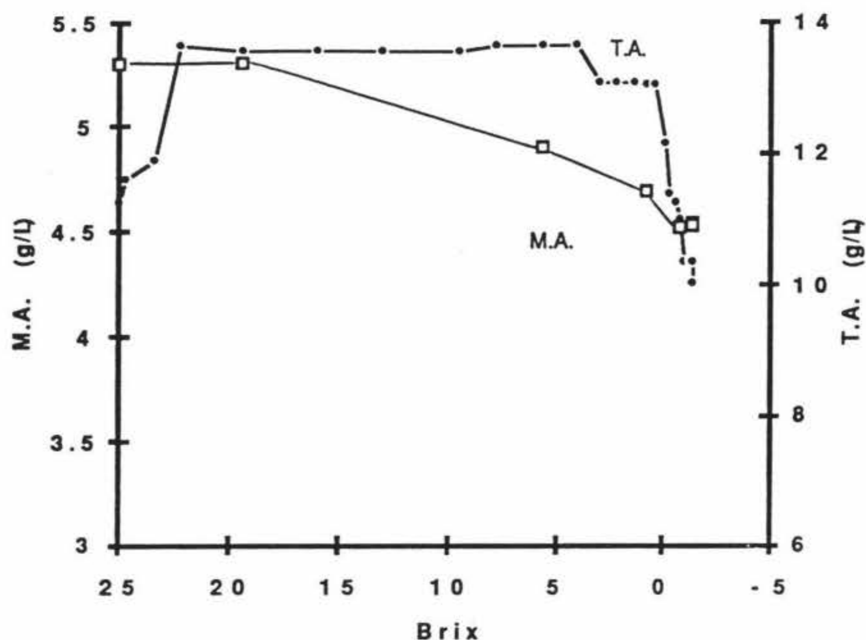


Fig.F7 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial pH was 3.0 and initial cell count was 5.8 x 10^6 cells/ml.

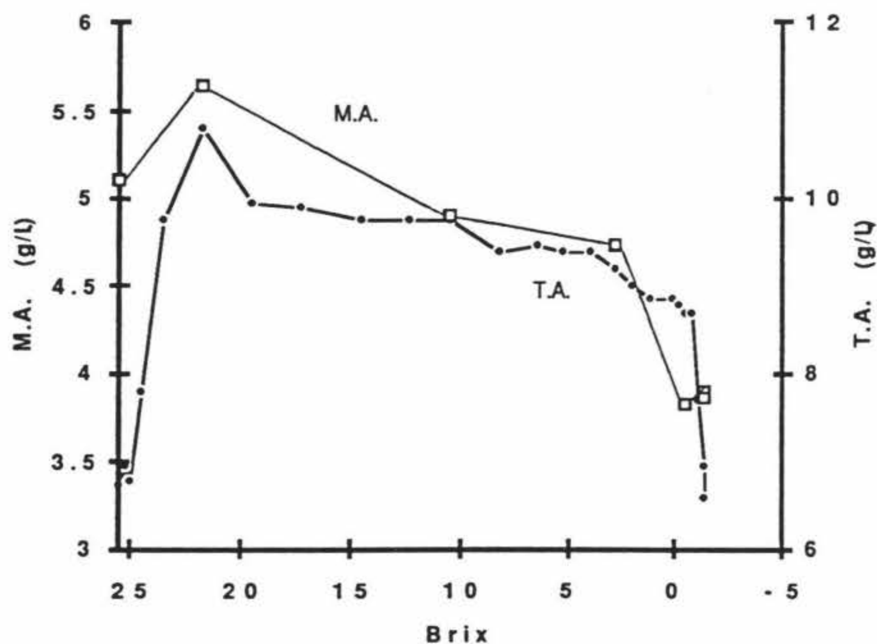


Fig.F8 Malic acid (M.A.) and titratable acidity (T.A.) concentrations during fermentation (18°C) of Simulated Grape Juice inoculated with yeast strain D432. Initial pH was 4.0 and initial cell count was 5.2 x 10^6 cells/ml.

