The Role of Selected Metal Ions in the Growth and Physiology of Wine Yeasts

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Presented in partial fulfilment
of the degree of
Doctor of Philosophy
of the
University of Abertay Dundee.

University of Abertay Dundee
in collaboration with:
Lallemand Inc., Montréal, Canada
and
Universita degli studi di Perugia, Perugia, Italy.

September 1997

I certify that this thesis is the true and accurate version approved by the examiners.

Signed [redacted] Date 9th October, 1997

Director of Studies

ABSTRACT

Wine production is a complex biochemical process carried out by the main protagonist, the yeast *Saccharomyces cerevisiae* and its related species. The industrial processes leading to the production of alcoholic beverages, e.g. wine, are diverse and complex and yeast, during these processes, are subjected to a variety of conditions, not least a range of environmental stresses. This study investigated various aspects of yeast cell physiology in relation to ionic influences. The roles of magnesium and calcium in cell growth and fermentation, commercial biomass production and environmental stress were considered. Elevation of magnesium levels in the growth medium improved yeast cell viability, growth and fermentation parameters of *Sacch. cerevisiae* and *Torulaspora delbrueckii* (wine strains) in complex, semi-synthetic and minimal media. Magnesium was also found to alleviate the problems associated with agglomeration in active dried yeast (ADY) production. The phenomenon of agglomeration was identified to be a consequence of a combination of protein (α,β & γ proteins) and ionic (Ca^{2+} induced) influences and elevation of magnesium levels (5/10-fold) of the cultivation medium resulted in reduction of grit levels by 40-60%. Nutrient limitation (organic and inorganic) resulted in induction of a stress response in yeast. Metal ion related stress, either by removal or limitation of essential ions provoked stress protein production, particularly of 46 and 26 kDa proteins. Heat ($42^{o}C$) and ethanol (10%) stress induced a wide range of stress proteins which were subsequently repressed on elevation of magnesium in the growth medium above 10mM. These Mg^{2+} levels improved cell viability and reduced gross cell surface damage of yeast on exposure to stress. A protective role of magnesium in the yeast stress response is thus proposed. Magnesium is believed to counteract the stress response with regard to heat and ethanol, by offering physiological protection to yeast cells rather than having a role as a repair mechanism, as is the case with heat shock proteins. It is hypothesised that the mode of action is stabilisation of cell membranes by Mg^{2+}.

ACKNOWLEDGEMENTS

My sincere and grateful thanks go to Dr Graeme Walker, my director of studies, for his support, supervision (by mail, fax and in person!) and encouragement throughout the term of my PhD, both at home and abroad.

I greatly appreciate the support of the academic and technical staff of the School of Molecular and Life Sciences, University of Abertay Dundee, for their help with various aspects of this experimental work. For his help with Western Blotting analysis and the provision of aliquots of hsp70, hsp60 antibodies and GroEL for use in the stress studies, my thanks go to Dr Eldridge Buultjens and also to Prof. R.J.B King (University of Surrey) for supplying a sample of hsp27 antibody. Thanks are extended to two Erasmus students Raffaele De Nicola and Luca Catasti, who, while visiting our lab carried out and verified some of the viability studies included in this work.

I am grateful to the Biotechnology and Biological Sciences Research Council and Lallemand Inc. for financially supporting this work through a BBSRC (CASE) Studentship. My thanks go to my industrial collaborator, Mr James McLaren for his support in administering my contacts with Lallemand Inc. I also acknowledge, with appreciation, financial assistance from the EEC: ERASMUS Project which enabled me to carry out some research at the University of Perugia, Italy.

Italy

I wish to express my gratitude posthumously to the late Prof. Gianfranco Rosini for his encouragement and interest in my work during my time in his lab in Perugia. My sincere thanks go to Dr Maurizio Ciani for his help and supervision during my visits to his lab and to Sig. Fausto Maccarelli for technical assistance, especially with the fermenter studies. I wish to thank Dr Gianluigi Cardinali for carrying out the analysis of CHEF gels, for his patience in helping me learn Italian and his friendship. To Profs. Ann Vaughan Martini and Alessandro Martini and the staff of the Sez. Microbiologia Applicata, Dipartimento di Biologia Vegetale, University of Perugia, I extend my warmest appreciation for making my time in Italy most enjoyable.

I acknowledge the staff of the Centro per Microscopio Electronico of the University of Perugia, for their patience and assistance with the SEM and TEM work, in particular Dr Marcello Tosti for arranging access and Sig. Pierluigi Rondini for technical assistance in sample preparation and developing of the photographs.

Canada

I express my appreciation to Lallemand Inc. for extending me the invitation to undertake some research in their R & D laboratory at the NRC Biotechnology Research Institute, Montreal, Canada. Thanks are extended to Dr Richard Degré, of Lallemand Inc., Montreal, for his supervision, to the R & D laboratory supervisor, Mme. Ann Dumont (Lallemand) for her help in the laboratory during my time in Canada and Thomas Leeuw for supplying the SDS-PAGE equipment used. I would also like to thank the many people at NRC: BRI who made my visit enjoyable. In particular, I would like to thank David Juck for his friendship, fun, laughter and encouragement.....and a fellow appreciation of snow.

I acknowledge how fortunate I have been to have travelled as much with my PhD studies, I wish to thank Dr. Graeme Walker for his support and encouragement of this (I don't think he was just trying to get rid of me!) and the University for their support also, in particular the School of Molecular and Life Sciences.

I would like to thank my friends, who have helped keep me insane during all of my PhD, humoured me, got me roped into many a harebrained scheme (usually D of E), joined me in excursions to study the winter programme of the University of Life (skiing!) and given me excuses to test the effects of ethanol stress on humans, as well as yeast! To thank them all by name would be impossible and I appreciate the friendship of each of them, however, in particular I would like to thank my current flatmates Jane, Cate and Jo for their patience with me as I wrote up my thesis, keeping me down on ground level when the stress levels rose and generally just understanding.

Last but by no means least, I would like to thank my family, in particular my parents for their constant support and encouragement and their never-ending love. A piece of this belongs to them too, since a piece of them resides with me always.

" ... the only thing we require to be good philosophers
is the faculty of wonder ... "

Early Greek Philosopher.
Anon.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF PUBLISHED PAPERS

BIRCH R.M. & WALKER G.M. (1996) The role of magnesium in yeast stress responses. In: *Abstracts of 9th International Symposium on Yeasts.* pp46-47 Sydney, Australia. August 1996

WALKER G.M., BIRCH R.M., CHANDRASENA G. & MAYNARD A.I. (1995) Magnesium, calcium and fermentative metabolism in industrial yeasts. *Journal of American Society of Brewing Chemists* 54:1:13-18.†

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(† Refereed)

A NOTE ON NOMENCLATURE

Throughout this thesis the term yeast is used to mean *Sacch. cerevisiae* unless otherwise stated.

CHAPTER 1

INTRODUCTION

Science is interwoven with human development throughout the centuries and none more so in the case of yeasts. Yeasts are unquestionably the most important, commercially exploited group of microorganisms, both quantitatively and economically. No other group of microorganisms has been more intimately associated with the progress and well being of the human race. For centuries now, man has been utilising and exploiting yeasts and the abilities of yeasts for various purposes, all be it in the first instance unwittingly.

From the dawn of civilisation, yeasts have been used in the production of alcoholic beverages and bread. Nowadays, with the onset of genetic manipulation, yeasts are exploited in many diverse areas of biotechnology, including production of heterologous proteins (e.g. Human α-interferon, insulin, etc.), enzymes (e.g. invertase, β-galactosidase, etc.) and lipids, as well as in the elucidation of basic biochemical, metabolic and genetic processes of cells (an area previously dominated by the bacterium: *Escherichia coli*) (Burden & Eveleigh 1990). The total amount of yeast produced annually, including that formed during alcoholic fermentation, is in the order of several million tons and it is the brewing, distilling and wine-making industries which maximise the use of this group of organisms.

1.1 WINE

Wine is simply the fermented juice of the grape; it is basically the simplest and most natural of alcoholic drinks. A very empirical definition of both a complex process and a complex product. There are, in fact, infinite variations within the scope of wine, dependent on natural and microbial factors, producing wines with distinctively different tastes and characteristics.

Three major factors determine the type and quality of wine; grape cultivar, quality of grape and the wine yeast strain used for fermentation of grape must. Obviously the choice of

grape variety or blend of selected varieties has profound effect on the taste of the wine produced, but quality and taste are certainly not based on this variable alone. Natural factors such as regional climate, soil type, drainage, extent of sun exposure and the individual micro-climate of the vineyard site, all have a great effect on the taste of the wine and from year to year each climate varies and therefore vintages are affected accordingly. A further factor which is involved, is the action of the producer in controlling the growth of the vine, *i.e.* the higher the vine yield, the more dilute (thin) the resultant wine would be. The microbiological aspects influencing the wine produced are many-fold; the choice of using a starter culture or allowing natural colonisation of the must from the winery microflora, the conditions of fermentation: temperature, pH, oxygen level, actual sugar content of the must, nutritional complexity of the must (both organic and inorganic) and the control of the fermentation process itself, all contribute to the product quality. Therefore, the making of this "simple alcoholic beverage" evolves from a complex ecological and biochemical process where many variables require to be matched.

1.1.1 BRIEF HISTORY

The history of wine and the history of mankind coincide frequently throughout antiquity. Commencing long before our scientific knowledge of wine and wine yeasts was born, the saga of wine-making is old and distinguished. The origins of wine-making are obscured in prehistory, however, fossil proof exists to show that wine production was known in Egypt and Mesopotamia from 3500BC. and presumably earlier. The fact that sugary fruits, such as grapes, when left enclosed in a vessel for any significant length of time, actually produced a nutritious and satisfying beverage was initially discovered by chance. However, by the time records began, this was no longer the case. The early Egyptians, at this time, were producing wine on a large scale and making use of knowledge of irrigation and œneological practices such as pressing, racking and filtration, all be it a crude technology compared to modern times (Younger 1966). The concept of the commercial aspect of wine production was also understood by these early œnologists, with the development of full-scale wineries,

storage, labelling and even transportation of wines being recorded (Kunkee & Goswell 1977).

Following on from this, around 1400BC. the Greeks had developed a wine culture and expanded it to commercial export; carrying wine to the new settlements they colonised and in doing so, naming Italy's southern lands "Oenotria" - the land of wine. In the hills of central Italy, the Etruscans were also practitioners of the art of wine-making, from the evidence of art and artefacts discovered in archaeological digs of their tombs (Anderson 1992). The Romans, however, were responsible for spreading this 'cult of Bacchus' throughout the empire and by the 4th century AD. wine was produced in every region of the Roman world which could support growth of the vine, including Britain (Kunkee & Goswell 1977). Wine-making even survived through the Dark Ages without too much of a set back to progress, thanks to the monastic movement producing sacramental wines and in some cases much more than was required for mass; the commercial aspect of wine and wine production was also surviving this desperate period.

It was not until the 17th or 18th Century that the techniques of viticulture and œneology were improved or even equalled, such had been the extent of the knowledge of the Roman œneologists. In reality, this change only came about when wine-making was no longer regarded as a mystique, but as a science. Throughout the 19th Century, wine-making progressed in terms of trade and quality. The loss of quality, which had come with the demise of Roman control of the wine trade, was re-established and new areas opened up for production. Gone were the myths that vines could only yield good grapes and therefore good wines be produced, in the classical areas of central Europe. With travel and trade becoming more extensive vines were introduced into a wide range of countries, opening up the wine market considerably. Not everything went well from this point on, for the wine producers. Around 1870, disaster struck in the form of Phylloxera (*Dactylosphaera vitifoliae* or Root Louse) introduced from the 'New World' to the 'Old', destroying vines and drastically reducing the number of varieties which could be used for production. Recovery came, and with the new scientific knowledge available, in terms of microbiology,

the age old industry did not die out. The science of wine-making (scientific viticulture) was now recognised as important and the old methods of leaving the processes to chance were being changed. In particular, following Prohibition in USA (1918-1933) and the subsequent government regulations imposed, the resultant analyses required meant that science (biology and chemistry) was to be relied upon more and more. Leading up to the present day, with the technology explosion in the fermentation processes and the influence of biotechnology, the microbiological considerations for wine are of major importance.

1.1.2 GEOGRAPHY

The grape genus *Vitus* encompasses more than 50 species, however, one species *Vitus vinifera* is responsible for the majority of wine produced worldwide. Vines of the family *Vitaceae* are native to North America and the Far East and *Vitus vinifera* has been introduced to a large number of European countries, Australia and Eastern Europe. The geography of wine, as with a lot of things with a commercial aspect, has been influenced over the centuries by politics and economics. Politics can influence the choice of trading partner and subsequentially influences the general tastes of a nation, for example, the Methuen Treaty of 1703 and the anti-French polices of the then King of England: William, favoured Portuguese wines thus revolutionising the wine-drinking habits of the English (Kunkee & Goswell 1977). This change had some major benefits in terms of the quality of wines subsequently produced, with a restoration of the concept of ageing wine to improve its complexity. This technique had been abandoned following the classic period and consequently wine quality had deteriorated over the centuries passed.

The geography of wine production has changed over the years and now we have a definite split in terms of techniques, legislation and regulations between the "Old World" and "New World". The "Old World" - those established wine producing countries, namely the core of Europe; the route the Romans conquered: Italy, France and Germany (rather to name them by wine regions: Medoc, Burgundy, Champagne and Rhine-Mosel, etc.) remain, with Italy

and France being the top two wine producing countries in the world (Table 1.1). In these major wine producing countries there has been little change in the extent or technologies of wine production over the last few decades, where the development of new vineyards is forbidden or severely restricted. Wine laws created to control and protect the origins and names of wines have also greatly restricted the viticulturists to using only traditional methodologies and techniques.

Created by the Romans as a form of delimitation, modern legislation is not so far removed from that of the Ancients. Several countries use denominations to identify the zone of wine production, thereby classifying wines of "particular reputation and worth" to come from that region, after meeting many criteria. In Italy, the classifications are DOC (Denominazione di Origione Controllata) and DOCG (Denominazione di Origione Controllata e Garantita). There are 240 DOC designated zones, geographically spread throughout the country and twelve of these zones have the higher distinction of DOCG, these being: Barbaresco, Barolo, Brunello di Montalcino, Chianti (7 sub-zones), Vino Nobile di Montepulciano, Albana di Romagna, Gattinara Carmignano (Red), Torgiano Rosso *riserva,* Taurasi, Sagrantino di Montefalco and Moscato d'Asti/Asti Spumante (Anderson 1992). Within DOC and DOCG zones further classification exists either by colour, type, grape variety or age, to distinguish the hundreds of types of wines produced within these zones. These laws specify the delimited area the wine originates from, permitted grape varieties, permitted vineyard practices and ageing requirements. France uses a similar system with the classification of wines: AC or AOC (Appellation Contrôlée or Appellation d'Origine Contrôlée) the highest of the categories, followed by VDQS (Vin Délimité de Qualité Supérieure) and Vin de Pays, the lowest of the three categories. Germany's wines are similarly controlled with QmP (Qualitätswein mit Prädikat) being the highest with five regions; Kabinett, Spätlese, Auslese, Eiswein, Trockenbeernauslese and Beernauslese, followed by QbA (Qualitätswein bestimmter Anbaugebiete) encompassing eleven regions and the lowest category being Landwein. Following the radical changes to the wine laws in 1992, classified wines are now allowed to display names of geographically important areas or communities, or name areas of historical importance, in labelling the

product (Anderson 1992). The names of individual vineyards of recognised esteem may also be displayed and the European Community designations for spumante (VQPRD or VSPRD), frizzante (VFQPRD) and liquoroso or fortified (VLQPRD) wines included on the labelling. These classifications, whether they be the Italian, French, German or any wine producing country's demarcation system, only include a small proportion of the wines produced in a particular country (12-15% of Italy's production in recent times, for example: Anderson 1992). The majority of wines produced remain unclassified and must be labelled as table wine (vino da tavola, vin de table, deutscher tafelwein) with no specification of grape variety, vintage or place names. The new laws allow the better table wines of Italy to qualify under a category; IGT (Indicazione Geografica Tipica) created to classify wines by colour or grape variety. This Italian IGT category will be equivalent to the French; Vin de Pays or German; Landwein. Classification however, does not restrict good wines to only those within DOC or AC mark, many excellent wines remain unclassified and in fact these strict laws and regulations on processes and viticultural techniques sometimes constrict these countries of the "Old World".

The shift in the geography of viticulture occurred not to shut out the "Old World" countries, but instead to add to its richness, with a "Pandora's Box" of possibilities offered by the "New World" producers. In the so called New World, the legal restrictions are less profound and the fight against tradition does not exist. The shift has been due to several factors, including the greater desire to experiment with new ideas and technologies. New World wine-makers are unconstrained by the regulations, spatially delimiting cultivable areas, prescribing grape variety, yield and cultivation techniques. They have been free to experiment with various vine varieties and hybrids to match them to the soil and climatic conditions of the vineyard, disregarding the myth that the grape must 'struggle against nature' (terroir) if it is to produce a good wine and more importantly they have utilised scientific viticulture to its fullest extent in providing them with knowledge the traditionalists would only find through evolution. The emphasis in the Old World is that of tradition, whereas in the New World the emphasis is on innovation and the concept that it is; Old

World versus New: tradition versus innovation: nature versus science, although a simplistic view, holds elements of truth (Dickson 1992).

The New World of wine production includes: California (USA), Southern Australia, Ontario (Canada), South America, Eastern Europe, Bulgaria, Chile, South Africa and New Zealand, to name a few. It is not in reality that viticulture is 'new' in these countries, vines are native to North America for example, only that the recognition and status of them on the world wine map is new. In the past 10-20 years the acceptance and reputation of these areas has increased, as have the production figures and volumes of wine produced increased dramatically, placing several of these wine-producing countries of the so called New World within the top ranks of the world's wine producers (Table 1.1).

Table 1.1 The world's top ten wine producing countries and their exports (in hectolitres $x10^{-3}$)

Country	Production	Export
Italy	58.026	14.033
France	55.182	12.989
Spain	33.711	5.520
CIS	16.000	0.346
USA	15.420	0.972
Argentina	13.845	0.319
Germany	11.381	2.767
Portugal	9.684	1.609
South Africa	9.377	0.650
Romania	4.666	0.400

(From: Anderson 1992)

It is not so much that the New World has new vines and different soil ecology and environment, but that together with the scientists, the viticulturists have introduced classic grape varieties (chardonnay, cabernet sauvignon, etc.) and experimented with new varieties and blends (e.g. shiraz, zinfandel, etc.). Experimentation with better irrigation, better trellising, improved varieties, selected clones, better pest control, etc. in research trials, has resulted in extension of these trials to large scale, with commercial acceptance and adoption of the results.

Innovations in the New World have not only restricted themselves to viticultural practices but also improved œnology. Technological improvements in terms of; grape treatments (carbonic maceration, cold maceration, heat maceration and graduated pressing), must treatments (enzymatic treatment, cross-flow membrane filtration, hyperoxygenation, reverse osmosis and flotation), fermentation (temperature control, dried selected yeast starters and immobilised organisms) and process improvements (automation of control systems, insertion of robotics and informatics), are being developed and considered for use in the œneological industry, to ameliorate the systems currently used for wine production (Colagrande *et al.* 1994). For example, cold fermentation of clarified white musts, use of selected dry yeast inocula, malo-lactic fermentation control and improved management of barrel ageing, etc., have been mostly pioneered in California (Singleton 1992) and are applied in wineries around the world. Many institutes and universities around the world are carrying out œnological research and with this continuing research into viticulture and œnology, the opportunities which exist are vast.