APPENDIX G

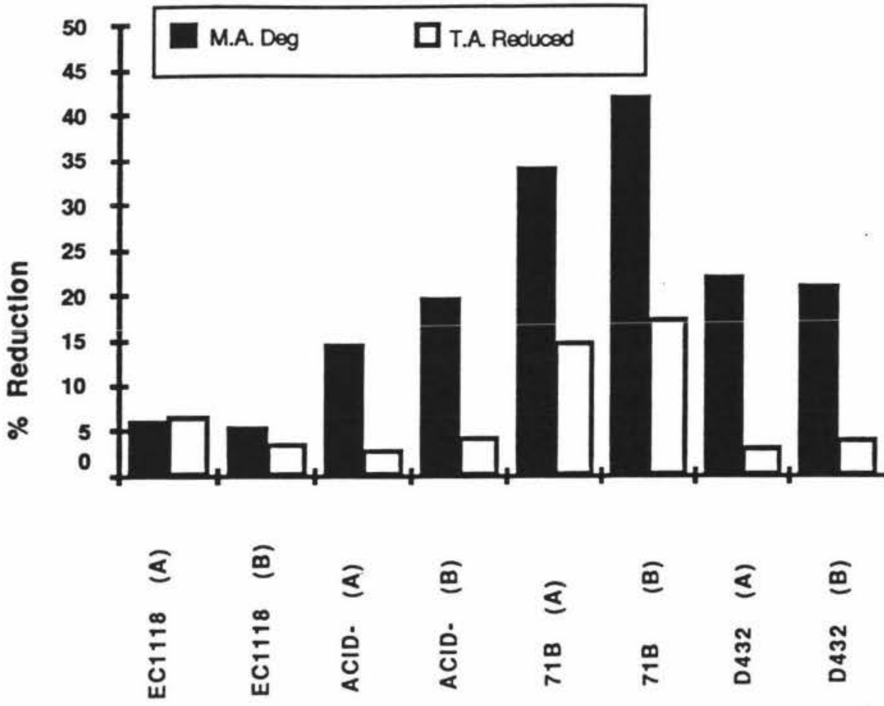


Fig.G1 Percentage reduction in malic acid (M.A.) and titratable acidity (T.A.) of wines vinified in duplicate from commercial Chardonnay juice with strains EC1118, ACID-, 71B and D432.

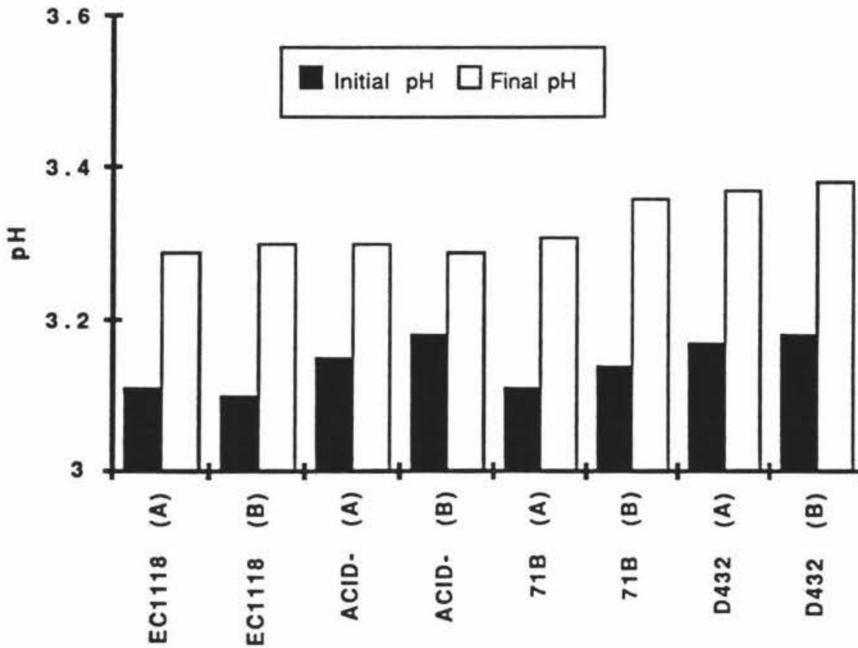


Fig.G2 Initial and final pH values of commercial Chardonnay juice vinified in duplicate with yeast strains EC1118, ACID-, 71B and D432.

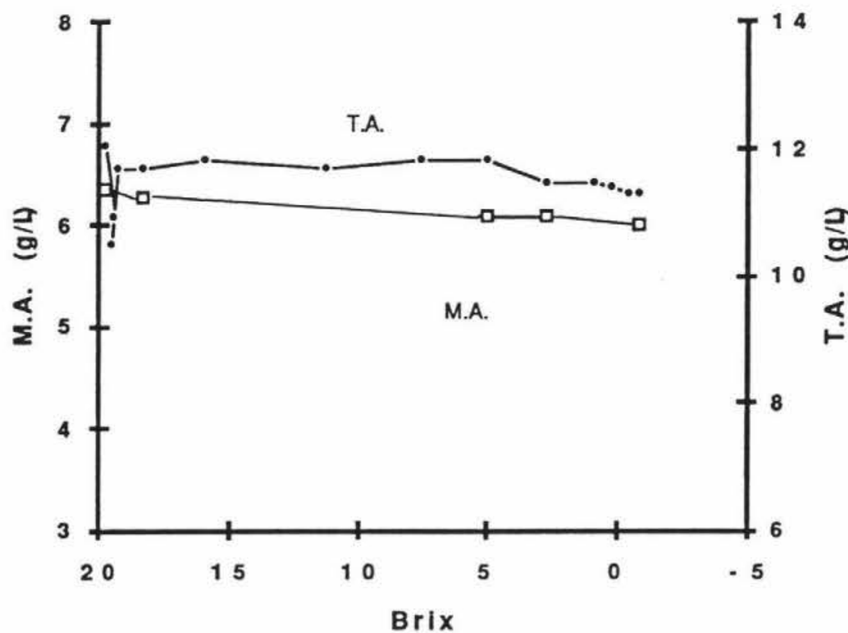


Fig.G3 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain EC1118 (a). Initial cell count was 3.4 x 10^6 cells/ml.

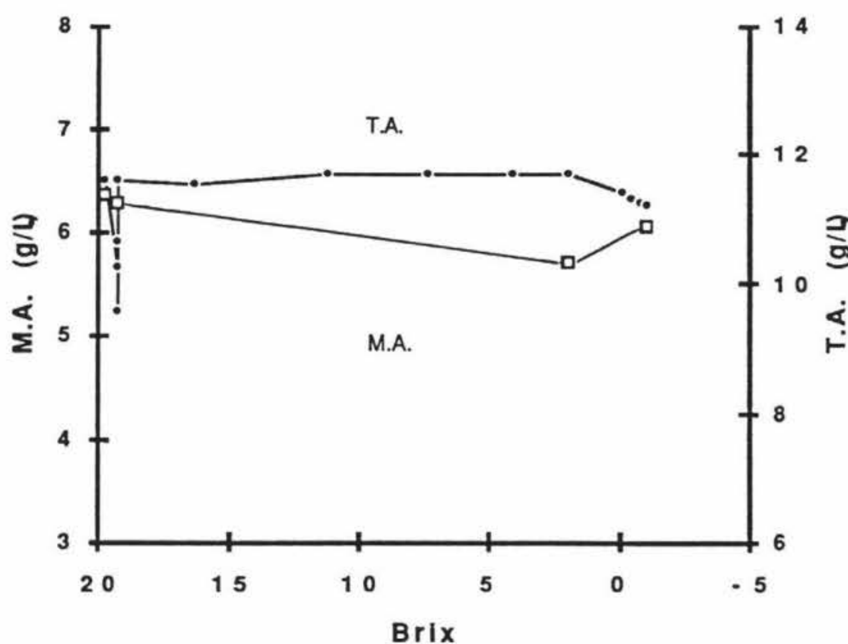


Fig.G4 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain EC1118 (a). Initial cell count was 4.3 x 10^6 cells/ml.