New World wines still face competition from European (Old World) wines, even those of lesser quality, simply because foreign wines from these established countries are automatically thought to be prestigious. Competition will continue and in future the requirement will be, to base this competition on value rather than price. This is becoming possible due to the occurrence of the qualitative revolution in the New World, with most areas having improved from the initial low grade wines to being producers of independently recognised wines with much varietal dependence, e.g. Australian Chardonnays, Californian Cabernets and New Zealand Sauvignon Blancs. Although these are classic grape varieties they are also able to experiment, being free of EC regulations, to create their own blends or specialities, e.g. Australian Semmilion-Chardonnay blends, Australian Shiraz, Bulgarian Mavrud and Californian Zinfandel. Specific areas of quality wine production are emerging in the importance of world wines, *i.e.* Napa Valley in California, USA and Hunter Valley in New South Wales, Australia. Wines from these areas are now regarded as being equal competition to the "classic" regions of the Old World, producing some prize-winning vintages.

Economic change and scientific changes in the newer wine regions have great influence on each other. The concept of small vineyards is disappearing, with large conglomerates influencing the wine industry either in the form of co-operatives, producer companies or transnational beverage corporates (TBC's). Initially of influence only in the brewing and spirits sector, these TBC's are expanding into wine (Dickson 1992) and creating "brand" wines, e.g. Piat d'Or and Blue Nun. Companies are expanding and taking over control of existing small wineries, for example, as of 1992 E. & J. Gallo provided one quarter of wine output in the US (Dickson 1992). Present day wine production, therefore, is spread throughout the world; all tastes are catered for and with the rapid expanse of wineries in the New World, those producers of the "Old" are possibly now realising more and more how important scientific and in particular, microbiological research is for œnology - without research their market hold will slip.

The way forward for both the Old and New Worlds is research and the utilisation of new technology. Competition between these two "worlds" should be encouraged if only to inprove, generally, the quality of wine being produced overall. The complacency of the established producer countries is being shaken, wine is as it should be, produced wherever the vine can grow and the technology and research exists to produce good wine. The geography of wine, therefore, is worldwide and with the developments in research and technology, it should remain this way for some time.

1.1.3 PRODUCTION OF WINE

Wine-making, although conceptually simple, involves a complex process in order to produce a quality wine. The procedures for producing red and white wines differ somewhat. The treatment of grapes with potassium metabisulphite prior to crushing, to inhibit oxidative browning, is however, a technique applied to grapes in both processes. For white wine production, the first step is pressing and extraction of the must, often followed by a clarification procedure. Clarification, either by chilling or centrifugation, removes

unwanted solids including particulate browning enzymes and residual elemental sulphur, thus preventing subsequent formation of hydrogen sulphide during wine-making (Kunkee & Goswell 1977). In red wine production, the skins are kept in contact with the must, sometimes until the end of alcoholic fermentation. This maceration allows ethanolic extraction of pigments and tannins from the grape skins. Dependent on the type of wine being produced, this period varies and once the desired level of maceration has been reached (*i.e.* colour and tannin levels acceptable) the mixture is pressed and the must extracted.

Alcoholic fermentation of the must, either by the addition of selected starter cultures or by making use of the natural microflora of the grape or winery, is the most important stage of the production. Several factors affect fermentation, the most important of these being; yeast strain, temperature, pH, initial sugar concentration and nutritional content of the must. Of major concern to the wine producer is the heat produced during the fermentation, as a by-product of the conversion of sugar to ethanol. Methods of cooling are employed to slow the rate of fermentation, somewhat more important in white wine production but also required for some red wines. Without cooling, temperature rises may lead to 'stuck fermentations' or cell death. For dry wines, the fermentation results in all the sugars being converted into alcohol, in the case of sweet wine production either the fermentation is stopped (before all the sugar is fermented out) naturally, *i.e.* alcohol content is detrimental to viability, or by chilling, filtering or the addition of brandy.

Following fermentation, the new wine is susceptible to spoilage and it must be racked and chilled awaiting further processes. Some wines undergo a malo-lactic fermentation, (usually utilising *Leuconostoc oenos*) in which the tart malic acid present in the wine is converted to smoother lactic acid, dependent on the style of wine being produced. The addition of sulphur dioxide (and thiamine) to non-malolactic wines is required to complex with the free acetaldehyde produced during fermentation and prevent oxidative browning of the wine (Usseglio-Tomasset 1992) which would impair the taste.

Maturation is required for most wines, although the extent of this varies. It can either take the form of storage in large stainless-steel vats, ageing in oak barrels or bottle ageing in the case of "nouveau" wines. The ageing process adds complexity to the wine thereby improving its flavour and quality. Wood ageing of premium wines is generally done in new barrels of oak, for a period of six to twenty-four months, followed by clarifying and bottling. Bottle ageing, either straight from fermentation or after wood ageing, allows the development of special flavours and odours; "bottle bouquet", this is required for many fine wines, to allow the tannins and flavour compounds to complex and soften. The extent of bottle ageing varies with the wine and a fine wine is one with the potential to improve with time, reaching a peak, after which point an irreversible decline of wine quality and taste occurs. Less expensive wines are desired to be drunk young and fresh and these require little ageing, generally being bottled following clarification, within the same year of production.

Sparkling wines require a second fermentation of the still wine to take place. In the case of the Champenois method, this is achieved by adding sugar and yeast to the bottled wine with a secure closure to trap the carbon dioxide as bubbles in the wine and prevent leakage of this important gas. Once the second fermentation is complete (30-60 days) and the wine has been in contact with the yeast for at least a year, the bottles are slowly turned (remuage) to allow the dead yeast deposit to collect in the neck of the bottle, then they are immersed in freezing brine and the frozen sediment plug is removed under pressure (degorgement) and the clear, sparkling wine is re-corked and wired. Sparkling wines can also be produced by a Charmat process, where the second fermentation takes place in large tanks under pressure, then the wine is filtered, fined and bottled (also under pressure).

So, for a "simple" alcoholic beverage there are several complex processes to go through from the vine, to achieve the wine (Figure 1.1). The most important factor of these processes is the microbiology of such and this shall be discussed in more detail.

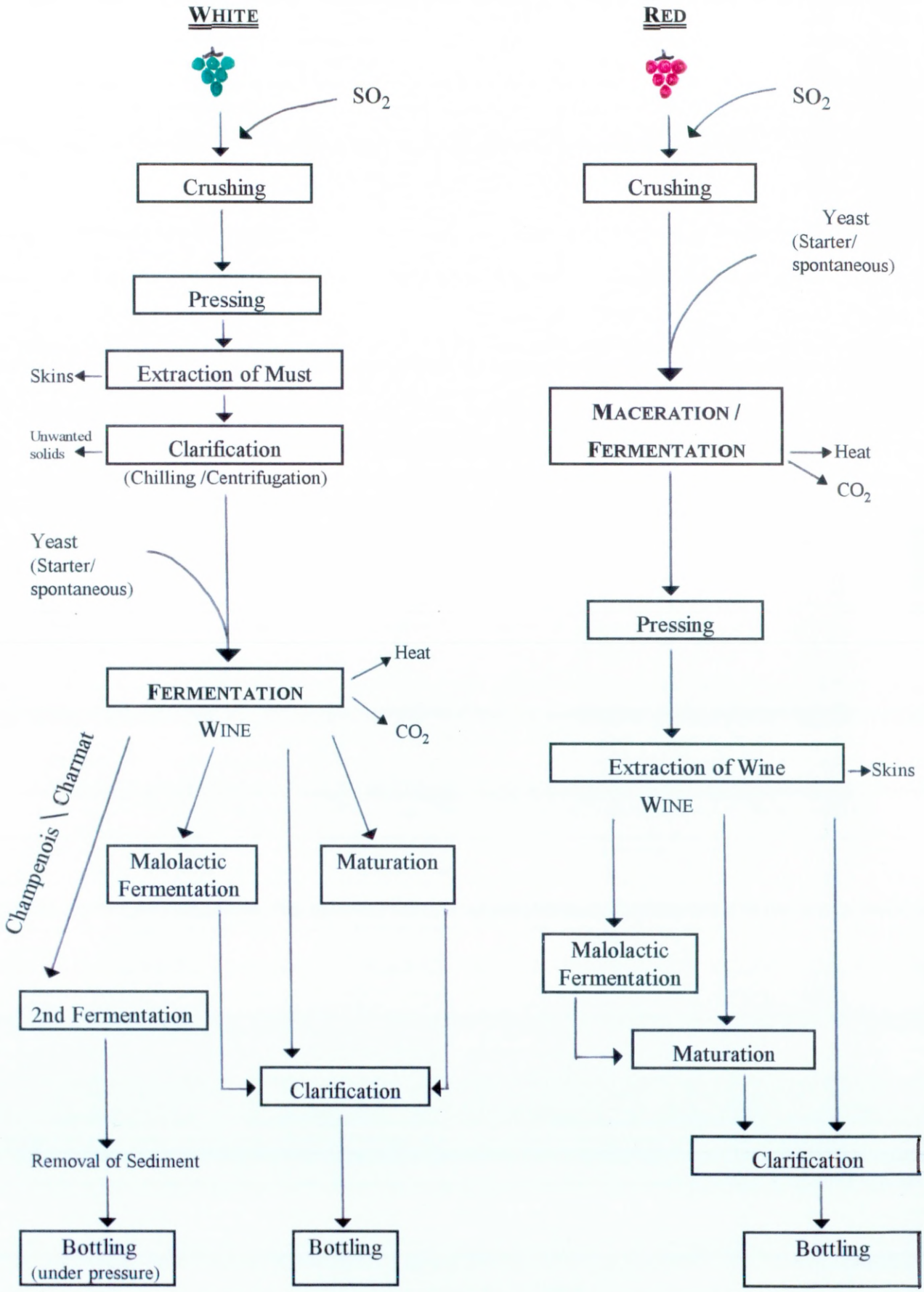


Figure 1.1 Industrial process of red and white wine production

1.2 MICROBIOLOGY OF WINE

The alcoholic fermentation of grape must into wine is a complex biochemical process involving interactions between many microorganisms; yeasts, bacteria, fungi and viruses (Fleet 1993). Dependent on its composition, a number of microorganisms can grow in grape must, however, due to the high sugar content (16-18%), low pH (pH 3.0-3.5), low concentration of nitrogenous compounds, phenolic content and alcohol content during fermentation, the number of microbial species able to proliferate in must and wine is limited to; yeasts, lactic acid bacteria, acetic acid bacteria and moulds. Being almost completely restricted to yeasts, the capacity to produce ethanol by fermentation of simple sugars, restricts further the variety of microorganisms involved in each stage of wine-making, quite simply due to the specific metabolism of these microorganisms.

1.2.1 BRIEF HISTORY

Despite microscopic observations of yeasts by Antonie van Leeuwenhoek in 1680, the concept of a 'living' organism being associated with fermentation dates back only to 1818 and the studies of Erxleben (Phaff *et al.* 1978), a stark contrast to the actual history of fermentation (circa. 3500BC. onwards). The link between yeast cells and fermentation transpired in 1837 (Cagniard de la Tour 1838) and through further work by Schwann, power was given to the proposal that fermentation was due to the growth of, and action of yeasts on a sugar substrate, producing alcohol and carbon dioxide as by-products. This theory and much of the early work on yeast was opposed by many biologists, chemists and alchemists of the time, however, Pasteur in his classic "Memoire sur la fermentation alcoölique" (1860) presented convincing proof of the yeast's role, undoubtedly illustrating yeasts as fermentative agents. Following on from this in the monograph "Etudes sur le vin" (1866) Pasteur convinced the majority that the alcoholic fermentation of grape must was in fact carried out by the "sugar fungus" described by Meyen in 1838 (Martini 1993) and

named *Saccharomyces*. Pasteur went on to carry out various experiments in relation to fermentations, concluding in the late 1800's that grape juice was unable to undergo fermentation unless it was 'contaminated' by fermenting yeasts resident on the epidermis of ripe grapes: spontaneous fermentation (Martini & Martini 1990).

The early investigations into wine yeasts and on the whole, yeasts in general, continued with research into pure cultures and the use of selected starter cultures in the brewing industry by Hansen in 1883 (Stewart & Russell 1986). Studies into the microbial ecology of sugary fruits in the early 1900's, lead to Hansen's theory of yeast cycling in nature. This model of yeast circulation was supported, for apiculate yeasts by the work of Müller-Thurgau and Berlese at the end of the 19th Century and challenged by Boutroux, proposing only a different mode of transportation (insects) rather than disproving or discounting Hansen's theory completely (Martini 1993). All these early studies including those by Martin and Rietsch indicate that apiculate yeasts are associated with the sugary fruits in their natural environment (Martini & Martini 1990).

With this significant progress toward understanding the biology of yeasts, in particular with the extensive work of Pasteur, an appreciation of yeast physiology and biochemistry developed. Based on these contributions to yeast microbiology, the elucidation of biochemical pathways, e.g. glycolysis, followed in close succession, with the work of the Buchner brothers in 1897 (Stewart & Russell 1985) and that of Harden and Young in 1906, in which cell-free extracts were found to ferment, proving the existence of enzymes (e.g. zymase) and the metabolic roles of phosphorylated sugars (Burden & Eveleigh 1990).

From that point on, yeast research went from strength to strength and this group of microorganisms has been associated with the elucidation of many biochemical and genetic mysteries over the years. In terms of the involvement of yeasts in fermentation processes, in particular wine fermentations, succeeding the research of Hansen in 1883 and the use of starter cultures in the brewing industry, pure cultures were developed for use in wine production around 1890, a practice which despite adoption in the other alcoholic beverage

industries, after a brief period of acceptance, the early vintners rejected. It may also be surprising to note that the commercial aspect of yeast was appreciated at this early stage, with the operation of a compressed yeast plant by the American company Fleishman commencing around 1868 (Burden & Eveleigh 1990).

1.2.2 WINE YEASTS

Although many species of microorganisms may be associated with grape must, yeasts have been unequivocally proven to be the causal agents of fermentation (Pasteur 1866). Many yeast species are known to carry out this transformation of simple sugars, however, only a few are able to yield significant amounts of ethanol during the natural fermentation of the juices of various sugary fruits and of these only a handful are commercially exploitable as potential selected starter cultures. The sources of yeasts responsible for the alcoholic fermentation of must therefore are, either: i) surface of grape, ii) surfaces of winery equipment and/or iii) inoculum culture.

In ancient times, wine-makers left the ethanolic fermentation of grape juice (must) to spontaneous transformation caused by the resident yeast flora of the grape surface (Martini & Martini 1990) even when selected yeast starter cultures were available, since it was thought by the early vintners that the natural microflora, although less efficient, gave the wine greater complexity. Recent studies have shown, however, the absence on the surface of grapes, of the principle yeast species associated with wine fermentations, suggesting the source of the fermenting yeasts is not the fruit itself (Rosini *et al.* 1982a). In fact, of the hundreds of yeast species isolated only a few play an important role in œnology. Ecological investigations of the indigenous microflora of grapes and wines have been carried out by many research groups and throughout the various studies of yeasts associated with sugary fruits and in particular, grapes (Hansen 1881: see Martini 1993; Barnett *et al.* 1972; Davenport 1976; Phaff *et al.* 1978; Rosini *et al.* 1982a; Holloway *et al.* 1990) the predominant yeast associated with grape surfaces and must (>75%) are the low ethanol-

tolerant, lemon-shaped (apiculate) yeast *Kloeckera apiculata* (and its perfect form *Hanseniaspora uvarum*). Other species have also been found, chiefly of the genera *Candida, Trichosporon, Pichia* and *Hansenula.* The total yeast population and the proportions of individual species found on grapes are affected by a number of factors including: œnological and cultivation techniques (Reguerio *et al.* 1993), climatic conditions (Kunkee & Goswell 1977), degree of maturity at harvest (Rosini *et al.* 1982a), physical damage (Guerzoni & Marchelli 1987) and grape variety. It would appear, from the evidence of these studies, that species of the genus *Saccharomyces*, so strongly associated with the fermentation industry, do not find favourable growth conditions on the surface of sugary fruits and despite yeast cell numbers reaching as high as 10^6 per gram, this strict "wine species" is consistently absent (Barnett *et al.* 1972; Rosini *et al.* 1982a).

The term "wine species" is an all encompassing term, covering a vast range of yeast species. Commercially, only strains of the genus *Saccharomyces* are utilised as selected starter cultures, although, Zambonelli *et al.* (1989) catalogue *Schizosaccharomyces pombe, Torulaspora delbrueckii* and *Saccharomyces cerevisiae* (all synonyms *sensu* Yarrow) as yeast species of strict œnological relevance to the production of alcoholic beverages. Other workers (Ciani & Ferraro 1996) have considered *Candida spp.* to possess useful œnological characteristics. *Schizosaccharomyces pombe*, due to its malo-alcoholic fermentation abilities has been proposed for the biological deacidification of musts containing high levels of malic acid (Zambonelli *et al.* 1989; Rosini & Ciani 1993), whereas *Torulaspora delbrueckii* has been considered for use in the wine industry due to its high "fermentation purity" (low acetic acid, acetaldehyde and ethyl acetate) and application of this yeast in the production of base wines for sparkling wine production has been proposed (Ciani & Piccotti 1995). The principal limitation of *Candida stellata*, for use in wine-making is its low fermentation rate (Ciani & Ferraro 1996) however, the benefits of this yeast would be for increased glycerol production in wines. Despite the possible applications of these "alternative" species, implementation has rarely gone beyond the laboratory or pilot plant levels.

In practical terms therefore, "wine species" may be defined as: the species of the genus *Saccharomyces* that always predominate in fermenting grape must. *Saccharomyces cerevisiae* and its related species (*Sacch. bayanus*, *Sacch. chevalieri*[1], *Sacch. oviformis*[1], *Sacch. pastorianus*, *Sacch. italicus*[1], *Sacch. uvarum*[1] and so on, *ad nauseam*) are the main protagonists of alcoholic fermentation and have been regarded as such for many decades. These strains of the old epithets, previously distinguished by their sugar fermenting abilities, were found to frequently exhibit random fermentative abilities (Rosini *et al.* 1982b). In a major review of the taxonomy of *Saccharomyces* (*sensu* Yarrow) using molecular taxonomy, it has been demonstrated that *Saccharomyces* sensu stricto is composed of four separate species: *Sacch. cerevisiae, Sacch. bayanus, Sacch. pastorianus* and *Sacch. paradoxus* (Rosini *et al.* 1982b; Vaughan Martini & Kurtzman 1985; Vaughan Martini & Martini 1987; Vaughan Martini - personal communication). Three of these (excluding *Sacch. paradoxus,* the only species habitually isolated in the environment: Vaughan Martini & Martini 1989) are strictly exclusive of the alcoholic fermentation environment. As a result of this revision of taxonomy, the old epithets have been abolished and the "wine species" concerned are defined, therefore, as: *Saccharomyces cerevisiae*Hansen, being the single species encompassing strains previously known as *Sacch. cerevisiae, Sacch. italicus*[1], *Sacch oviformus*[1] and *Sacch. chevalieri*[1] (Yarrow 1984); *Sacch. bayanus,* the species to which *Sacch. bayanus* and *Sacch. uvarum*[1] have been assigned (Vaughan Martini & Kurtzman 1985) and *Sacch. pastorianus* the recently restored (Vaughan Martini & Martini 1987) species to which *Sacch. carlsbergensis* has been assigned. Thus, in commercial terms, it is still *Sacch. cerevisiae* which is the most important yeast species, with its involvement in all of the alcoholic beverage and baking industries. An opinion strengthened by the fact that, according to recent ecological and technological evidence (Rosini *et al.* 1982a/1982b; Vaughan Martini & Lai 1984) *Sacch. bayanus* and *Sacch. pastorianus* are apparently not related to the environment of wine (*Sacch. pastorianus* being of use exclusively to the brewing industry and *Sacch. bayanus* finding only occasional use in Champagne production and as a finishing yeast). It can be assumed therefore, the strains

[1] Denotes nomenclature now obsolete

associated with wine-making belong to the species *Sacch. cerevisiae*, which is characterised by a large spectrum of technological properties distributed randomly among its strains.