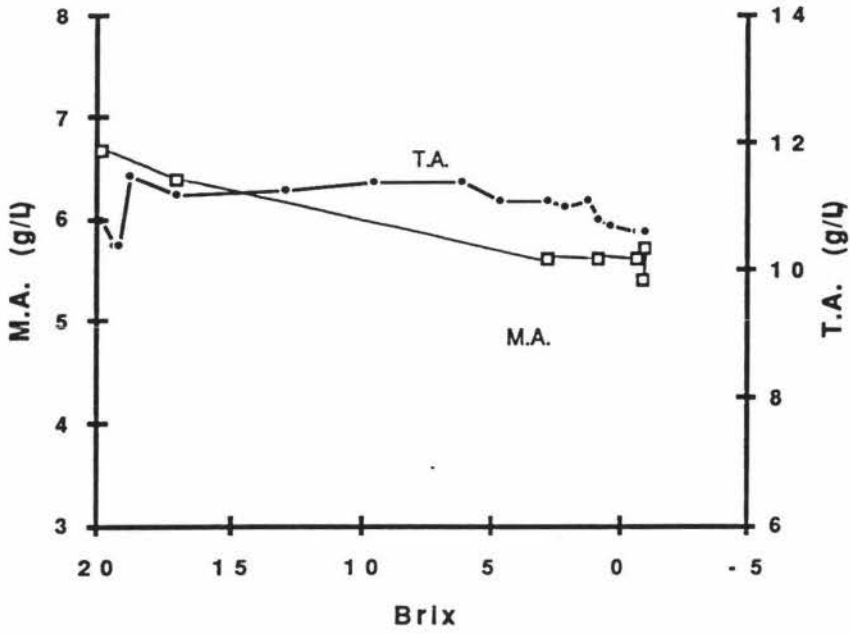


Fig.G5 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain ACID- (a). Initial cell count was 2.8 x 10^6 cells/ml.

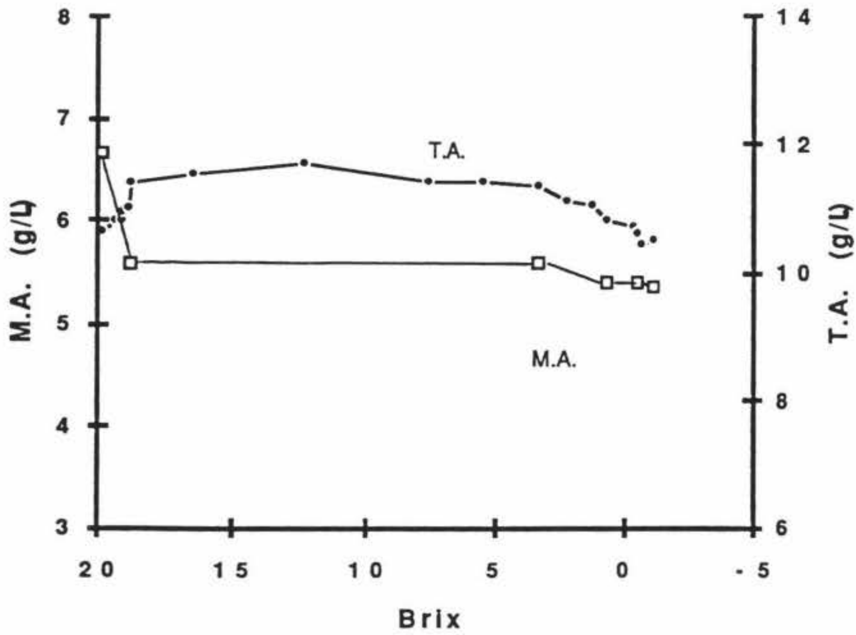


Fig.G6 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain ACID- (b). Initial cell count was 1.6 x 10^7 cells/ml.

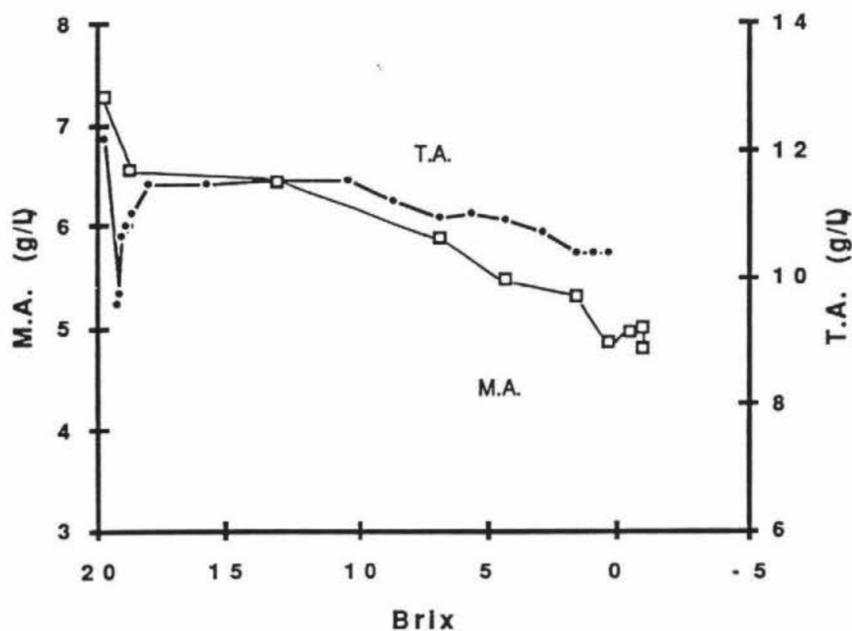


Fig.G7 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain 71B (a). Initial cell count was 5.6 x 10^6 cells/ml.

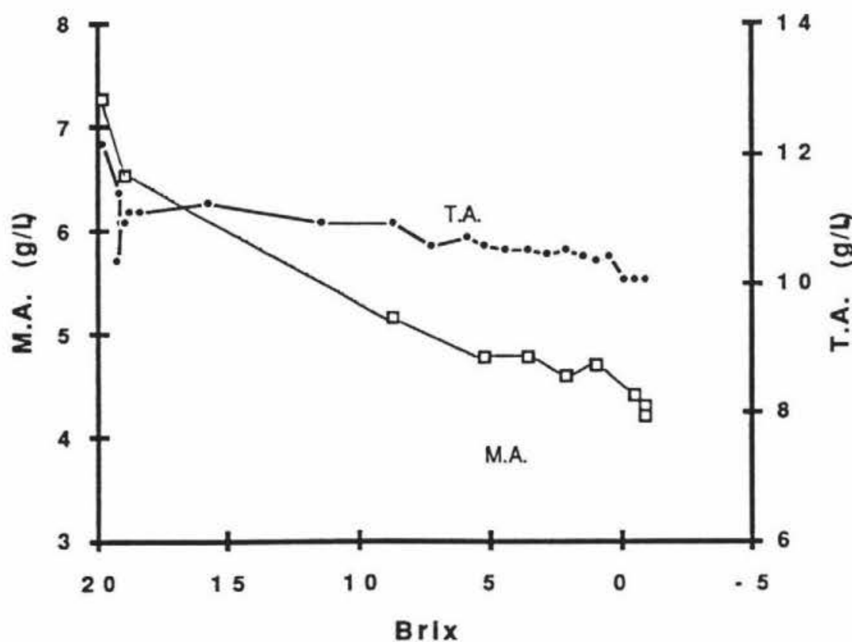


Fig.G8 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain 71B (b). Initial cell count was 6 x 10^6 cells/ml.

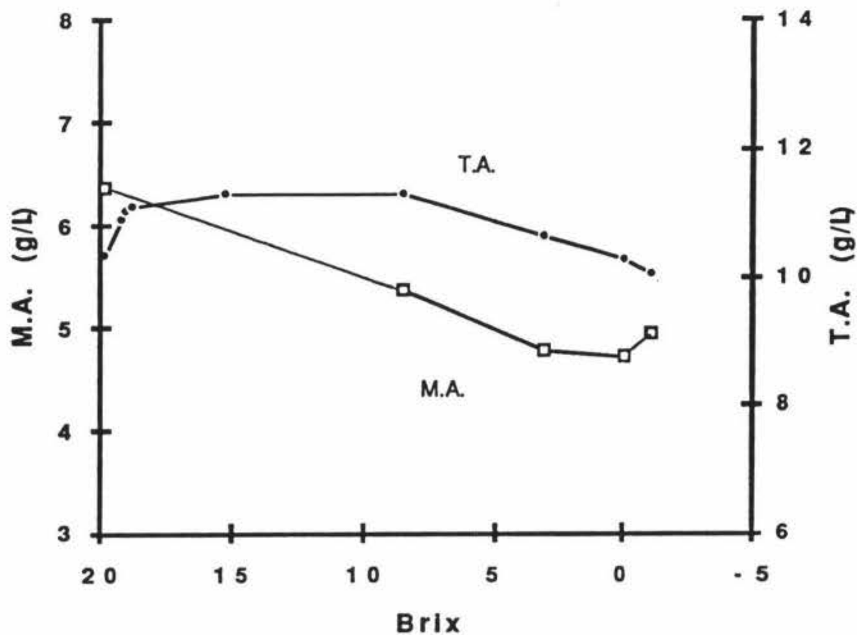


Fig.G9 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain D432 (a). Initial cell count was 1.6×10^7 cells/ml.