In "natural" fermentations of grape must, *i.e.* spontaneous fermentation initiated by the microflora 'naturally' present in the environment, a succession of different yeast populations results. The fermentation process is carried out and completed by a limited number of dominating strains. *Sacch. cerevisiae* in the early stages of fermentation is always underrepresented but during the following stages it constitutes almost exclusively the totality of the isolated colonies (Smart *et al.* 1994; Versavaud *et al.* 1995). The initial phase of fermentation is predominated by apiculate yeast (*Kl. apiculata*) however, this domination is short lived and by the end of fermentation, species of the genus *Saccharomyces* predominate. The nearly monospecific population of *Sacch. cerevisiae* which develops during the mid to final stages of fermentation is polyclonal, *i.e.* constituted of several genetically distinct strains growing simultaneously with one or two being predominant (Versavaud *et al.* 1995). The predominant strains can be considered the most adapted to conditions of wine production, a criterion recently used in programs of œnological strain selection. This monospecific biomass is, at the end of fermentation, representative of the population diversity (Versavaud *et al.* 1995) showing natural variability of *Sacch. cerevisiae* strains within specific wine-producing regions. Therefore, strains widespread throughout a wine producing area could be considered as representative of an œnological region or "terroir". The explanation of this substitution, from apiculate to *Saccharomyces* strains, is difficult since, as has been noted (Rosini *et al.* 1982a) these strict wine species (*Sacch. cerevisiae*) do not originate from the fruit itself. Mortimer *et al.* (1994) have developed a model called "genome renewal" to explain the rapid evolution of wine yeast strains. Since all œnological strains are at least diploid, they have proposed that new genotypes could arise from diploid homothallic strains changing multiple heterozygotes into completely homozygous diploids, which may exhibit greater fitness and will replace the original strains (Mortimer *et al.* 1994). Also, strains important in wine fermentation have been isolated from vats and other winery equipment (e.g. crushers, presses, tanks, fermentors, barrels, pipes, pumps, filtration and bottling units, etc.: Rosini 1984).

Saccharomyces cerevisiae is prevalent on these surfaces and following the work of Rosini (1984) and the use of a marked strain to trace its origin, Martini and Martini (1990) concluded that winery surfaces were the main source of indigenous *Sacch. cerevisiae* in such fermentations.

In the present day, all phases of vinification can be conducted and controlled by using selected and specific cultures and the process is no longer left to chance. Although, despite this present level of control and selection, the microbiology of wine-making has not evolved at the pace of the technologies supporting it, revealing it to be a complex situation. The "wine species" responsible for the biotransformation of grape must to wine, contribute more to vinification than solely the ethanol formed through the fermentation of grape sugars. Yeasts utilise sugars and other components of grape must during fermentation, transforming them into ethanol, carbon dioxide (by-product) and other metabolic end-products which contribute to the chemical composition and sensory quality of the wine. These important metabolic end-products vary dependent on the composition of the grape must, but also to a greater extent, it depends on the strain of yeast used.

1.2.2.1 Characteristics of Wine Yeasts

Although some similarity exists, generally the desired characteristics of wine yeasts differ somewhat from those of brewing and distilling yeasts. Yeasts utilised in these industries are characterised by i) good utilisation of sugar, ii) alcohol production and tolerance, iii) flocculation, iv) cropping ratio, v) O_2 requirements and sterol biosynthesis, vi) maltase activity and vii) formation of higher alcohols and other flavour components For distillery yeasts the ability to utilise trisaccharides, dextrins and starches is also required (Spencer & Spencer 1983). The desired characteristics of wine yeasts, however, can be divided into two main categories: technological characters affecting the wine-making process and qualitative characters affecting wine quality (Table 1.2).

Table 1.2 Characteristics desired of wine yeasts.

Technological Characters	
Fermentation Properties:	Rapid initiation of fermentation
	High fermentation efficiency
	High ethanol tolerance
	High osmotolerance
	Low temperature optimum
	Moderate biomass production
Technological Properties:	High genetic stability
	High sulphite tolerance
	Low sulphite binding activity
	Low foam formation
	Flocculation properties (Champagne yeast)
	Compact sedimentation/Rapid sedimentation
	Resistance to desiccation
	Zymocidal (Killer) properties
	Genetic marking
	Proteolytic activity
	Low nitrogen demand
Qualitative Characters	
Flavour Characteristics:	Low sulphide/DMS/thiol formation
	Low volatile acidity production
	Low higher alcohol formation
	High glycerol production
	Glycosylated-flavour precursor hydrolytic activity
	Enhanced autolytic activity
	Modified esterase activity
Metabolic Properties:	Low sulphite formation
(Health Implications)	Low biogenic amine formation
	Low ethyl carbamate (urea) potential

(from: Henschke & Lee 1991)

The qualities sought after in wine yeasts are, first and foremost, the ability to grow and ferment efficiently in must of high sugar content. As a result of the high sugar and acid concentration of grape must, wine yeast do not demonstrate the 'Pasteur Effect'. Even when exposed to high oxygen levels, diauxie does not take place when ethanol is utilised (Cantarelli 1989). Fermentation prevails over respiratory glycolysis given the condition of the substrate and the physiological features of the yeast. This predominance of fermentation and rapid onset of such a process also serves to counteract and suppress the growth of spoilage organisms, thus reducing possible problems later on and ensuring the taste of the final product is not corrupted.

The resistance of the wine yeast to sulphur dioxide is a requirement which must be considered at the initiation of the wine-making process, since it is commonplace for vintners to add SO_2 (30-90mg/L) during the process of crushing or to add it to the resultant must. The rational for sulphite addition is two-fold; this dosage is required for its inhibitory or cidal effects on the wild yeast populations present in must or on winery equipment, which would compete with selected inoculum cultures and as a secondary effect, it is used for the control of spoilage microorganisms, as well as affording some protection against oxidation. The use of 'killer' yeasts as selected starter cultures could essentially effect a reduction or elimination of the dosage levels of SO_2 utilised in wine production (popular 'killer' strains used in wine-making are: Champagne 111 and Montrachet 1107 (Prisse de Mousse): Vine 1993).

The progress of a non-foaming fermentation, with low residual sugar (<5g/L) and rapid sedimentation following fermentation is essential to attempt to overcome the problem of 'stuck' fermentations; that is when substantial quantities of residual sugar are left in the wine, due to premature arrest of fermentation, a major problem in commercial terms for the wine producer. Rapid sedimentation following fermentation also helps in further processing of the wine, making the clarification step much easier. The ability of the yeast to grow and ferment at low temperatures is essential for white wine production and in cold-climate wine producing areas e.g. Ontario, Canada. The optimal temperature for fermentation (15-

20°C) is different from that of growth (25-30°C) (Castelli 1941) and therefore methods of cooling during fermentation systems must be able to set an optimum to allow these events to occur. All of these characteristics go some way towards influencing the process or ultimately the final product, however, the most important attribute of a wine yeast is its capacity to produce alcohol. Wine strains must have the capacity to produce high levels of ethanol (80-90% theoretical) and be both osmotolerant and ethanol tolerant, since ethanol is the major end-product of the whole wine-making process. Sensory neutrality of yeast is important to the final product also, it is important that spent yeast does not impart off-flavours to the wine and it is essential that the formation of volatile acids (particularly acetic acid), hydrogen sulphide, sulphurous acid (20-30mg/L) and acetaldehyde (<20-30mg/L) must all be kept low (Spencer & Spencer 1983) since these compounds would serve to taint the flavour and quality of the wine.

With all these desired characteristics of wine yeasts, it is a yeast of complex calibre which is required for a particular wine fermentation and to rely on indigenous microflora to supply this range of attributes which the modern vintner wants, would be to turn the technology of wine-making back to being a mystique, rather than the complex science it actually is. Advances in the study of the microbiology of wine lead to the development of selected starter cultures for use in the wine industry, allowing the producer to select a yeast exhibiting the desired characteristics they require and allowing the production of wine of uniform quality.

1.2.2.2 Selected Starters

Many wineries of the world still rely on the indigenous microflora to initiate fermentation of grape musts, however despite this, the use of pure starter cultures is increasing significantly. Wine fermentations in Europe and elsewhere now rely on selected inocula and around 10% of wine produced in the world is fermented by special wine yeast strains (Maráz & Deak 1990). Pure culture yeasts are deemed necessary for efficient production of everyday wines

and desirable for the production of fine wines. Virtually all commercial yeast strains are naturally occurring variants that have been empirically selected because they have the fermentation characteristics required. Grape must itself is a selective medium, with its acid pH and high concentration of sugar and therefore the yeast which excel have already undergone a process of selection. During fermentation there is a constant selection for more ethanol tolerant yeasts; basically adaptation of a natural progenitor to conditions in industrial substrates - natural selection (Johnston & Mortimer 1986). The methods of yeast selection for the preparation of starter cultures now include: clonal strain selection, selective hybridisation, induction of mutants (Zambonelli *et al.* 1989), spheroplast (protoplast) fusion; the later having greater applicability to wine strains, and genetic engineering.

Some œnologists thought the use of starter cultures unacceptable for the making of great wines, since it was believed a particular yeast originating from the area of production was completely adapted to that particular type of must and that a starter culture could not mimic this quality. Good results, vintners noted, could also be obtained with selected yeast starters originating from the micro-area where these great wines are produced, although there are those who still believe they are only good for producing ordinary wines and indeed there are cases where strains isolated from winery equipment posses technological properties on the average comparable with those of commercial selected starters. (Rosini *et al.* 1988). Inherent in the use of pure cultures is the decision as to which yeast strain to use in order to produce a desired wine type, since within genetically identical species, strains exist with fermentative versatility and variability useful for the yeast to adapt to various environmental conditions. Aspects of wine production influence the choice of strain and in the selection of yeast for starter cultures several things must therefore be taken into account; desired yeast characteristics, particular qualities of region or must from a particular grape variety etc. and it is now possible for the selection of strains better adapted to different regions of the world and their respective grape varieties e.g. Lallemand "Côtes du Rhone" L-2226 (Degré 1993). Commercial strains available throughout the world number only around 10-20 (Reed & Nagodawithana 1988) however, with the technology available

to the wine microbiologists now, avoidance of a standardisation of yeast cultures in wine-making is possible. Selection of yeast cultures for œnological use, either through the use of dominant strains isolated from natural wine microflora (Ciani & Rosini 1990) or genetic development, e.g. protoplast fusion (Yokomori *et al.* 1990) to create new strains, or a combination of various techniques, is being carried out. Presently the number of commercially available strains are increasing and as such definitive techniques to identify commercial wine yeast strains, e.g. PCR and DNA fingerprinting (Lavallée *et al.* 1994) must be implemented. These techniques can also be used as selection tools, in quality control and the monitoring of strain stability during industrial propagation. Strains selected nowadays have more than a simple role of fermenting sugar into ethanol and they may even (due to the advances of genetic engineering) now be selected for very specific enzymatic activities e.g. strain VL1 has significant β-glycosidase activity which can release bound terpenes in muscat and sauvignon grape varieties (Degré 1993). These strains are not only being selected for their own particular properties but on the interaction of selected strains relying on the exaltation of the varietal specificity and harmonisation of the relationship between the yeast strain and the grape variety itself. Care must be taken with the use of genetically engineered strains however, since wine is an end-product produced for human consumption. In enhancing a particular characteristic one must look at the overall physiological effect and metabolic processes which consequently may be affected by the genetic alteration. If the final taste or wine quality is adversely affected or reduced by a significant degree, or more importantly, if the health implications of the resultant wine are affected due to the genetic alteration made, then this would render the strain unfit for wine production.

The use of selected yeasts has evolved from the use of pure culture slants and lyophilised cultures to the use of dry fermentation starters. The greatest technological breakthrough in the microbiology of wine has been that of the development of active dry yeast (ADY) to be used as selected starter cultures for winery fermentation. The advent of ADY in the major wine producing regions of the world makes it easier to achieve efficient wine-making with concomitant microbiological purity. The use of ADY relieves the vintner of the process of

maintaining pure cultures throughout the year and the preparation of a "pied de cuve". This has the further benefit of improved productivity since active dry yeast show increased fermentation capacity compared to a "pied de cuve" from the same strain at the same concentration. This may be due to the fact that dried yeast are derived from strongly aerated cultures, hence they are much richer in sterols, saturated fatty acids and trehalose and thereby the yeast has an improved ability to start fermenting, an essential consideration when one must remember the starter must compete with the natural microflora (wild yeast) present in the must.

Production of dry wine yeast is traditionally associated with baker's yeast production and originally baker's yeast were used to restart 'stuck' fermentations (Degré 1993). In the early years, strains marketed as wine yeasts were the same as used for bakers yeast production (Rosini 1980) although nowadays specific wine yeasts are propagated. Large-scale production of commercial dry (ADY) wine yeasts (*Sacch. cerevisiae* and occasionally *Sacch. bayanus*: Champagne) occurs usually in bakers yeast plants of the major yeast companies, as the physiological and technological principles of production are essentially the same as in bakers yeast production (Degré 1993) although, several technical properties of wine yeasts are influenced by the way the yeast is grown and dried. Generally, these cultures are grown aerobically, using fed-batch procedures, on molasses (as main carbohydrate source) supplemented with ammonium hydroxide (as nitrogen source), phosphoric acid (phosphorous source) and other growth factors, vitamins and minerals. Commonly a mixture of cane and beet molasses is employed to attempt to compliment the initial vitamin and mineral contents of the base medium e.g. use of at least 10-20% cane molasses to overcome the biotin deficiency of beet molasses (Degré 1993). Chapter 4 of this Thesis focuses on certain aspects of industrial yeast physiology during propagation on molasses.

1.2.2.3 Nutritional Influences

Strain selection has assisted in industrial fermentation (wine) problem solving, however, problems still occur in the industry including; apparent sporadic occurrence of incomplete fermentation ('stuck' fermentation), hydrogen sulphide formation and excessive volatile acidity (Henschke & Lee 1991). It must be recognised that, although strain selection may help in solving these problems, vinification is a complex interaction between yeast, must and physical conditions. The importance of yeast is displayed in terms of strain and the physiology of such. Physical conditions such as fermenter design, temperature control and level of agitation during fermentation, all play an important part, however the composition of grape must plays the greatest part in œnology. Nutritional composition of the grapes and thus the fermentation medium can vary extensively as a consequence of normal seasonal variation, growing regions, cultivation conditions or grape variety and even varying for one area and grape type, from season to season and cultivar to cultivar.

The influence of the chemical composition of grape must in determining wine flavour is well known. Of greater importance though, is the fact that the nutritional complexity of must is critical to yeast growth and metabolic activity. The composition of grape must cannot be exactly defined, it is a complex media and each grape variety has a unique chemical composition. The climatic and soil conditions which affect growth of the vine influence the composition of the grape and hence the organic and inorganic composition of the must varies, dependant on the vintage and climate the vine is grown in, for example, levels of organic acids are higher in vines grown in cold-climate regions.

The role of inorganic nutrition in the case of wine yeasts, as with other microbial species, has generally been overlooked, despite the appreciation of the role of these elements in cellular biochemistry. It is beginning to be understood that more research must be done into the area of nutrient availability in various grape juices and strain differences in response to nutrient limitation, to determine the factors affecting glucose utilisation and control of fermentation rates (Bisson 1986). The story is much wider than this and little research has

been carried out on the inorganic nutrition of yeasts, especially in the case of the metal ions: magnesium and calcium. Fundamentally, one must look at the effect of inorganic nutrition at the level of the yeast and the response this causes in terms of growth and physiology of wine strains, so as to have an understanding of the fermentation process and for maximisation of the yeasts' abilities. As Fleet and Heard (1993) state; to fully understand the role of yeasts in fermentation it is essential to know:

- the taxonomic identity of each species contributing to the fermentation
- the kinetics of growth of each species
- the biochemical properties of these yeasts and the chemical changes they produce
- the influences of vinification factors upon the kinetics of growth and chemical change.

1.3 THE ROLE OF METAL IONS IN YEAST PHYSIOLOGY

The nutritional complexity of grape must plays a large part in the wine produced, in terms of growth of the yeast strain and therefore the sensory complexity of the wine. Many studies have been carried out into the environmental and nutritional factors influencing yeast growth and metabolism, with sugar metabolism (glucose and fructose in the case of grape must) and oxygen availability being the most documented. Metal ions play important roles in brewing. In relation to yeast growth and fermentation, zinc and calcium are significant. Calcium is important in wort pH regulation, oxalate precipitation and in governing yeast cell-cell flocculation in brewing yeasts. The roles of magnesium ions in industrial fermentations, however, have not been fully appreciated (Walker *et al.* 1996). Little attention has been paid, at all, to inorganic nutritional factors in influencing the growth and physiology of wine yeasts.

Yeasts, like most microorganisms, have a strict nutritional requirement (both organic and inorganic) in terms of macronutrients (required at a level of approx. 10^{-3}M): carbon,

nitrogen, hydrogen, oxygen, phosphorous, potassium, magnesium and sulphur, and micronutrients (required at approximately the 10^{-6}M level): calcium, copper, iron, manganese, zinc, nickel and molybdenum (the so-called "trace" elements). An understanding of how these elements are supplied and metabolised by the organism, is essential in order for the optimisation of growth and metabolic activities. Often neglected in this consideration are the inorganic/ionic constituents of a particular growth or fermentation medium. The requirement of yeast for minerals is similar to that of other cells, with a supply of potassium, magnesium and several trace elements being necessary for growth, in fact metals are directly and/or indirectly involved in all aspects of microbial growth, metabolism and differentiation (Gadd 1992). Metal ions are essential to yeast growth and physiology and out of the 109 elements known, 30 are believed to be necessary for the survival of living organisms (Dedyukhina & Eroshin 1991). Essential metal ions have many functions in microbial cells, not all of which are fully characterised. Such functions may reflect their fundamental chemical properties e.g. charge, size, redox properties and rates of ligand exchange (Hughes & Poole 1989). A balanced requirement for inorganic ions is exhibited by yeast cells and appropriate concentrations of these elements allow accelerated growth and increased biomass yield, accelerated ethanol production or both, with a higher final substrate to product yield. An imbalance in ionic nutrition is reflected in complex, and often subtle, alterations of metabolic patterns and growth characteristics (e.g. morphology and tolerance to the environment: Jones & Greenfield 1984). The roles of these essential minerals are extremely diverse, influencing all aspects of microbiology.

Categorisation of the roles played by these essential inorganic ions can be divided mainly into; enzymatic and structural. Considerable attention has been focused on metalloenzymes and it has been estimated that up to one third of all known enzymes contain a metal ion as a functional participant (Dedyukhina & Eroshin 1991). Ions function as the catalytic centre of a particular enzyme, as either an activator or stabiliser of enzyme function, or to maintain physiological control by causing antagonism between activators and deactivators. Zn^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} are common catalytic centres, whilst Mg^{2+} acts as one of the most common activators of enzymatic activity and K^{+} commonly functions in the role of a metal

coenzyme (Jones & Greenfield 1984). All ions appear capable of antagonism and inhibition at appropriate concentrations. The structural role is particularly relevant to the function of the plasma and other membranes and several studies link specific divalent metal ion interactions (mainly Mg^{2+} and sometimes Ca^{2+} in the range of 0.5 to 10mM) with enhanced tolerance to the inhibitory and deactivating effects of ethanol (Dombek & Ingram 1986; Jones 1987).

Important roles of ionic metals, therefore, include: maximising growth rate, formation of charge and concentration gradients across membranes which may be used in transport processes (Gadd 1992), intracellular compartmentation, osmotic responses and sensing (Borst-Pauwels 1981; Hughes & Poole 1989; Jones & Gadd 1990), stabilisation of cellular structures e.g. cell walls, organelles and membranes, synthesis and stabilisation of biomolecules e.g. enzymes, proteins and nucleic acids, heat tolerance and in redox catalysts (Hughes & Poole 1989). It has been suggested that cations are required to reduce electrostatic repulsion between lipids and proteins, thereby increasing membrane stability (Dedyukhina & Eroshin 1991). For polyphosphate, DNA, RNA and proteins, the ion species K^+ and Mg^{2+} are most commonly encountered in this role. The charged structural membrane phospholipids are shielded principally by Ca^{2+} and Mg^{2+} (in whole membranes Zn^{2+} also appears to play a role) but can be shielded by other multivalent cations. Cell wall phosphomannan is typically complexed to Ca^{2+} although other cations can freely replace this ion. Potassium, for example, plays a central role in the regulation of yeast growth and fermentation under aerobic and fermentative conditions: Na^+/K^+ pump (Jones & Greenfield 1984) and phosphorous plays a central role in both energy metabolism and in the biosynthesis of membrane phospholipids, with phosphorous deficiency limiting ethanol production, as does its excess (Jones & Gadd 1990). Both the structural and enzymatic roles of any ion are important in defining the optimum concentrations of growth and fermentation (Jones & Greenfield 1984). Ionic nutrition as a whole will consist of contributions derived from both the metabolic and structural events within the cytoplasm. Direct relations between ion transport and metabolic events may sometimes be obscured by these dynamic interactions (Jones & Gadd 1990), for example, the levels of Mg^{2+} and Zn^{2+}

in yeast cells are highly regulated due to their central role in glycolysis (Jones & Greenfield 1984).

Not all inorganic species are essential. Essentiallity can be defined as, the deprivation of an essential metal ion which will, by definition, ultimately, result in death. However, broader definitions of essentiallity may only refer to impairment of growth, reproduction or other function(s) in the absence of that 'essential' metal and the fact that its beneficial effects cannot be completely replaced by any other element (Dedyukhina & Eroshin 1991). Requirements for essential chemical elements depend on strain properties and on the environmental conditions of growth. Elemental composition of biomass depends to a great extent on the microbial growth rate (Dedyukhina & Eroshin 1991), which in turn depends on the oxygen supply to the medium and in fact, Jones and Greenfield (1984) noted a large variation in the biomass elemental composition of yeast. The intracellular ionic composition of yeast is different from that of the external environment (Jones & Gadd 1990). The importance of essential chemical elements for microbial metabolism is known, but little attention has been paid to the amount of trace elements in the medium nor have the strain properties and cultivation conditions been taken into account. Optimum concentrations of the various cationic species required for growth and fermentation of yeasts of the genus *Saccharomyces*, have been determined for defined media (Table 1.3: Jones & Greenfield 1984). This optimum concentration, however, depends largely on the concentrations of other ionic species present in the medium, due to a range of ion-ion interrelations which would affect this so-called "optimum". When any ion, either essential or toxic, is present at inhibitory levels, the required concentration of Ca^{2+}, K^{+}, Mg^{2+}, Mn^{2+} and Zn^{2+} will also be elevated (Jones & Greenfield 1984). The nature of the growth-limiting nutrient in the medium can exert a considerable effect on the composition of the yeast biomass (Dedyukhina *et al.* 1989). Metabolism of yeast depends substantially both on the nature of the growth-limiting component and on the medium concentration of mineral components which, while not growth-limiting, nevertheless influence the intensity of distinct metabolic processes (Dedyukhina *et al.* 1989). In addition, requirement for any one ionic element will decrease if, in a complex industrial medium, the end-product of a particular metalloenzyme

catalysed reaction is supplied by the medium itself e.g. purines, amino acids etc. (Jones & Greenfield 1984). The actual ionic composition of the medium, therefore, has significant effects on cellular composition and levels of Mg, K, Ca, Co, Cu, Mn, Zn and Fe vary significantly over a wide variety of complex environmental changes.

Table 1.3 Growth optimum and intracellular ion concentrations of various ionic species for yeast.

Ion	Growth Optimum (M)	Intracellular Concentration (mole/100g DW)
B^+	0.4-1.0μM	0.5μmole
Ca^{2+}	0.5-5.0mM	0-1.5mmole
Co^{2+}	0.1-1.0μM*	0.03-1μmole
Cu^{2+}	1.0-10.0μM*	8-200μmole
Fe^{2+}	1.0-10.0μM	36-180μmole
K^+	2.0-10.0mM	8-56mmole
Mg^{2+}	2.0-4.0mM	4-17mmole
Mn^{2+}	2.0-10.0μM	7-55μmole
Mo^{2+}	1.0-10.0μM	0.04-0.08μmole
Ni^+	1-50μM	0.03-2μmole
Zn^{2+}	5-15μM*	80-300μmole
Cl^-	~1mM	11-140mmole
I^-	~1μM	0.04-1μmole
SO_4^{2-}	~1mM	0.6-15.0mmole
$H_2PO_4^-$	2-4mM	40-65mmole
NH_4^+	~1mM	~0.7mole

* Ions most likely to be deficient in complex media. (Jones & Greenfield 1984)

In a complex inorganic medium such as grape must (and other industrial media, e.g. molasses) the scenario of supply and demand and in fact the applications of these recommended concentrations (optimum) in an industrial context is complicated further by the chelation of metal ions by organic components of the fermentation substrate, therefore limitation of inorganic nutrients may be more prevalent than is realised (Jones & Greenfield 1984). Most major industrial fermentation media such as molasses, malt wort and grape must consist of complex plant materials with a correspondingly more complex ionic composition than defined media and metal ion composition of wort will vary greatly depending on raw materials and process conditions (Walker *et al.* 1996; Saltukoglu & Slaughter 1983) a fact also true of grape must. Ionic imbalances occur and most industrial

media (malt wort excepted) have the incorrect balance of magnesium and calcium, such that the relative concentration ratios favour calcium rather than magnesium, which may not be conducive to fermentation (Walker 1994). Ionic deficiencies occur in the majority of natural sources of carbohydrates that are used as alcoholic fermentation feedstocks. The optimisation of ion concentrations in complex industrial media can be difficult.

Complications occur in industrial fermentation media due to the fact that they contain a range of chelating, sequestering and adsorbing materials which act to reduce the effective available ionic concentration (Lie *et al.* 1975; Jones & Greenfield 1984). Organic material such as; humic acids (Jones & Greenfield 1984), amino acids, proteins, organic acids, polyphenols, phytic acid, polyphosphates and other colloidal materials, etc. (Lie *et al.* 1975) act as a sink for removal of available ionic species. These materials chelate and sequester the various ions with differing affinities and rates of association. Ca and Mg, for example, are bound by the reactive groups in the order: $RCOO^- > RHN^- > RS^- > H_2O$ but these two metals have different reaction rates (in decreasing order: $Sr^{2+} > Ca^{2+} > Zn^{2+} > Mn^{2+} > Fe^{2+} > Co^{2+} > Mg^{2+} > Ni^{2+}$) therefore at equilibrium, Ca^{2+} and Mg^{2+} will usually be in the free form available to yeast. If complexed, calcium will rapidly be made available, magnesium, however, when complexed will only be slowly released (Jones & Greenfield 1984). Therefore the bioavailability of metal ions to yeast is governed by competition between insoluble and soluble chelators in wort (Lie *et al.* 1975; Jacobsen & Lie 1977). The imbalance of these ionic species leads to complex alterations of metabolic patterns and growth characteristics (Jones & Greenfield 1984), with an excess of most cations being inhibitory. The toxic effects caused by both the essential and non-essential metal ions are a result of their strong co-ordinating abilities; including blocking of functional groups of enzymes, of essential metal ions from cellular sites and transport systems, denaturation and inactivation of enzymes and disruption of cell and organelle membranes (Karamushka & Gadd 1994). The cations K^+, Mg^{2+} and Ca^{2+} directly influence sugar fermentative metabolism by yeast and the correct balance and the availability of these cations are vital for a successful fermentation process. Appropriate concentrations of these elements allows for accelerated growth and increased biomass production and ethanol fermentation (Jones &

Greenfield 1984). Although an appropriate value can be placed upon optimum requirements by yeast for metallic elements, there exist a wide range of ion-ion interactions, resulting in the optimum concentration being highly dependent on the concentration of other ionic species (Jones & Greenfield 1984). Cellular ionic composition of yeast is highly dependent on the yeast species and the type of environment in which the yeast is grown. The influence of other physico-chemical attributes, e.g. pH or ionic strength, can also affect ionic availability (Jones & Gadd 1990). Substitution, displacement dependant upon the strength of the individual ligand complexes (Irving-William Series) and on the concentrations of ligands and metal ions present in the system, can occur. Non-essential cations can substitute essential cations in biological systems e.g. metalloenzymes, with subsequent loss of activity of the enzyme (Jernelöv & Martin 1975).

Meeting minimum requirements for certain elements is complicated by their role as 'antagonists' to the inhibitory effects of other ionic species and by other metabolic interrelations which determine final uptake and effect, e.g. Ca^{2+} is an antagonist to the inhibitory effects of excess Mg^{2+}, and the uptake of $H_2PO_4^-$ (phosphate critical for cell growth: Soumalien & Oura 1971) is dependent upon the presence of Mg^{2+} (Jones & Greenfield 1984). Ionic relations illustrate complex factors that may govern the growth of yeast during a fermentation designed to produce ethanol. For example:

i) Mg^{2+} acts to stimulate both specific growth rate and cell division (replication)

ii) Ca^{2+} and Mg^{2+} are competitors/ antagonists to the action of each other and a 10x excess of Ca^{2+} over Mg^{2+} prevents cell growth, 3x excess increases lag phase and decreases specific growth rate (Saultukglu & Slaughter 1983) but has little effect on biomass yield.

iii) High concentrations of Cd^{2+}, Cu^{2+} or Zn^{2+} and other metal ions can be toxic and induce leakage of UV-absorbing materials and K^+ from yeast cells.

iv) Monovalent cations, e.g. K^+ may antagonise the uptake of divalent cations e.g. Mg^{2+}/Ca^{2+} and vice-versa (Borst-Pauwells 1981).

v) both intracellular and external pH can affect yeast ionic uptake and nutrition (Jones & Gadd 1990).

Virtually all metals whether essential or inessential can exhibit toxicity above certain threshold concentrations (Gadd 1992). Although specific ionic interactions may exist it is pointed out that media containing abundant organic material will be effectively buffered with respect to ionic availability of, especially, the toxic species (Jones & Greenfield 1984).

Metal Ion Uptake

Essential elements (and others) can interact with microbial cells and be accumulated as a result of physico-chemical mechanisms and transport systems of varying specificity, independent of, or directly and indirectly dependant on, metabolism (Gadd 1992). The cell wall is an important site of initial biological interaction between cells and external metal ions. This and the cell membrane form a selective barrier which can control both the influx and the efflux of metal ions and the uptake, excretion and cellular distribution of ions vary according to the specific metal and its reported roles in cell metabolism. Of central importance to the study of the physiological mechanisms involved in the uptake of ions by yeast, are the transport properties of the yeast plasma membrane, since this is the primary biological site for interaction with the external environment (Jones & Gadd 1990). The permeability of pure lipid bilayers is low (10^{-9} -10^{-1} cms^{-1} for OH^-, H^+ and Cl^-: Jones & Gadd 1990), biological membranes (protein and lipid) have permeabilities up to 100x greater, however, despite this additional mechanisms are required to facilitate transport of essential ions into the cell and act to regulate the intracellular ionic environment. The plasma membrane is a selective barrier which is permeable to H_2O, O_2, CO_2, free ammonia, ethanol and organic acids (unionised) by passive diffusion, in addition a variety of systems can be utilised for the uptake of ions into the cytoplasm (Jones & Gadd 1990). The main uptake systems are; diffusion channels or active transport. Diffusion channels exist in yeast for certain ions as voltage dependant "gates", to transiently move ions down concentration gradients. Active transport, on the other hand, is a concentrative, energy-dependent mechanism based on chemiosmotic principles which is responsible for the uptake of the

majority of nutrients into yeast cells, against a concentration gradient. This enables nutrients to enter with influxed protons "symport" mechanisms, or against effluxed protons as in "antiport" mechanisms.

Transport of monovalent cations (M^+) is linked to the action of the plasma membrane-bound H^+-ATPase that expels protons, thus creating a transmembrane electrochemical proton gradient ($\Delta\mu_{H^+}$), the membrane potential ($\Delta\Psi$) that drives electrophoretic monovalent cation transport into the cell (Jones & Gadd 1990). Divalent cations such as Mg^{2+}, Ca^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} are essential for growth and metabolism and therefore need to be accumulated from the external environment. Interrelationships exist amongst many monovalent and divalent cations for their respective uptake systems. Ca^{2+} and Mg^{2+} enter *Sacch. cerevisiae* as low affinity substrates of the monovalent cation transport system (diffusion channel). Energy dependent systems of *Sacch. cerevisiae* are utilised for the ions $Mg^{2+}/Co^{2+}/Zn^{2+}>Mn^{2+}>Ni^{2+}>Ca^{2+}>Sr^{2+}$ with decreasing affinity for systems (Fuhrmann & Rothstein 1968). Structural integrity and efflux of cations may also result in permeability changes. For the majority of divalent cations, though, transport is dependent on plasma membrane H^2-ATPase energising the cell membrane (active transport).

Uptake depends on the metabolic state of the cell and varies with growth media and conditions; differences in kinetic parameters, *i.e.* differences between strain, binding or complexation effects involving wall materials, components of buffer or suspending medium, toxic effects and differences in thermodynamic conditions. Competition between di- and mono-valent cations e.g. Ca^{2+}/Mg^{2+} and K^+ tends to be negligible, but di and polyvalent cations can affect monovalent cation uptake by binding to and screening negatively charged groups on cell membranes which decreases the negative surface potential (Ψ) (Jones & Gadd 1990). pH, both extra- and intracellular is another factor which would influence transport of ions into yeast cells. Therefore, the uptake of inorganic ions is a complex function of medium composition, including other ions, diffusible organic acid species and overall medium buffering capacity and ionic nutritional requirements should be varied dependent on media type etc. (Jones & Gadd 1990).

Metal ions are highly important nutrients for yeast growth, as are carbon, nitrogen, hydrogen and oxygen sources for growth and optimal functioning of cells. The recent literature has, however, paid little attention to inorganic nutritional factors in influencing the growth and physiology of wine yeasts. Some metal ions are deleterious or toxic to yeast viability, but a wider range of ionic substrates are essential in, at least, trace amounts, with the absence of some of these, from the culture medium, seriously inhibiting cell growth and development. The most noticeable effects on fermentation will be produced by deficiencies in Mg^{2+} and K^{+} (Jones & Greenfield 1984). Magnesium (Mg^{2+}) and calcium (Ca^{2+}) will be considered in fuller detail to give some background into two of the most important ionic nutrients in yeast physiology, given that they are essential elements (Jones & Greenfield 1984) and they are known to interact in cell physiological functions to the extent of antagonism (Williams 1976).

1.3.1 MAGNESIUM

Magnesium has long been recognised as an essential biological element and for the past 60 years it has been known to be essential for all living things (Wacker 1993). Historically, the biological role of magnesium was first acknowledged due to its pharmacological properties, in the form of Epsom Salts ($MgSO_4$) an osmotic purgative! Magnesium fulfils a requirement as a nutrient in all phyla and species, and it plays a role in enzymatic function; in all reactions requiring ATP and other nucleotides, including phosphorylation; it has a structural function in supporting tissues, proteins and membranes; is an active component in chlorophyll and is involved in modulating intracellular metabolism. It is an essential element in virtually all the major biological processes in many genera: DNA and RNA synthesis; protein synthesis; nerve conduction; muscle contraction; membrane transport; cell division; oxidative phosphorylation and photosynthesis (Wacker 1993).

The fifth most abundant element on the Earth's surface and the second most abundant cation in cells, magnesium is centrally important to cellular metabolic and physiological function.

It is, like calcium, a divalent cation (alkali earth metal) of the GroupIIA: transition metal cations. Mg^{2+} is a small ion (ionic radius = 0.65Å; $^2/_3$ of Ca^{2+}) and being six-co-ordinate, it forms regular structural complexes (Hughes 1981). It is the 'hardest' and most charge dense (Maguire 1990) of all biologically relevant cations exhibiting high electropositivity and low polarizability, with a tendency for electrostatic bonding (Gadd 1992) and in general, it is non-exchangeable; other cations can very seldom substitute for magnesium in any of its biochemical and physiological functions. Yeast cells have a high demand for Mg^{2+} ions and the growth requirements cannot be met by other metals. The growth optimum for Mg^{2+} is between 2.0 and 4.0mM, with a cellular concentration around 4-17 mmole/100g DW (Jones & Greenfield 1984). The high intracellular content of free Mg^{2+} (in the mM range) is manifested in the essential structural and metabolic functions of yeast and in fact, magnesium has a free ion concentration of approx. 1mM both inside and outside the cell (Williams 1974).

Simply due to the chemistry of Mg^{2+}, in terms of its relationships with other ions of interest, there will be circumstances in which Mg^{2+} will be in competition for binding sites with several ions. Competition arises between Na^+ and K^+ because these ions are 100 times more concentrated in biology, so the intrinsic weaknesses of general ion interaction between Na^+ and K^+, and anionic and poly-electrolytes are balanced in mass action effects, by the specific binding of magnesium ions. The competition with Mg^{2+} for organic anion sites is largely based on the general ionic atmosphere of monovalent ions, however with Ca^{2+} (and some other divalent ions) the competition for sites is based mostly on their specific and often selective bindings (McDonald & Martin 1988). Mg^{2+} does not bind to the vast majority of biological substrates, e.g. mono- and even many di-carboxylates, amino acids, mononucleotides, sugar phosphates, sulphates, etc. because the negative charge density on almost all small substrates with single anion centres (e.g. RSO_4^-, $R_2PO_4^-$, RO^-, RSO_4^{2-} & RPO_4^{2-}) is too low to bind Mg^{2+} (Williams 1993). In fact, Mg^{2+} is less comfortable than Ca^{2+} in binding to large multidentate anionic ligand groups (Martin 1990). In the mineral world, interactions between Mg^{2+} and Ca^{2+} are the second most common interactions (Williams 1993), and these two ions tend to act antagonistically toward each other

(Williams 1976). Equilibrium stability and structural differences account for the differentiation between Ca^{2+} and Mg^{2+} in biological systems.