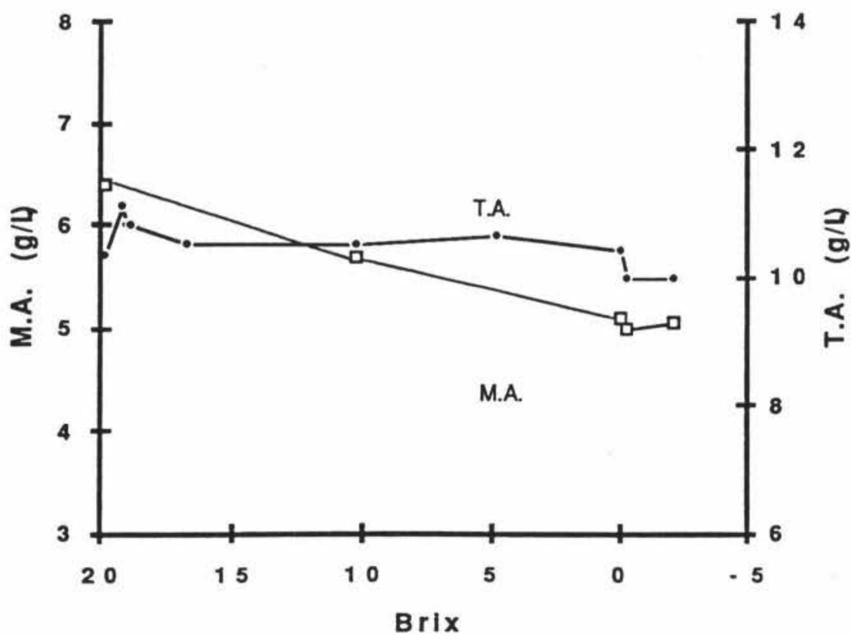


Fig.G10 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Chardonnay juice fermentations (18°C) inoculated with strain D432 (b). Initial cell count was 1.4×10^7 cells/ml.

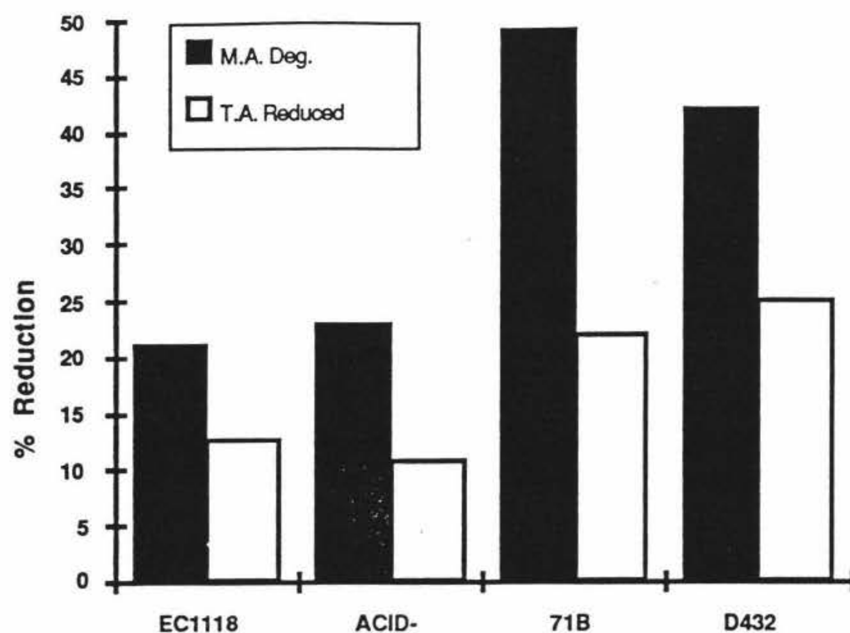


Fig.G11 Percent reduction in malic acid (M.A.) and titratable acidity (T.A.) of wines vinified from commercial Sauvignon Blanc juice with strain EC1118, ACID-, 71B and D432.