Magnesium Uptake in Cells

Biological uniqueness of Mg^{2+}, compared with other alkaline earth metal ions; Ca^{2+} included, is portrayed in the slow rates of ligand exchange in and out of the metal ion co-ordination sphere (Martin 1990). The stability of the structure (magnesium ions are held in an octahedral surround of six oxygen atom donors) is the cause of the slower on-rates in magnesium ion complex formation. Also, Mg^{2+} to ligand donor atom bond distances vary less than those of Ca^{2+}, *i.e.* Ca^{2+} forms looser complexes of higher and variable co-ordinate number without directionality and with variable bond lengths, hence Ca^{2+} will, in some cases, outperform Mg^{2+} due to its stronger affinity (Jones & Gadd 1990) yet, magnesium can antagonise calcium's action by de-stabilising stable calcium complexes (Williams 1970). Magnesium and calcium often exhibit an inverse functional relationship by acting antagonistically toward each other. Several chemical differences between them regarding complex formation and stability, serve to explain their competitive cellular interactions. Certainly the relationship between magnesium and calcium is complex; cells actively exclude calcium and actively include magnesium. Interesting differences exist in the structures of Mg^{2+} and Ca^{2+} relevant to the differing biological roles of the two cations, Mg^{2+} tends to be bound by nitrogen donors in biological systems, while Ca^{2+} prefers ligands with oxygen donors (Hughes 1981; Martin 1990). Magnesium acts as a slow control and may regulate many processes. Calcium is, in contrast, a fast-acting divalent ion (based on water exchange rates) and a principal second messenger in biology. Being an intracellular cation, magnesium is the activator of intracellular enzymes; calcium, being an extracellular cation, is the protector of some secreted (usually hydrolytic) enzymes. Generally speaking, therefore, magnesium is regarded as an intracellular activator and a structure-breaker (Williams 1970). It may also act as a second messenger and as a regulator in its own right, independent from calcium (Walker & Duffus 1983; Walker 1986; Maguire 1990). In fact, the increased

awareness of calcium has also drawn attention to the roles of magnesium, which in many situations have been shown to counter the actions of calcium.

Magnesium is present at a much higher intracellular concentration in yeasts than calcium (4-17 mmole/100g DW compared with 0-1.5 mmole/100g DW: Jones & Greenfield 1984) and it is *the* major intracellular divalent metal cation. It exists in two forms: free Mg^{2+} and bound Mg^{2+}, bound Mg^{2+} is thermodynamically unavailable for physiological and biochemical processes and free "activity" is relevant to intracellular control. Yeast tend to take up a constant amount of magnesium per cell, rather than absorbing magnesium in proportion to what is available in the medium and this seems to be consistent with the idea of magnesium as an important biochemical element (Saltukoglu & Slaughter 1983). Cells cannot absorb all the magnesium from the medium, even though as magnesium becomes limiting the proportion absorbed increases appreciably. Cellular content of Mg^{2+} changes less than two-fold in both eukaryotes and prokaryotes when the extracellular Mg^{2+} concentration is changed 10 000 fold (Webb 1948). The level at which magnesium becomes limiting will depend not just on the yeast but also on the composition of the medium, presumably due to the intrinsic characteristics of the magnesium pump and the chelating power of the medium (Saltukoglu & Slaughter 1983). Internal Mg^{2+} concentrations generally remain in the millimolar range regardless of the external concentrations (Frausto da Silva & Williams 1991). In the cytoplasm of cells, free magnesium ion concentration appears to be approx. 0.5mM (Grubbs & Maguire 1987) although this is inherently difficult to measure. Comparing the intracellular concentrations of Mg^{2+} and Ca^{2+}, it is recognised that there are characteristic differences between these two cations such that there exists a considerable Ca^{2+} gradient (high outside) across cell membranes and a less substantial Mg^{2+} gradient in the opposite direction. Most active cells maintain high (millimolar) concentrations of free Mg^{2+} and low (nanomolar) concentrations of free Ca^{2+} (Walker 1994).

In relation to cellular Mg^{2+} transport, active mechanisms have been demonstrated for yeasts (Fuhrmann & Rothstein 1968; Borst-Pauwells 1981). Mg^{2+} transport, as with other

essential nutrients, is strictly regulated in response to changing growth conditions, e.g. total intracellular Mg^{2+} ion concentrations remain relatively constant even when extracellular levels increase dramatically, however, when extracellular levels become limiting, yeast growth rate and cell Mg^{2+} contents are then found to be directly proportional to available media Mg^{2+} concentrations (Joho *et al.* 1991). Protein pumps which control cellular Mg^{2+} levels are involved in Mg^{2+} uptake (Maguire 1990). In yeast and fungi, Mg^{2+} uptake is a biphasic process - firstly biosorption occurs in which a metabolism-independent surface binding to fixed anionic groups on cell walls takes place (unspecific with Ca^{2+}, Mn^{2+}, and Sr^{2+}), this is followed by a slower, energy-dependent uptake at the level of the plasma membrane. Mg^{2+} can also be taken up by the K^{+} transport system, but affinity for this system is low (Borst-Pauwells 1981). In *Sacch. cerevisiae*, divalent cations are actively transported according to the affinity series: Mg; Co & Zn > Mn > Ni > Ca > Sr (Fuhrmann & Rothstein 1968) and both surface binding and membrane uptake of Mg^{2+} are inhibited by Ca^{2+} ions (Borst-Pauwells 1981). In addition to this, Mg^{2+} uptake is well known to compete with that of Ni^{2+} and Co^{2+} in *Saccharomyces* (Fuhrmann & Rothstein 1968). This apparent exchangeability suggests that while many metabolic reactions have an absolute requirement for Mg^{2+} ions, there are many others where the exact cation is not crucially important (Walker 1994). In *Sacch. cerevisiae* Mg^{2+} transport appears to correlate with fermentative activity (Maynard 1993). The decline in fermentation rate that begins at low alcohol concentrations appears to be related, in part, to magnesium deficiency and cells in magnesium-supplemented cultures were shown to maintain a higher fermentation rate as ethanol accumulated (Dombek & Ingram 1986). The bioavailability of metal ions to yeast is also governed by competition between soluble and insoluble chelators in the medium (Jacobsen & Lie 1977; Jacobsen *et al.* 1981) and in turn the biological effects of Mg^{2+} are caused by its ability to form chelates. The chelate-forming properties of Mg^{2+} are related to substrates, enzymes and to substances of cell structure, e.g. proteins, phospholipids and nucleic acids (Günther 1981). Due to its effects on enzymes and cellular structures, Mg^{2+} is involved in most reactions of carbohydrate, lipid, nucleic acid and protein metabolism, as well as in energy-producing (glycolysis, oxidative phosphorylation) and energy-consuming (active transport, muscle contraction) reactions.

Cellular Role of Magnesium

Magnesium is essential for multicellular organisms, but more important are the functions of magnesium at the intracellular and sub-cellular level (Table 1.4) where it serves absolutely essential biochemical roles (Walker 1994). Many of these roles have now been identified clearly as regulatory ones, specific and independent of calcium. Magnesium is unquestionably essential for the growth of cells, exhibiting in *Sacch. cerevisiae* for example, a K_S value (the concentration of Mg^{2+} that limits growth to half its maximum specific rate) equalling 35.7μM [Mg^{2+}] (Maynard 1993; Walker & Maynard 1996). Magnesium is involved in almost every metabolic and bioenergetic pathway in the cell. Whereas Ca^{2+} needs calmodulin to activate many cellular processes, Mg^{2+} requires no intermediary to exert its co-ordinate control, but can produce broad metabolic and growth effects based on its own intrinsic properties (Sanui & Rubin 1982b). The physiological role of magnesium is primarily intracellular where it serves as a cofactor for enzymes to maintain conformation of nucleic acids, to stabilise ribosomes and generally to maintain the structural integrity of membranes in cells and organelles.

Table 1.4 The roles of magnesium in yeast physiology.

GROWTH:	Cell division control		(Walker & Duffus 1979;1980;1983)
	Cell size mediation		(Walker & Duffus 1980; Walker 1986)
	Cell-Cell flocculation		(Nishihara *et al.* 1976)
CARBOHYDRATE METABOLISM:	Glycolytic activity		(Walker *et al.* 1990a;1990b)
	Mitochondrial function		(Walker *et al.* 1982)
FERMENTATION:	Progress of ethanol production		(Dombek & Ingram 1986)
	Progress of higher alcohol production		(Gildenhuys & Slaughter 1983)
	Protection of cells from:		
		ethanol	(Dombek & Ingram 1986)
		high temperature	(D'Amore *et al.* 1989)
		high osmotic pressure	(Dasari *et al.* 1990)

Magnesium is reportedly required for the functionality of more than 300 enzymes (Günther 1981; Saltukoglu & Slaughter 1983; Walker *et al.* 1995). Conformational changes of enzymes are caused by Mg^{2+} during the catalytic process, resulting in increases in catalytic activity proportional to the logarithm of concentration of free Mg^{2+} (Günther 1981). Mg^{2+} is an intracellular activator and regulator of enzymes and works by two major mechanisms; either by the formation of an active substrate, e.g. $Mg(ADP)^-$ for ATP synthetase (Heaton 1990) or by Mg^{2+} binding directly to an enzyme to produce the conformational changes essential for catalytic activity, although this second mechanism is less common. The activities of the key enzymes of the Embden-Meyerhof-Parnas pathway (Glycolysis), *i.e.* hexokinase, phosphofructase, phosphoglycerate kinase, enolase and pyruvate kinase possess an absolute requirement for magnesium and in yeast, carbohydrate catabolic pathways are strongly influenced by Mg^{2+} depletion (Walker *et al.* 1982; 1990b). In addition to this, virtually all enzymes with phosphate cofactors require Mg^{2+} for their function, *i.e.* kinases, synthetases and phosphatases all require Mg^{2+} for activation. The strict magnesium dependence of phosphate transfer enzymes, particularly those involving ATP, and of enzymes involving the synthesis, expression and translation of genetic information, serves to highlight the biological essentiallity of this metal (Walker 1994).

Magnesium is vitally important in stabilising the structures of nucleic acids, proteins, polysaccharides and lipids, in relation to the maintenance of cell integrity. It binds in cell walls, to membranes and to many proteins and enzymes, but some of these properties also happen to be shared with calcium. However, it is the one metal ion which can interact directly with DNA and RNA. Magnesium is involved in cell envelope stabilisation and in preservation of permeability barriers (Brock 1962). The ion is involved in the structure of ribosomes and neutralises electrostatic forces present in polyphosphates, DNA, RNA and proteins (Jones & Greenfield 1984) and magnesium also provides shielding to ionic sites. The denaturation of DNA is associated with a release of magnesium and tRNA can only bind amino acids when magnesium is present (Hughes & Poole 1989). Due to this, intracellular integrity is highly dependent on magnesium, with its involvement in sub-cellular structures and organelle stabilisation, *i.e.* ribosomes, mitochondria (Walker *et al.* 1982;

Walker & Birch-Andersen 1984) and microtubules (Prescott *et al.* 1988) all require magnesium, also it is essential in the nucleus for maintaining structural integrity of polynucleotides and chromosomes (Walker 1994). In yeast, when cells are deprived of Mg^{2+} they lose their normal shape due to cell surface (and cytoskeletal) de-stabilisation effects. In the case of environmental stress, magnesium may act in its capacity as a stabiliser of membrane structure effecting protection against, for example, high temperature or ethanol.

Magnesium plays a central role in fermentative metabolism, the importance of this ion in yeast fermentation is indicated by its effect on cellular metabolism, it directly influences fatty acid synthesis, activates glycolytic enzymes and stimulates uptake of phosphate in such a way to increase the fermentation, activates the enzyme plasma membrane ATPase enabling it to transport more cations which are required for fermentation, is involved in protein-protein membranes and in the protection of yeast cells from adverse effects of high temperature and high osmotic pressure. Cells growing in Mg^{2+}-supplemented media show a prolonged exponential rise in cellular protein and the addition of magnesium was also shown to enhance the rate of glucose consumption and consequent ethanol production (Dombek & Ingram 1986). Walker *et al.* (1996) found that by artificially elevating the level of magnesium in various fermentation feedstocks, improvements in both rates and yields of ethanol were observed. Such influences of Mg^{2+} ions may be expressed at the level of glycolytic enzyme activation (Mg^{2+} is known to specifically activate pyruvate decarboxylase) and cell membrane stabilisation. Maintenance of high Mg:Ca concentration ratios may serve to alleviate biological antagonism of essential Mg^{2+}-dependent functions by Ca^{2+} (Walker *et al.* 1995). It would appear, therefore, that straightforward addition of Mg^{2+} (in the form of magnesium salts) may be all that is required to ameliorate the inhibitory effects of excess Ca^{2+} commonly encountered in fermentation media (Wolniewicz *et al.* 1988; Walker *et al.* 1996), and by enhancing the bioavailability of Mg^{2+} corresponding enhancement of yeast fermentation performance is achieved. The presence of excess Ca^{2+} ions and low Mg:Ca concentration ratios may be counter-productive in terms of ethanol production by yeast. Perhaps then, the positive beneficial effects of Mg^{2+}

on yeast fermentation performance are expressed at two levels, namely: enzyme activation and membrane stabilisation.

Regulatory Roles of Magnesium

This involvement of magnesium in yeast fermentation and yeast physiology has as its basis a strict intracellular effect and it is most probable that this ion plays a major part in cell regulatory roles. Cell regulation can take many forms and many cellular processes require to be regulated for normal and efficient cell growth and metabolism to occur. Magnesium, it has been suggested plays a role in the regulation of mitochondrial activity and glycolysis, hence it is involved in regulating catabolic metabolism in cells. Another major regulatory role of magnesium is the control the ion exerts on cell cycle processes and sporulation. Both Ca^{2+} and /or Mg^{2+} have been proposed as the responsible agents for sporulation enhancement. Suizu *et al.* (1994) found that the addition of Ca^{2+} or Mg^{2+} was apparently effective in inducing sporulation in yeast cells in both nutrient-deficient and nutrient-rich media.

Respiratory activity of cells may be considerably affected by the presence of divalent cations in the incubation medium and Šubík & Kolarov (1970) demonstrated that Ca^{2+} inhibited the oxidation of physiological substrates, hence affecting respiration. Magnesium, on the other hand, is known to be important in maintaining mitochondrial structural integrity and bound magnesium is a necessary component of the mitochondrial coupling apparatus. Inorganic pyrophosphataseII in yeast mitochondria has an absolute need for Mg^{2+} up to concentrations equivalent to those of PPi (Uribe *et al.* 1993) and Mg^{2+} is a cofactor of the absolute majority of reactions occurring with the participation of ATP (Zvyagil'skaya *et al.* 1987). Since the mitochondria deal with respiration, a process involving the synthesis of ATP, Mg^{2+} is, therefore, an essential requirement of the mitochondria. Stimulation of respiration by Mg^{2+} may be due to adenylate kinase, or Mg^{2+}-ATPase, another Mg^{2+}-dependent system which catalyses the hydrolysis of ATP (Zvyagil'skaya *et al.* 1987). Zvyagil'skaya *et al.* (1987) however suggest that yeast mitochondria do not posses an

effective system of active transport of Mg^{2+}, but amounts uptaken are significant for the stabilisation of membranes, regulation of ionic permeability and to provide for effective occurrence of Mg^{2+}-dependent processes. So despite initial ideas that regulation of Mg^{2+} levels in cells may be linked to mitochondrial activity in higher eukaryotic cells (Crompton *et al.* 1976; Zvyagil'skaya *et al.* 1987) this role in yeast cells is most likely played by the vacuolar system (Okorokov *et al.* 1980). Mg^{2+} may play a role as a "chronic" regulatory agent, setting the 'gain' or sensitivity of the overall response system and it might thus play a role in the sensitisation or desensitisation of cellular response systems (Grubbs & Maguire 1987).

Role of Magnesium in Cell Division

Magnesium plays a fundamental role in cell division. Most structural and metabolic events that determine cell division can, at least in part, be explained on the basis of changes in the levels of intracellular free Mg^{2+} ions. Mg^{2+} plays a central regulatory role in the cell cycle and regulates the co-ordination of growth with karyokinesis (nuclear division) and cytokinesis (cell division). When cells of *Schizosaccharomyces pombe* are propagated under magnesium-deficient conditions they fail to undergo karyokinesis and complete cytokinesis but do continue for finite periods to synthesise protein and grow in size (Walker & Duffus 1980; Walker *et al.* 1982) suggesting a dissociation of the 'growth cycle' from the 'DNA-division cycle' (Mitchison 1971). This implies that the two cycles possess different magnesium dependencies, with maintenance of the growth cycle requiring only a threshold magnesium level that would be necessary for essential biosynthetic reactions, e.g. protein synthesis. Low intracellular magnesium levels may permit continuous protein synthesis associated with cell growth, whereas periodically high intracellular magnesium levels may operate to promote cell division (Walker 1985). In *S. pombe*, cell division can be synchronised by limiting magnesium availability to the extent that specific magnesium depletion appears to arrest cells late in the cell cycle, with the transition point most likely at the G_2/M boundary, unusual in eukaryotes (Walker & Duffus 1980). This arrest is reversible on the restoration of the magnesium supply (Walker & Duffus 1979). Cell

magnesium analysis in synchronised cultures of *S. pombe* has revealed that magnesium fluctuates in a manner which points to a regulatory role for this cation in the cell cycle (Walker & Duffus 1980;1983). Walker (1985) has demonstrated that total cell magnesium 'content' remains constant throughout most of the cell cycle, but that cell magnesium 'concentration' falls with increasing cell volume until the end of the cell cycle and a transient influx of magnesium, which may reach as high as 15mM, is observed at a period coinciding with cell division. Following completion of cytokinesis, controlled loss of magnesium occurs which effectively re-sets intracellular magnesium concentration of newly divided daughter cells in preparation for their next cell cycle (Walker & Duffus 1983). Cyclic fluctuations in intracellular magnesium during the cell cycle represent the fundamental means of co-ordinating cell growth and cell division (Walker & Duffus 1983; Duffus & Walker 1985; Walker 1986).

Walker *et al.* have over the years hypothesised that magnesium concentration acts as the fundamental transducer of cell size in control of the cell cycle and in particular of nuclear division (Ahluwalia *et al.* 1978; Walker & Duffus 1979;1980;1983) and that cell division possesses an absolute requirement for magnesium (Walker & Duffus 1983). The proposal that magnesium operates as a transducer of cell size which correlates cell growth to mitosis and cell division (cell cycle progression), the periodic variations in intracellular free magnesium acting to control the timing of chromatin condensation and mitotic spindle assembly (Staron & Jerzmanowski 1981), has been supported by studies using the ionophore A23187 (Walker & Duffus 1980). This revealed that selective reduction of intracellular free magnesium, but not calcium, homeostasis resulted in a re-timing of the cell cycle. Walker & Duffus (1983) deduced that cellular magnesium serves to regulate the polymerisation and depolymerisation of tubulin (microtubule proteins) and hence their subsequent assembly into a functional mitotic apparatus. Cell-cycle-specific changes in magnesium during mitosis act to promote the sequences of chromatin condensation-decondensation and elevation of ionised Mg acts as a trigger of metaphase chromatin condensation (Jerzmanowski & Staron 1980) and mitotic spindle assembly-disassembly (Jerzmanowski & Staron 1980; Staron & Jerzmanowski 1981; Walker & Duffus 1983).

Magnesium may be involved as a "second messenger" for growth stimulants in releasing cells from proliferative rest in the quiescent (G_0) phase of the cell cycle (Sanui & Rubin 1982b). Jazwinski *et al.* (1976) have shown that magnesium is required for the activity of a cytoplasmic initiator of DNA synthesis in extracts of proliferating cells (cancer) and thus could also play a role in removal of the G_1 phase block in the cell cycle. Increased magnesium ion concentration would then bypass cell cycle restraint at G_1 and G_2 and promote entry into DNA synthesis and mitosis (Walker 1985). This phenomenon has also been shown in organisms apart from the fission yeast *S. pombe*. Magnesium has also demonstrated cell cycle control in *Sacch. cerevisiae* (Walker *et al.* 1990b) and in fact, the hypothesis that magnesium is the fundamental cell cycle regulator can be applied to higher cells (Walker & Duffus 1983). Other organisms possess an absolute requirement for magnesium (but not calcium) for cell division and DNA synthesis (Walker 1986) suggesting a possible universal involvement for magnesium in microbial proliferation and further suggests that regulation by magnesium may have been conserved during evolution. The full story of how cells regulate magnesium must be fully understood before categorical statements can be made concerning the causative roles of magnesium in the cell cycle, however the variety of studies carried out to date lend much credibility to the view that magnesium is universally important in cell cycle control and general cell physiology.

Magnesium, due to its heterogeneous functions in yeast cell physiology, is without a doubt classed as an essential ion for yeast growth. Although some of its métiers are exchangeable with other biologically important cations, the majority of its roles are non-interchangeable making it a quintessential ionic nutrient. It must, therefore, be supplied in sufficient quantities in culture media for optimal growth and fermentation of yeast. In defined laboratory media this is usually not a problem, however in the case of complex industrial media, magnesium is usually in deficit and in grape must, is certainly inferior to calcium (Walker 1994). Consideration must therefore be given to the interaction of these two ions and the influence magnesium can exert on yeast physiology and metabolism under a variety of growth conditions: aerobic, fermentative and stressed, to be able to fully appreciate the influence of this salient ion on yeast.

1.3.2 CALCIUM

Calcium ions are essential for cell growth, performing a number of functions in biology and controlling very different biological actions from those controlled by other cations, for example, magnesium. Being ubiquitous in biological systems, the Ca^{2+} ion executes a variety of roles that include functioning as a "second messenger" in numerous cellular processes such as, cell division and growth, secretion, ion transport, etc. (Chazin 1995).

As with magnesium, calcium is a divalent cation (alkali earth metal) of the Type A transition metal cations, which are relatively small (calcium has an ionic radius of 0.99Å) and exhibit high electronegativity and low polarizability (Gadd 1992). Interesting differences in the structures of Mg^{2+} and Ca^{2+} *i.e.* the irregular geometry complexes which Ca^{2+} forms, are relevant to the differing biological roles of the two cations, with Ca^{2+} ions tending to be bound by oxygen donors (oxyanions) in biological systems (Hughes 1981). Selective binding of magnesium or calcium can be very carefully controlled through a combination of concentration and binding strength differences and is not simply dependent on charged groups, since then the problem of competition of different cations would be simplified. Calcium has a more variable range of structures than magnesium due to its higher co-ordination number (Hughes 1981) and its binding to proteins is often much stronger than that of magnesium, allowing calcium to frequently act as a cross-linking agent. In fact, calcium will almost always displace magnesium from a binding site composed of a complex set of multidentate and flexible ligands (Williams 1974).

The function of calcium is not just dependent on the relationship between function and structure. Variations in calcium ion concentration in different parts of the cell are controlled by metabolism, plus the action of calcium can be antagonised by a variety of cations, of both inorganic and organic origin. In prokaryotes, diverse and important roles have been attributed to calcium, these were initially shown by the effect of variations in the level of the ion in the external medium on bacterial growth rate (Shanker & Bard 1952; Aranha *et al.* 1986; Onoda & Oshima 1988; Perry & Brubaker 1987). Calcium flux is a

major determinant in the prokaryotic cell cycle (Norris *et al.* 1988) however, in bacteria, the role of calcium is not limited to cell cycle alone, Ca^{2+} is implicated in many other systems, both extracellular and intracellular (Vyas *et al.* 1987; Bradbeer *et al.* 1986) including involvement in environmental response e.g. chemotaxis (Matsushita *et al.* 1989). In fact calcium has been reported to be required for sporulation and proteolysis in *Bacillus spp.* (O'Hara & Hageman 1990) and as an important factor in the regulation of the cell cycle in *E. coli* (Norris *et al.* 1991) therefore, calcium plays disparate roles in prokaryotes. Diverse roles for calcium exist in eukaryotes, as well as in prokaryotes.

Ca^{2+} is required for the growth of microorganisms, but this does not mean that no other element can replace it and growth can still occur without Ca^{2+}. Calcium is known to have only a few specific biochemical functions in eukaryotes and many cells actively extrude this element (Helin & Slaughter 1977; Silver 1977). Some workers (Carafoli *et al.* 1970) have reported a slight increase in growth of *Sacch. cerevisiae* with the addition of calcium but since yeast have been shown to be able to grow in calcium deficient media, the ion may be replaced by other cations, e.g. Mn^{2+}, and in fact Mn^{2+} is at least 500fold more effective than free Ca^{2+} in supporting yeast cell-cycle progression (Loukin & Kung 1995). Despite this, the ion is required in some form for biological function. The optimum Ca^{2+} concentration (growth optimum) is 0.5 - 5.0mM (Jones & Greenfield 1984) however, the apparent requirement for calcium is dependant on the conditions of growth (Youatt 1993). It has been reported that the presence of optimal concentrations of calcium in fermentation media with high concentration of sugars (300g/L) led to the production by *Sacch. bayanus* of higher final concentrations of ethanol and to a lower percentage of unfermented sugars during batch alcoholic fermentations. The absolute cell growth requirement of calcium and its role as an intracellular regulator has been based, in many cases, on observations with chemical agents employed to alter cellular Ca^{2+} homeostasis (Youatt 1993). However, the "calcium" chelator EGTA and the "calcium" ionophore A23187 are not specific for calcium, A23187 also affects membrane permeability to Mg^{2+} ions and several biological processes are influenced primarily by the action of this ionophore on cellular Mg^{2+} homeostasis (Walker & Duffus 1980; Vidar & Rubin 1984; Walker 1994). This does not deny the cells

requirement for calcium and the role of this ion in cellular processes, but it does question the emphasis which has been placed on calcium as a cellular regulator to the detriment of magnesium and therefore allows the consideration of important cellular roles for magnesium, also. The reported calcium effect was generalised to strains of the species *Sacch. cerevisiae* and *K. marxianus* (Nabis *et al.* 1988). Addition of high levels of calcium to complex industrial and laboratory growth media may, however, have a negative effect. This negative effect was shown with the addition of 1.5 and 5mM calcium to molasses of 22°Brix in batch fermentation using *Sacch. bayanus* in work by Nabis *et al.* (1988). Based on the same study, the positive effect of calcium was expected to become more significant in continuous fermentation or in batch fermentations involving the production of high concentrations of ethanol (e.g. during the secondary fermentation in the sparkling wine industry). Complex media, however, generally do not exhibit optimal concentrations of inorganic ions, in fact calcium levels in most industrial media, e.g. molasses, grape must etc. are highly predominant (Wolniewicz *et al.* 1988; Walker 1994-review for all ions) and since high concentrations of Ca^{2+} are known to inhibit yeast growth (Tajima *et al.* 1996) a problem therefore exists. Inorganic phosphatase in yeast cytoplasm (Ridlington & Butler 1972) and mitochondrial matrices (Uribe *et al.* 1993) for example, are inhibited by Ca^{2+}; the mechanism of inhibition involving competition with Mg^{2+} for the substrate pyrophosphate (PPi) (Uribe *et al.* 1993).

Calcium Uptake in Cells

Ca^{2+} uptake was initially thought to be inversely related to the degree of stress placed on the yeast (Lentini *et al.* 1990) however, this theory has been disputed by Mochaba *et al.* (1996). Vacuolar membrane vesicles of *Sacch. cerevisiae* catalyse active transport of Ca^{2+} (Ohsumi & Anraku 1983) regulating Ca^{2+} in the cytoplasm and magnesium tends to regulate the medium calcium composition (Wolniewicz *et al.* 1988). In general, Ca^{2+} antagonises many Mg^{2+} dependent functions of yeast growth and metabolism, through inhibitory competitive binding mechanisms. High Ca^{2+} levels are known to curtail cellular Mg^{2+} uptake and cell membrane proton-pumping ATPase activity (Ohwaki & Lewis 1971)

which is essential for nutrient translocation and subsequent cell growth. Elevating Ca^{2+} may also have an impact on reduction of media pH, which may slow yeast growth and fermentation.

Cellular Role of Calcium

Ca^{2+} plays an important regulatory role in *Sacch. cereviase,* the calcium ion is involved in controlling steady-state structures of cell membranes and walls, cell-cell conglomerates and structural units inside the cell (Williams 1974), functions which Mg^{2+} ions antagonise. The Ca^{2+} messenger systems are finely tuned for rapid response to transient variations in Ca^{2+} concentration with a central role often being played by a family of highly homologous, Ca^{2+}-modulated proteins e.g. Calmodulin, Troponin C (Chazin 1995). Strictly controlled uptake of Ca^{2+} ions and binding (e.g. by calmodulin) may trigger certain cellular processes. In eukaryotic cells, the paradigm of calcium control is a calcium induced conformational change of these proteins which translates the transient influx of Ca^{2+} into metabolic or mechanical responses (Ca^{2+} sensors: da Silva & Reinach 1991) e.g. kinase activation and substrate phosphorylation, however, it is known that Ca^{2+} binding to calmodulin does not affect vegetative cell growth (Dunn *et al.* 1994).

The importance of calcium ions is reportedly highest for the stabilisation of the plasma membrane (Karamushka & Gadd 1994; Nabis *et al.* 1988; Ohsumi & Anraku 1983). Membrane-bound calcium appears to be central in regulating lipid-protein interactions with activation of plasma membrane ATPase and maintenance of the membrane permeability barrier under adverse conditions. Calcium, therefore, is involved in membrane stability, whereas ethanol, high temperature and other stresses interfere with membrane organisation, increasing its fluidity and permeability to ions and small metabolites (Salguerio *et al.* 1988; Nabis *et al.* 1988) and inhibiting the transport of nutrients (van Uden 1985). Ca^{2+} ions have been shown to protect cells against ethanol toxicity (Nabis *et al.* 1988) however, such cells would clearly be under osmotic stress (due to high glucose levels in medium) and it is recognised that any influences of calcium in ethanol protection depend on the physiological

state of the cells (Jones 1987). Ca^{2+} acts in the maintenance of the membrane permeability barrier under adverse conditions by shielding the charged structural membrane phospholipids and regulating lipid-protein interactions. Calcium is also one of the typical cations that make complexes with yeast wall phosphomannans and since, at growth temperatures, the target sites for ethanol-induced death are most likely located in the plasma membrane (Sá-Correia & van Uden 1986) the protection exerted by calcium could be related to its action in decreasing the extent of the release of intracellular compounds stimulated by ethanol (Nabis *et al.* 1988). The calcium ion could increase plasma membrane stability (Jones & Greenfield 1984) either by decreasing the ethanol-induced passive proton influx or stabilising ATPase activity which is inhibited by ethanol. Membrane bound Ca^{2+} is central in the regulation of lipid-protein interactions with activation of ATPase occurring at Ca^{2+} concentrations between 1 and 10mM (Ohsumi & Anuraka 1983), inside the cytoplasm of cells the calcium concentration is usually 0.1μM or lower (Williams 1974).

Calcium may be critical to other intracellular events apart from stabilisation of membrane structures. A physiological role in the metabolic control of yeast mitochondria has been suggested (Uribe *et al.* 1993) and Carafoli *et al.* (1970) reported that Ca^{2+} is beneficial to, but not essential for, mitochondrial function in yeast and that calcium may be important in the regulation of nitrogen metabolism, possibly at the level of the mitochondrion. Calcium ions are also involved in the mechanisms of flocculation in brewing strains of *Sacch. cerevisiae* (Miki *et al.* 1980; Stratford 1989). Nishihara *et al.* (1982; 1994) reported that Ca^{2+} activates the protein component essential for floc-forming ability. A further employment for this cation is in meiosis and/or sporulation, Suizu *et al.* (1994) demonstrated the importance of Ca^{2+} and Mg^{2+} in sporulation enhancement of *Sacch. cerevisiae*.

Role of Calcium in the Eukaryotic Cell Cycle

A fundamental role played by calcium is in the regulation of the eukayotic cell cycle and transient increases in the level of intracellular free calcium are believed to control passage

through three of the four cell-cycle transition points: G_2-M, M-G_1, G_1-S (Whitaker & Patel 1990) although the actual role of Ca^{2+} during the cell cycle in *Sacch. cerevisiae* is still unknown (Iida *et al.* 1990). Yeast cells can grow indefinitely in Ca^{2+}-deficient medium (Kovãc 1985; Iida *et al.* 1990) however, Ca^{2+} (or Mn^{2+}) deficiency causes cell cycle arrest (Loukin & Kung 1995). Genetic studies have suggested the involvement of Ca^{2+} in mitotic and G_1 events in yeast (Ohya & Anraku 1989; Ohya *et al.* 1986). Ca^{2+} deprivation first inhibits the initiation of bud emergence and DNA synthesis and then inhibits S and G_2/M traverses (Iida *et al.* 1990) however, it is unclear whether the requirement of Ca^{2+} for S and G_2/M traverses is a direct or an indirect effect of Ca^{2+} deprivation. In G_1, the deprivation results in uncoordinated initiation of bud emergence and DNA synthesis, events which in normal growth conditions are initiated co-ordinately (Iida *et al.* 1990). The resultant effect on G_1 (transient G_1 arrest) is not due to an indirect effect of Ca^{2+} deprivation on cell cycle progression through the inhibition of protein accumulation, however Ca^{2+} may positively control G_1 events by mediating the intracellular cAMP level in yeast cells (Iida *et al.* 1990) although, it is unclear if this is the result of a direct effect. Iida *et al.* (1990) suggests that Ca^{2+} is required at all the stages of the cell cycle except for the initiation of DNA synthesis and that G_1 events may be mediated by biochemical communication between Ca^{2+} and cAMP pathways. Intracellular pools of Ca^{2+} and Mg^{2+} are essential for cell division in yeast, possibly to promote the breakdown of microtubules involved in nuclear division and cell plate formation (Duffus & Patterson 1974). Calcium is involved in the regulation of cell proliferation and Ca^{2+} may play a role in the control of initiating cell proliferation from the lag phase (Friis *et al.* 1994). Calcium and its primary receptor protein; Calmodulin, are essential for cells to survive and grow and they have been implicated in the re-entry of quiescent cells into the proliferative cycle as well as for traversing the G_1/S, G_2/M and metaphase/anaphase boundaries of the cell cycle (Means 1994). In the yeast *Sacch. cerevisiae* calmodulin can function in chromosome segregation and polarised growth for bud formation independently of Ca^{2+} binding (Geiser *et al.* 1993), however, Ca^{2+} is known to be necessary for budding yeast proliferation (Anraku *et al.* 1991; Dunn *et al.* 1994). Bud development is tightly co-ordinated with other aspects of cell cycle progression and Ca^{2+}

and intracellular Ca^{2+} pools may also play important roles in cell proliferation independent of calmodulin (Means 1994).

These diverse functions of Ca^{2+} give fuel to the case that calcium is an essential ion for yeast growth. In the case of calcium, it can be seen from the literature that essentiallity is a relative term, in the fact that the roles of Ca^{2+} can be fulfilled by another ion, yet it is difficult to fully rid a medium of Ca^{2+} and therefore, at least trace levels of the ion must be required. In complex industrial media, calcium is usually in excess of most other ions and in the case of grape must, certainly in excess of magnesium (Walker 1994). What must be considered, therefore, is the antagonism between Mg^{2+} and Ca^{2+} and the effects this has on yeast growth and metabolism under natural growth conditions, fermentative conditions and stress conditions, to be able to fully understand the influences and effects of these important ions on yeast.

1.4 RESEARCH OBJECTIVE

An understanding of fermentation, the complexities of grape juice composition and the control of yeast metabolism and growth, is an essential prerequisite for successful wine production. The aim of this research is, overall, to attempt to increase the knowledge of the ionic nutrition of yeasts, and the effects selected ions can have on wine yeast in particular. Magnesium is accepted to be an essential ion for yeast development and it has been observed to exert a positive effect on both growth and fermentation properties. Yet still, complex fermentation media, and laboratory defined media for that matter, are neglected in terms of the consideration of their actual ionic levels, in particular, those of magnesium and calcium. In complex media, e.g. grape must, Mg:Ca ratios typically favour calcium and in high concentrations this ion is believed to have a detrimental effect on yeast performance.

This research, therefore, aims to establish the roles of magnesium and calcium in the growth and physiology of wine yeasts by examining their effects on proliferation and fermentation performance of selected wine yeasts in a variety of lab-based and complex media and analysing their influence on cellular ion homeostasis. The effects of the ions will also be assessed at the industrial level with the yeast *Sacch. cerevisiae*, in an attempt to utilise alterations in the inorganic content of the growing medium and yeast itself, to help alleviate problems which may occur in the commercial production of yeast for the wine and baking industries. Furthermore, the effects of these ions on the physiology of wine yeasts will be examined in more detail by considering the role played, if any, by magnesium when yeasts are subjected to environmental stress, a factor which, in the wine industry and fermentation industry in general, is not an uncommon occurrence. Due to the inherent nature of fermentation, yeast cells are subjected to high levels of ethanol, possibly high temperatures and nutrient stresses throughout varying stages of the alcohol production process.

It is hoped that this outlook and fundamental research will give an interesting and novel perspective on yeast cell physiology by examining a previously unconsidered aspect of ionic nutrition and its consequences for the wine industry.

CHAPTER 2

GENERAL METHODS

2.1 YEASTS

Yeast strains used within the context of this work were obtained from a variety of sources. The strain *Saccharomyces cerevisiae* DCL'M' (Quest Int., Menstrie, Scotland) was used for comparative studies as the industrial standard. The majority of experiments were carried out with the wine yeast *Saccharomyces cerevisiae*, strains DBVPG2168 (DBVPG) and L-2226 (Lallemand Inc.), with some additional work using a *Torulaspora delbrueckii* strain DBVPG6168 (DBVPG). Agglomeration studies were carried out with a bakers strain of *Saccharomyces cerevisiae*: Industrial Strain C (Lallemand Inc.). Strains prefixed DBVPG were obtained from the Industrial Yeast Collection: University of Perugia, Italy (DBVPG) and those prefixed L were supplied by Lallemand Inc., Montréal, QC. Canada.

Saccharomyces cerevisiae DCL'M' - A distilling strain with generally good fermentative qualities. It is conventionally utilised as the standard industrial strain for comparative studies.

Saccharomyces cerevisiae DBVPG2168 - This *Sacch. cerevisiae* strain (Meyen ex Hansen) held by DBVPG was isolated from a Sagrantino winery, Italy (Ciani & Rosini 1986). It is capable of producing levels of ethanol up to 15.2% and is used in wine-making and in sparkling wine for bottle fermentation.

Saccharomyces cerevisiae L-2226 - Lalvin L-2226 is a strain selected for the fermentation of red wines of rich constitution, good body and fine aroma, with the typical characteristics of Côtes-du-Rhone wines. It originates from a selection in a Côtes-du-Rhone vineyard by the Comité Interprofessionel des Côtes-du-Rhone du Ventoux et du Tricastin (CICDRVT). It possesses some resistance to alcohol and therefore can be used in grape musts very rich in sugar and it exhibits good fermentative qualities (Lallemand Inc: Technical Data Sheet).

Torulaspora delbrueckii DBVPG6168 - Originally named *Torulaspora fermentati* and now *Torulaspora delbrueckii* (*sensu* Lodder 1984), this strain held by DBVPG was isolated

from sorghum brandy and corresponds to strain CBS818 (Centraalbureau voor Schimmelcultures, Delft, Netherlands) (Martini *et al.* 1987).