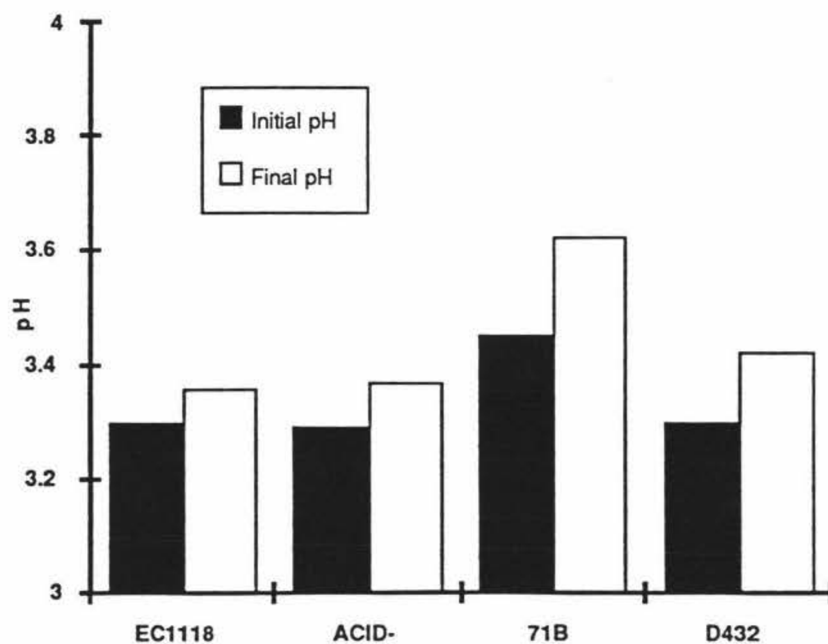


Fig.G12 Initial and final pH values of commercial Sauvignon Blanc juice vinified with yeast strains EC1118, ACID-, 71B and D432.

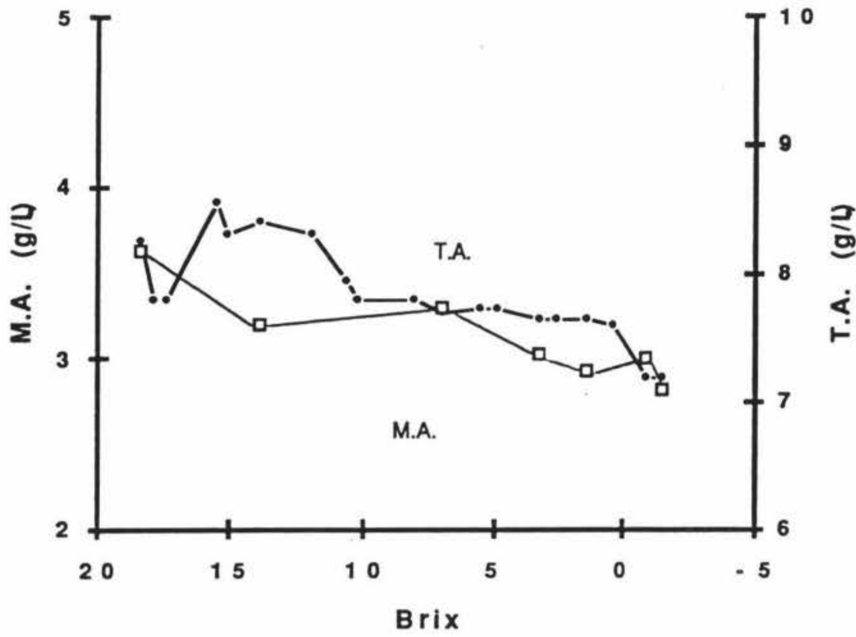


Fig.G13 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Sauvignon Blanc juice fermentations (18°C) inoculated with strain EC1118. Initial cell count was 1.6×10^7 cells/ml.

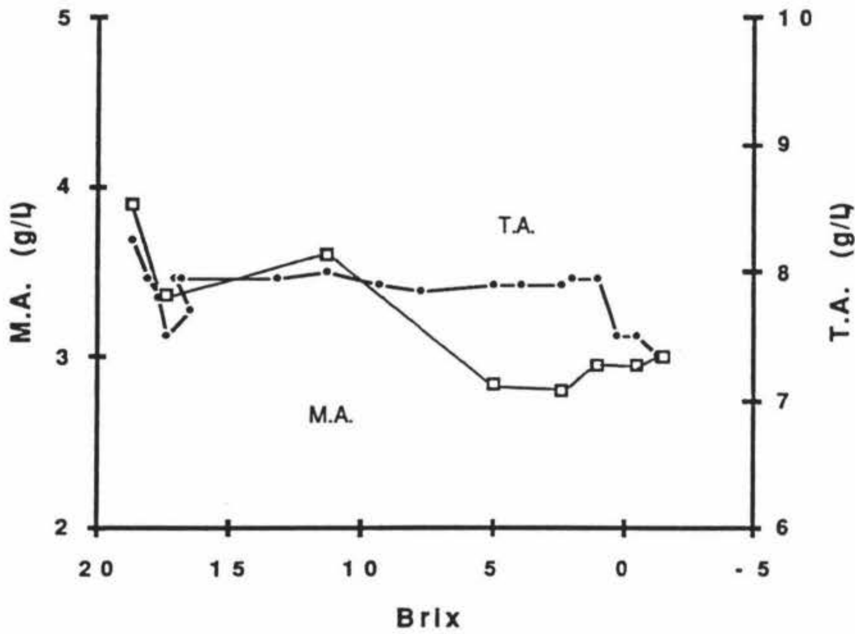


Fig.G14 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Sauvignon Blanc juice fermentations (18°C) inoculated with strain ACID-. Initial cell count was 3.9×10^7 cells/ml.