Saccharomyces cerevisiae Industrial Strain C - A commercially supplied bakers strain of the yeast *Saccharomyces cerevisiae*. Supplied either as Active Dry Yeast (ADY), pressed or fresh cream yeast (Lallemand Inc.).

N.B. Throughout this thesis the term yeast is used to mean *Sacch. cerevisiae* unless otherwise stated. The acronym DBV used in figures/tables corresponds to that of DBVPG.

2.2 CULTURE STORAGE AND MAINTENANCE

Cultures were maintained on Malt Extract Agar (Oxoid) or Yeast Extract Agar (Oxoid) at 25^{o}C and stored at 4^{o}C until required for inoculum preparation or sub-culturing. Sub-culturing was performed at regular intervals and stock cultures of each yeast were freeze-dried to maintain the original strains in a more permanent manner.

2.3 INOCULUM PREPARATION

Inoculum preparation depended greatly on the ensuing experiment and any variations to the methods are noted in the respective chapters. Generally, however, a loopful of stock culture was aseptically removed and inoculated into a small volume of the respective liquid medium (inocula being prepared in the experimental medium), and incubated at 25^{o}C overnight, in a shaking (orbital) incubator.

Either, a set volume of inoculum was added to the fermentation or growth medium to give the required percentage volume of inoculum; usually 3% v/v inoculum level, or the fermentation seed amount was calculated using the following equation to determine the volume of seed required:

$$\frac{\text{Initial Cell Number required}}{\text{Inoculum Cell Number}} \times \text{Volume of Final Medium} = \text{Volume of Seed Required}$$

2.4 EXPERIMENTAL MEDIA

Modified Minimal Media (CMMM)

(Modified from EMM3: Fantes & Brocks 1993 & Wickerhams YNB Media: Van der Walt & Yarrow 1984).

Base and Salts media were made up separately in 500ml deionised water (18ohms), the pH brought up to pH 5.5 and then autoclaved. Base medium contained: 2% w/v Glucose (20g/L); 5g $(NH_4)_2SO_4$ and 1.5g Na_2HPO_4, and was autoclaved at 110°C/15min. The Salts medium containing: 0.5g KCl; 0.5g $MgSO_4.7H_2O$ and 0.015g $CaCl_2.6H_2O$, was autoclaved at 121°C/15min. These two components were then mixed aseptically to 1L and 1ml each of vitamin and trace element stocks added. Vitamin and trace element stocks were made up as 1L of x1000 concentrated stock solutions and filter sterilised through a 0.45μm cellulose acetate filter (Whatman). Final amounts in the resultant media were as follows:

Vitamin Stock: 10mg Inositol; 10mg Nicotinic Acid; 1mg Calcium Pantothenate; 0.01mg Biotin; 0.4mg Pyridoxine-HCl; 0.4mg Thiamine-HCl and 0.2mg p-Aminobenzoic Acid.

Trace Elements Stock: 0.5mg H_3BO_3; 0.4mg $MnSO_4.H_2O$; 0.4mg $ZnSO_4.7H_2O$; 0.2mg $FeCl_3.6H_2O$; 0.16mg $H_2MoO_4.H_2O$; 0.1mg KI; 0.04mg $CuSO_4.5H_2O$ and 1.0mg Citric Acid.

Magnesium, calcium and glucose levels were varied in this medium dependent on the conditions required of a particular experiment.

Semi-Synthetic Media

PYN Media: Semi-synthetic PYN media containing: 3.5g Peptone; 3.0g Yeast Extract; 2.0g KH_2PO_4; 1.0g $(NH_4)_2SO_4$; 2% w/v Glucose (20g/L); variable $MgSO_4.7H_2O$ and $CaCl_2.6H_2O$ was prepared in 1L of deionised water (18Ω) and corrected to pH 5.5. Sterilisation of this medium was carried out at 121°C/15min. Magnesium and calcium levels were altered dependent on the requirements of the experiment.

YPD Media: Semi-synthetic YPD media was prepared to 1L in deionised water (18Ω), pH adjusted to pH 5.5 and autoclaved at 121°C/15min. YPD medium contained: 5g Yeast

Extract; 1% w/v Glucose (10g/L); 5g Mycological Peptone and 15g Agar (if required). (Campbell 1988)

Complex Media

White grape must of the grape cultivar *Trebbiano toscaio*, from the region of Deruta (PG), Umbria, Italy was utilised as the complex medium for wine yeast strains. Magnesium and calcium contents of the must (pH 3.1; 16% w/v fermentable sugar; specific gravity 1060^{O}Plato) were determined and supplementations with $MgSO_4.7H_2O$ (CarloErba Analyticals) or $CaCl_2.2H_2O$ (CarloErba Analyticals) were made dependent on the requirements of the experiment. Sterilisation of this complex medium was carried out at 100^{O}C/10min

Molasses was the complex medium employed in agglomeration studies (Chapter 4). 100% Cane Molasses was diluted to 40-45^{O}Brix (Reichert ABBE Mark II Digital Refractometer, Buffalo, NY, USA) in 5L distilled H_2O, autoclaved at 121^{O}C, then racked. Following settling overnight, the racked molasses was then decanted into a sterile feed flask for the fermentation.

Malt Extract Broth: This medium, supplied by Oxoid, is effectively a dilute version of brewers wort supplemented with mycological peptone and containing 1% fermentable sugar. Made up using 20g Malt Extract Broth in 1L deionised water (18Ω) and pH adjusted to pH 5.5 (optimum for yeasts) the medium was sterilised at 115^{O}C/10min. An agar version of this media is also supplied by Oxoid.

2.5 FERMENTATION CONDITIONS

Fermentation conditions and experimental procedures are best described in each individual results section. Generally, small scale batch fermentations were carried out as 300ml volumes in 500ml conical flasks (with or without baffles). Fermenters, when used, were

either of 2L (Bioindustrie Mantovane: BM300, Italy) or 20L (Bioengineering AG: D391, Switzerland) volume, with fully automated controller units attached for the control of temperature, pH, dissolved oxygen and antifoam, etc. Fermentations were generally allowed to proceed until a total sugar concentration of <1% was achieved, analysed by a refractometer or Fehlings Test. Fermentation samples were routinely analysed for: cell number/biomass, residual sugar, ethanol, intracellular and extracellular magnesium and calcium.

2.6 ANALYSIS OF FERMENTATION SAMPLES

2.6.1 Sampling Procedure

10ml samples were removed from the experimental culture in 5ml aliquots, using a 5ml automatic pipette (Finnpipette) with sterile tips and placed into a sterile pre-labelled 10ml plastic centrifuge tube. All sampling was carried out in a flow cabinet, previously ethanol sterilised. Samples were then either analysed, or frozen immediately for future analysis.

2.6.2 Cell Numbers

Cell numbers were determined at various stages throughout the experiments. Samples were removed from the experimental medium and diluted as required. 100µl sample was added to 20ml Isoton (Coulter Electronics Ltd, UK) and mixed carefully by inversion. Samples were then counted electronically using a Coulter Counter (Multisizer II: Coulter Electronics Ltd) fitted with a 100µm probe and the results recorded. Acceptable counts were those lying within a 5% coincidence correction, anything above this required dilution with deionised water (18Ω) and re-counting. A background count of isoton was also prepared and the resultant counts adjusted accordingly. The analytical volume per sample was 500µl, therefore a correction was made to achieve counts per ml.

2.6.3 Viability

Percentage viability was calculated using the vital staining technique of Methylene Blue Staining (Mills 1941; Lee *et al.* 1981) and counting by haemocytometer (Improved Neubauer), under light microscopy. Due to the ultrastructural changes which occur in the cell wall on death, non-viable cells are able to take up the methylene blue dye, viable cells remain unstained. Using the counting chamber, one can therefore determine the number of live and dead cells (total count) per ml of sample and from this calculate the percentage viability of the culture.

2.6.4 Biomass

10ml samples were removed from the fermentation vessel, at various time intervals throughout the course of the fermentation. Cell biomass was determined by vacuum filtering the samples through pre-weighed filter papers (Whatman GF/C: 1.2μm), washing with an equal volume of deionised water (18Ω) and then filters were dried to a constant weight using a moisture analyser (Mettler PC180/LP15 or Mettler LP16). Biomass was determined in terms of: g DW/L of culture sample.

2.6.5 Sugar (Total Sugar) Determination

Bioanalytical Methods

Analysis of filtrates for total sugar, *i.e.* glucose or glucose and fructose, dependent on the media type, was carried out using enzymatic bioanalytical methods. Glucose was analysed for using either the D-Glucose Diagnostic Kit (Boehringer Mannheim) or the Glucose Hexokinase (HK) Kit (Sigma Diagnostics). These methods were satisfactory for determining the total or residual sugar of synthetic and semi-synthetic media. Complex media, e.g. grape must, have a correspondingly more complex sugar composition, therefore glucose is not the only sugar present. Glucose and fructose concentrations in complex media, were analysed using the D-Glucose/D-Fructose Kit (Boehringer Mannheim) for the estimation of total and residual sugar levels in must. These kits measure an enzymic

reaction resulting in an altered spectrophotometric reading (UV spectrophotometer: LKB Biochrom).

Fehlings Test

Total reducing sugar levels were analysed for, at the end of fermentation, using this test. 5ml Fehlings Reagent A and 5 ml Fehlings Reagent B were added to 40ml deionised water (18Ω) in a conical flask, the mixture heated until boiling and then removed from heat. From a burette containing diluted sample, dropwise additions were made to the solution. The solution was heated and additions were continued dropwise until a bright red precipitate was formed, the amount of sample solution added was recorded. The percentage concentration of sugar was then calculated using the following equation:

$$\frac{0.0515*D*100}{\Delta \text{ Volume}}$$

[D = Dilution of sample solution
[ΔVol = Volume required for change

[Fehlings Reagent A =	$CuSO_4$	69.28 g/L]
[Fehlings Reagent B =	Rochelle Salt (NaKTartrate)	325 g/L]
	KOH	154 g/L]

HPLC

Sugar profiles were determined for final fermentation samples, from certain experiments, using Isocratic High Pressure Liquid Chromatography (HPLC). Sample filtrates were injected, without prior dilution, through a 0.22μm Millipore filter onto an Aminex® Ion Exclusion HPX87H: Fermentation Analysis Column (Biorad) and run at $45^{o}C/0.6mls.min^{-1}$ (Pump 1330, Biorad), using an eluent of 0.05N H_2SO_4 and a running buffer of ultra pure H_2O. Detection from the HPLC system (Biorad) was by a Knauer High Temperature Differential Refractometer (Biorad) and peaks were recorded on an integrator (Hewlett Packard: HP3396A). A three point calibration of external standards was used to calibrate the system, at concentrations of 0.5mg/ml, 1mg/ml and 2mg/ml. The 12 Std. Wine Standard contained: glycogen, trehalose, citric acid, tartaric acid, glucose, fructose, succinic acid, lactic acid, glycerol, acetic acid, acetaldehyde and ethanol, and the profile and

retention times can be found in Figure 2.1. Identification of sample components was based on corresponding retention times.

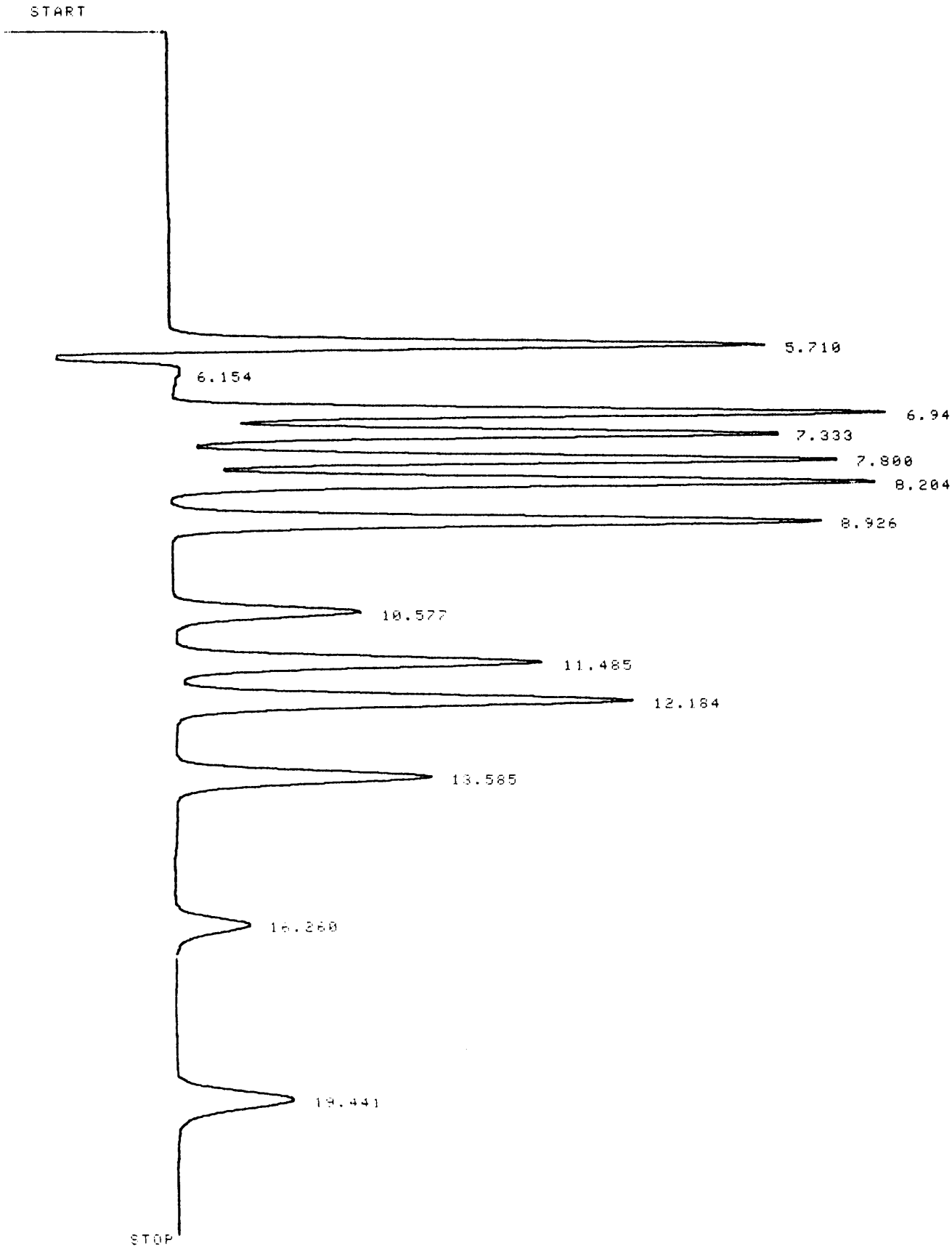


Figure 2.1 HPLC Calibration Profile for Wine.

Table 2.1 HPLC Wine Calibration Data.

Sugar/Ethanol Analysis
ESTD-Height

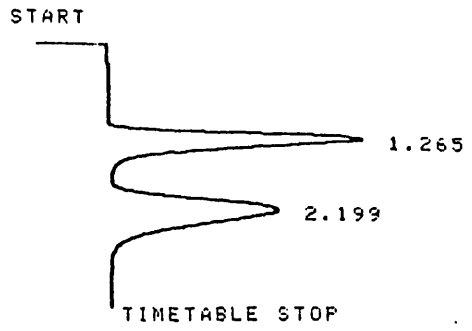
RT	Type	Area	Width	Height	Cal#	MG/	Name
5.710	BP	2378949	.320	123808	1R	.672	Glycogen
6.945	BV	1293967	.192	112454	2R	.927	Trehalose
7.333	VV	1126464	.193	97404	3R	.921	Citric Acid
7.800	VV	1217092	.189	107069	4R	.917	Tartaric Acid
8.204	VP	1386112	.206	112089	5R	.925	Glucose
8.926	PP	1368712	.221	103196	6R	.923	Fructose
10.577	PV	468113	.261	29941	7R	.929	Succinic Acid
11.485	VV	906071	.259	58360	8R	.925	Lactic Acid
12.184	VB	1139460	.264	72015	9R	.915	Glycerol
13.585	BP	682875	.282	40357	10R	.924	Acetic Acid
16.260	PP	244667	.338	12058	11R	.940	Acetaldehyde
19.441	PV	452975	.406	18611	12R	.924	Ethanol

Total Height = 911955
Mul Factor = 1.0000E +00

2.6.6 Ethanol Determination

Ethanol levels in sample filtrates were determined in one of two ways: Gas chromatography or enzymatic kits. The vast majority of samples were analysed by Gas Chromatography (GC). Using Flame Ionisation Gas Chromatography (Hewlett Packard 5710A: Integrator HP3396A) with N_2 as the carrier gas, flame H_2/Air and a column selective for organic compounds. Oven temperature was set at 130^{o}C and the injection and detector ports were set at 200^{o}C for all runs. Samples were analysed for ethanol levels over a 3 minute run period. Calibration of the GC was carried out using 6% ethanol containing an internal standard of 5% Isopropanol (Fig. 2.2). 0.5µl of filtered sample containing a 5% internal standard of isopropanol was injected onto the column and the amount of ethanol calculated using a reference standard as % v/v based on retention times, with a 5% window of error.

A small proportion of samples were analysed using spectrophotometric enzymatic bioanalytical methods; Ethanol kit (Boehringer Mannheim).
Dilutions were required of the samples, for the functioning of both of these methods.




```
RUN#  1953          MAR 30, 1993  17:37:29

ETHANOL ANALYSIS

ISTD-AREA
      RT        AREA TYPE  CAL#       AMOUNT
   1.265    27584656 ISHH    1R        5.973
   2.199    29503600 ITBB    2&

TOTAL AREA=5.7088E+07
MUL FACTOR=1.0000E+00
ISTD AMT=5.0000E+00
```

Figure 2.2 GC Calibration Profile for Ethanol

2.6.7 Metal Ions

Cell Washing

Cell washing was carried on pellets for the analysis of intracelluar magnesium or calcium, to remove surface bound ions prior to analysis. Washing was effected until no further residual cations remained, as determined after analysis by atomic absorbance spectrophotometry (AAS). A variety of samples from experiments with varying concentrations of magnesium and from various time points during the course of experiments, were selected to determine the washing procedure required prior to the analysis of cellular magnesium.

1ml samples of culture were removed and microcentrifuged (805G/5min). Media (supernatant) was removed and analysed, the cell pellets were resuspended in 1ml deionised water (18Ω), then microcentrifuged and the supernatant removed and analysed. This washing procedure was repeated eight times and each wash sample was analysed using

AAS. From the graph of the results (Fig. 2.3) it can be deduced that following the third wash, the concentration of magnesium in the washings was relatively constant and minimal and therefore analysis would be of intracellular magnesium only, not extraneous media. This is assumed to be true for calcium also. The initial high levels of magnesium in early washes can be accounted for as traces of media magnesium or loosely bound extracellular surface magnesium. Experimental samples, therefore, required three washings of cell pellets prior to analysis.