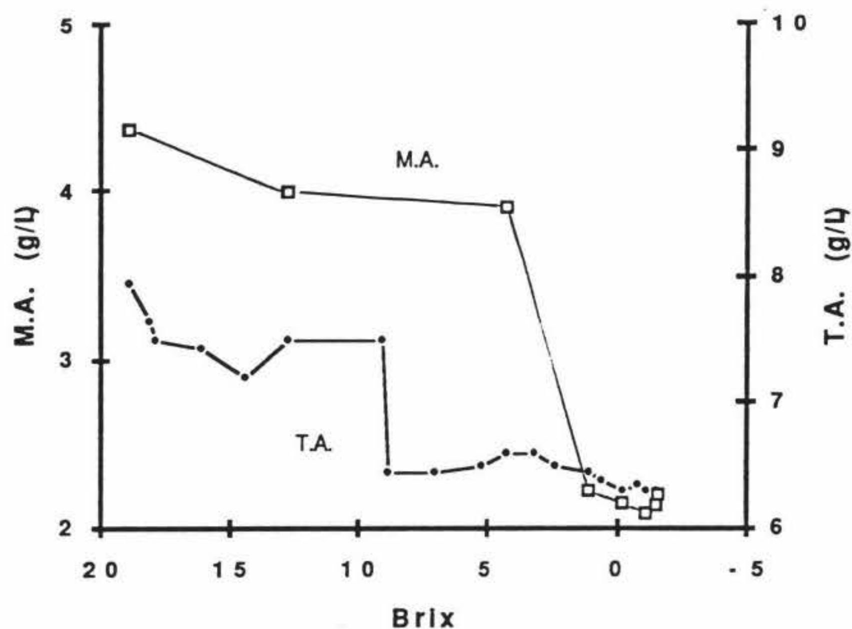


Fig.G15 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Sauvignon Blanc juice fermentations (18°C) inoculated with strain 71B. Initial cell count was 5.7×10^7 cells/ml.

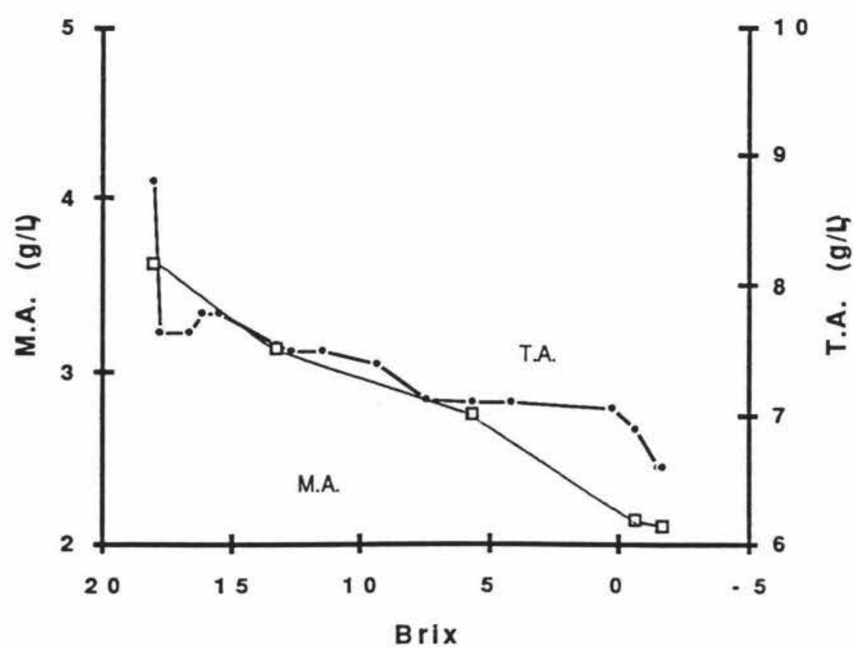


Fig.G16 Malic acid (M.A.) and titratable acidity (T.A.) in commercial Sauvignon Blanc juice fermentations (18°C) inoculated with strain D432. Initial cell count was 4.9×10^7 cells/ml.

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