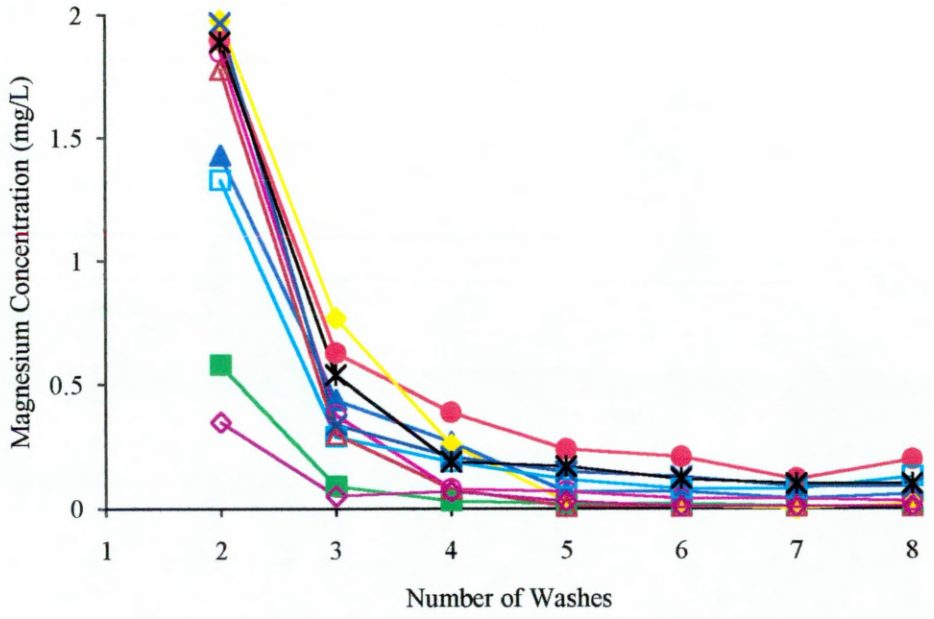


Figure 2.3 Magnesium content of cell washings. -●- *T. delbrueckii*: Must (2L) @ 30h, -■- *T. delbrueckii*: Must (2L) @ 118h, -○- *T. delbrueckii*: Must + [Mg]x10 (2L) @ 95h, -□- *T. delbrueckii*: Must + [Mg]x10 (2L) @ 240h, -▲- *S. cerevisiae*: Must + [Mg]x10 (2L) @ 6h, -◆- *S. cerevisiae*: Must + [Mg]x10 (2L) @ 116h, -Δ- *S. cerevisiae*: Must (300ml) @ 56h, -*- *S. cerevisiae*: Must (300ml) @ 72h, -◊- *C. stellata*: Must + [Ca]x10 (300ml) @ 9h, -×- *C. stellata*: Must + [Ca]x10 (300ml) @ 96h.
(Samples removed from fermentations of a selection of yeast in various media, as stated).

Atomic Absorption Spectroscopy (AAS)

Analysis of magnesium and calcium by Atomic Absorption Spectroscopy (AAS), (Perkin Elmer 1100B) using a Magnesium/Calcium lamp, was carried out on extra- and intra-cellular samples.

Conditions set for the AAS were as follows:

	Magnesium	Calcium
Fuel:	Acetylene (C_2H_2)	Acetylene (C_2H_2)
Flow Rate:	2.5ml/min	2.5ml/min
Oxidant:	Air	Air
Flow Rate:	8.0L/min	8.0L/min
Lamp:	Ca/Mg	Ca/Mg
Current:	12mA	12mA
Wavelength:	285.2nm	422.7nm
Slit:	0.7nm	0.7nm
Energy:	68	71
Background Correction:	ON	OFF
Calibration:	Linear AA-BG	Linear AA

Calibration of the AAS was done for each metal using standard solutions of;
for magnesium: 0.05, 0.1, 0.2, 0.3, 0.4, 0.45, 0.5 ppm Mg and for calcium: 0.05, 0.1, 0.2, 0.3, 0.4, 0.45, 0.5 ppm or 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 ppm Ca.

The technique of atomic absorption is based on the phenomenon that ground state atoms (M^o) of an element absorb radiation of discrete wavelengths and the fact that the amount of radiation absorbed can be related quantitatively to the number of atoms present. The fundamental instrumentation designed to utilise the potentialities of this phenomenon was first developed by Walsh in Australia in 1953 (Sanui & Rubin 1982a). Atomic absorption spectroscopy is especially suited for the measurement of Mg^{2+} because of the stability of readings, high sensitivity and relative freedom from interferences (Sanui & Rubin 1982a).

Analysis of magnesium and calcium concentrations by atomic absorption spectroscopy (AAS) was carried out on extracellular filtrates following dilution and on intracellular samples following the sample preparation and calibration programmes as described.

Magnesium & Calcium Determination

Several methods of analysis for magnesium and calcium have been employed dependent on conditions, type of samples and equipment available. The techniques employed have been; diagnostic kits, AAS and Ion Chromatography.

Extracellular Samples

Extracellular magnesium and calcium levels were analysed either by AAS, or diagnostic kits utilising spectrophotometric analysis.

AAS: Methodology of analysis by AAS is described above, dilution of filtrates was, however, required prior to reading samples.

Diagnostic Kits: Filtrates were analysed for extracellular magnesium (Mg) and calcium (Ca) content using Magnesium (Calmagite) Diagnostic Kit (Sigma) and Calcium (Cresophtalein) Diagnostic Kit (Sigma), respectively. Both kits measure metal ion concentration by measuring a change in absorbance of a colour reaction at a specified wavelength on a spectrophotometer (LKB Biochrom). Dilutions were required of most samples.

Intracellular Samples

Preparation of Samples: Cell Digestion

Intracellular magnesium and calcium concentrations were determined from 1ml cell pellets removed at various stages of the fermentations/ experimental cultures. 1ml samples were microcentrifuged (805G/5min), supernatant removed and the cell pellet washed three times with deionised water (18Ω). Pellets were then resuspended in 2ml deionised water and transferred to a 10ml tube. 2ml conc. H_2SO_4 was added and the solution mixed, then left for 24h, followed by a 10min boil to completion of cell hydrolysis. Cell hydrolysates were made up to 10ml total volume, using accurate pipettes (Grade A Bulb pipettes). If further dilutions were required, cell hydrolysates and washings were transferred to a 25ml volumetric flask (Grade A) and made up to a 25ml volume.

Analysis: Samples are then read by the methodology of analysis by AAS, described above.

2.7 STATISTICAL METHODS

All analysis was carried out in triplicate and data sets shown are the average of the sum of these sets, unless otherwise stated.

2.8 CHEMICALS

Media components were obtained from Oxoid, UK or from Difco Laboratories, Detroit. Michigan. Chemicals were obtained from Sigma/ Aldrich, Fissons or BDH. AnalaR grades were used where possible and for HPLC and GC the purest quality grades were employed. In Italy, grape must was supplied by a local vineyard and inorganic chemicals supplied by Analyticals Carlo Erba, Milano. Italia. Diagnostic Kits were supplied by Sigma, UK or Boehringer Mannheim, Germany.

2.9 GLASSWARE

All glassware/sample bottles were deionised prior to use, using an overnight soak in 2% Nitric Acid, followed by two washes in deionised water (18Ω H_2O), once with 0.1M EDTA then rinsed four times with deionised water and dried in oven (160^oC).

2.10 ADDITIONAL METHODS

Methods described in this general methods chapter were typical to the majority of the work covering various aspects. Additional methods specific to certain sections of work and as such described in the respective chapters include:

Ion Chromatography

Trehalose Determination -Extraction

Determination by HPLC

Yeast Cell Disruption

Electrophoresis - SDS-PAGE

Autoradiography

Immunoblotting - Western Blotting

DNA Extraction

Chef Analysis of DNA

Electron Microscopy - Scanning Electron Microscopy

Transition Electron Microscopy

CHAPTER 3

EFFECTS OF MAGNESIUM AND CALCIUM ON GROWTH AND FERMENTATION

3.1 INTRODUCTION

Various aspects of metabolism and physiology in yeast cells are still poorly understood from a fundamental standpoint, despite the magnitude of work presently undertaken on yeasts. This is particularly true for yeasts exploited in industries which employ complex organic feedstocks (e.g. grape must, molasses, malt wort, etc.). For example, for those involved in alcohol manufacture there is a need to minimise yeast cell growth and maximise fermentation productivity; whilst for producers of yeast biomass, the converse is true. A pre-requisite in preventing unnecessary wastage and enabling more efficient usage of plant is to gain a firm understanding of fundamental metabolic regulation so that, ideally, the operator dictates the process of the fermentation, rather than the yeast cells themselves. An understanding of the environmental and nutritional factors influencing yeasts would, therefore, be fundamental to the science of wine making and other yeast-based technologies.

3.1.1 YEAST METABOLISM

Metabolism describes all the enzymatic reactions which occur within the cell and the organisation and regulation of those reactions. Metabolism of yeast depends, substantially, both on the nature of the growth-limiting component and on the medium concentration of mineral components which, while non-growth-limiting, nevertheless influence the intensity of distinct metabolic processes (Dedyukhina *et al.* 1989). Of the environmental factors which influence yeast growth and metabolism, glucose concentration and oxygen availability are the most documented and are linked to the expression of regulatory phenomena, such as the Pasteur and Crabtree effects, the fundamental basis of which are still not fully understood, despite many years of research. Very little attention has, however, been paid to inorganic nutritional factors in influencing yeast fermentations (Jones & Gadd 1990) in spite of the fact that the importance of ions like Ca^{2+} and Mg^{2+} in cell physiology and biochemistry has long been recognised.

Yeasts will grow in simple media which contain fermentable carbohydrates to supply energy and "carbon skeletons" for biosynthesis, adequate nitrogen for protein synthesis, mineral salts and one or more growth factors (Young 1987), although they are mostly grown on cheaper, complex media. The complex media employed for yeast production is molasses, whereas the wine industry utilise grape must as the medium for alcohol (wine) production. The composition of grape must is generally selective to wine yeasts (Rosini & Ciani 1992) due to its acidic pH and high levels of fermentable sugars (mainly glucose and fructose), and supply of nitrogenous compounds, growth factors and minerals (mainly potassium, calcium, magnesium, phosphates and sulphates). It, however, contains many compounds with high chelating powers and the accessibility to the metal ions actually present may be restricted, thus compounding the issue of metal ion availability. Most major industrial fermentation media, such as molasses, malt wort and grape must, consist of complex plant materials with a correspondingly more complex ionic composition than defined media and the metal ion composition will vary greatly, depending on raw materials and process conditions (Saltukoglu & Slaughter 1983; Walker *et al.* 1996).

The fermentation of grape must is a complex biochemical process (typically anaerobic), as is aerobic growth in this medium. Growth cannot be separated from the fermentation process and is, in fact, necessary to the production of both wine and possibly fresh yeast for use in subsequent fermentations. *Sacch. cerevisiae*, the main "wine yeast", may be classed as a glucose-sensitive yeast exhibiting aerobic ethanol production in the presence of excess glucose (Käppeli 1986), however, yeast cells also proliferate during grape juice fermentation, with their kinetics of growth following a typical microbial growth curve (lag, log, stationary phases). During batch alcoholic fermentation, the yeast *Sacch. cerevisiae* exhibits a long growth phase, elevated rates of glycolysis and consequently elevated ethanol production. During stationary phase these rates gradually decrease leading occasionally to "sluggish" or "stuck" fermentations in media containing high initial sugar concentrations (Salmon & Mauricio 1994). Such fermentations result in the premature cessation of yeast growth and alcoholic fermentation, giving a wine with residual unfermented sugar and less than expected concentrations of ethanol and creating a sporadic, but serious, problem in

wine-making. The nutritional complexity of grape must plays a large part in the quality and type of wine produced in terms of the growth and fermentation characteristics of the yeast strain and therefore, the sensory complexity of the wine. Throughout fermentation, yeasts utilise sugars and other constituents of grape must as substrates for their growth, converting these to ethanol, carbon dioxide and other metabolic end-products which contribute to the chemical composition and sensory quality of the wine. The chemical and physical composition of the must affects the rate and completeness of fermentation, as well as the concentration of many aroma and flavour constituents in the wine. Important substrate variables that are likely to affect yeast growth and condition fermentation include; sugar concentration, supply of nitrogenous substances, presence of adequate vitamins, concentration of dissolved oxygen, pH, ionic nutrition and concentration of insoluble solids. In most instances, grape musts possess a full compliment of nutrients necessary for yeast growth and complete fermentation, although it is important to remember that must composition will vary according to grape variety, soil condition, use of fertilisers and the maturity of the grape at harvest. In addition, processing operations such as maceration, pressing, clarification, ion exchange, desulfiting and blanketing with nitrogen or carbon dioxide can affect the nutritional composition of the resultant must. An appreciation of the nutrient composition of the medium is important in understanding sugar metabolism during grape must fermentation due to the impact of nutrient availability on metabolic activities.

Metabolism of growing yeasts depends on the yeast strain, the carbon source and the physicochemical factors in the environment. Unlike most other yeasts, *Sacch. cerevisiae* catabolises hexoses mainly to ethanol (fermentation) even under aerobic conditions (Gancedo & Serrano 1989) in the presence of a high sugar concentration. After exhausting the exogenous sugar, this yeast can then adapt to catabolise ethanol aerobically, by the tricarboxylic acid cycle resulting in diauxic growth (Entian & Barnett 1992; Münch *et al* 1992). In *Sacch. cerevisiae,* during fermentation the level of glucose-6-phosphate dehydrogenase is very low (Young 1987) and it is probable that most of the yeast's requirement for pentose sugar is met by the action of transketolase on fructose-6-phosphate and glyceraldehyde-3-phosphate produced by glycolysis (Fig 3.1). Gluconeogenesis also

allows some fermentative yeasts to utilise ethanol, the major product of glucose fermentation. The metabolism of glucose by *Sacch. cerevisiae* in aerobic cultures is carried out by a complex network of catabolic and biosynthetic pathways, involving initial sugar transport and then the actions of several enzyme systems on the transported sugars. Sugar transport in *Saccharomyces* is quite complex, and is now known to involve multiple carriers (Kruckeberg & Bisson 1990). An apparent loss of sugar transport activity in *Sacch. cerevisiae* was systematically observed during alcoholic fermentation under conditions where the substrate was not a limiting factor. This decline was independent of the amount of ethanol produced, but influenced the ethanol production rate (Mauricio & Salmon 1992). Such a decrease of sugar transport activity has been attributed to a phenomenon of catabolic inactivation requiring a fermentable substrate (Busturia & Lagunas 1986) involving glycolytic enzymes and factors affecting their activity. Once transported into the cells, the major route of glucose and fructose utilisation in *Saccharomyces* is glycolysis (Fig 3.1). Regulation of glycolysis and therefore cellular metabolism is of fundamental importance to the cells and the major points of regulation of sugar utilisation in yeast are at the steps of transport or phosphorylation, or both. Magnesium is an essential cofactor for many of the glycolytic enzymes and has also been identified as a limiting nutrient in fermentation broths (Dombek & Ingram 1986a; 1986c) thus, as such, sugar metabolism, and yeast growth and metabolism in general, is affected by the ionic nutrition of the growth medium. Yet little attention has, however, been paid to inorganic nutritional factors in influencing the growth and physiology of wine yeasts.

3.1.2 Role of Inorganic Ions in Metabolism

Metals are involved in all aspects of microbial growth, metabolism and differentiation (Gadd 1992) and these ions play important roles in brewing and fermentation in general. Yeasts require a number of inorganic ions in various concentrations for optimum growth and fermentation and an imbalance in ionic nutrition is reflected in complex and often subtle alterations of metabolic patterns and growth characteristics (Jones & Greenfield 1984).

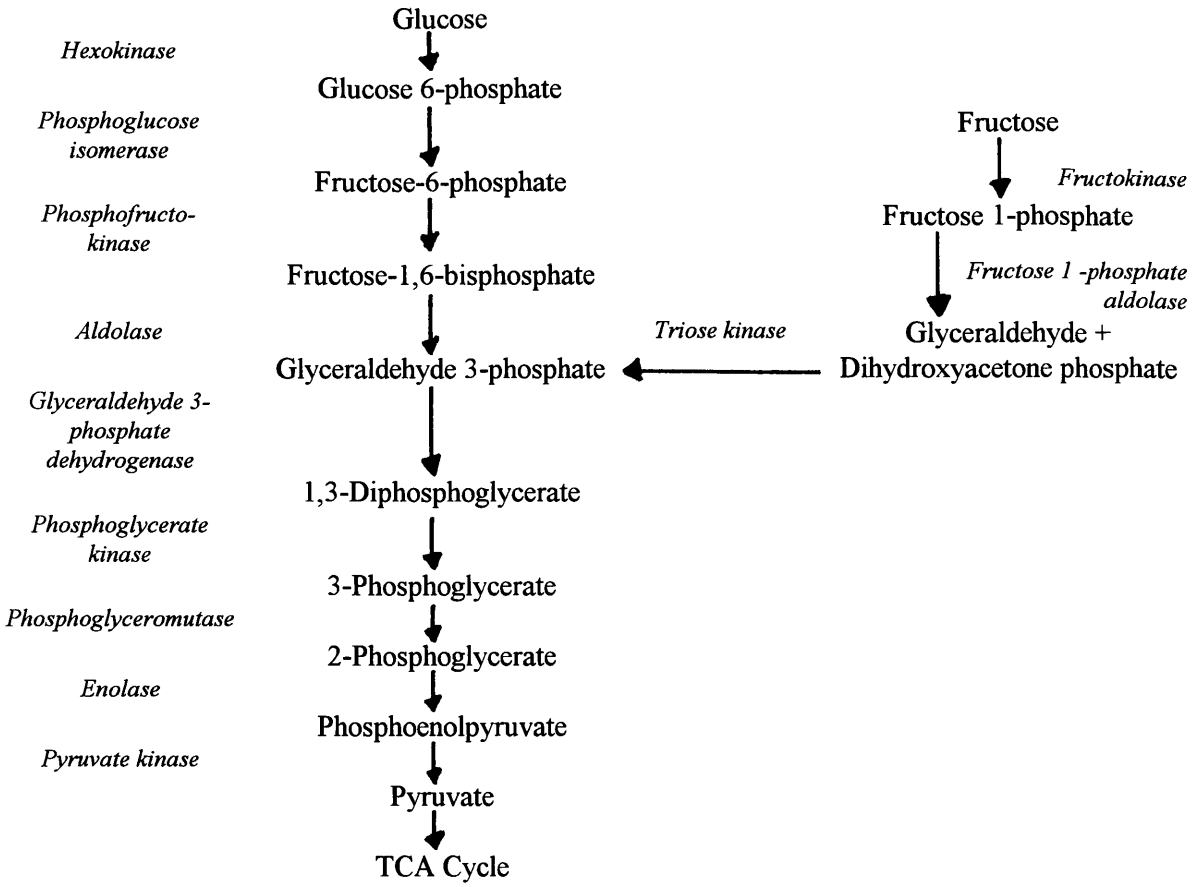


Figure 3.1 The pathway of glycolysis for glucose and fructose.

The regulatory roles of metal ions in fermentation and in particular, the effects of inorganic ion nutrition on wine yeasts, have not been investigated fully. Most industrial media have a complex ionic composition and ethanol yields in industrial fermentations have been shown to be affected by the various ionic constituents in the media. Analysis of the effect of the ionic environment upon ethanol production is complicated by the relationship which exists between specific ethanol production rate, yeast specific growth rate and cell and ethanol yield. Specific fermentation rate, specific growth rate and viability are individually dependent on the environment, each with different requirements.

Magnesium plays a central role in fermentative metabolism. The importance of this ion in yeast growth and fermentation is indicated by its effect on cellular metabolism. It is involved in numerous biochemical functions essential to yeast growth including, governing

cell division and growth (Walker 1986), mitochondrial structure and function (Walker *et al.* 1982) and respirofermentative metabolism (Walker *et al.* 1990). A further involvement of magnesium ions more directly pertinent to industrial processes follows the implications that magnesium can ameliorate the detrimental effects of both ethanol toxicity and high temperature inhibition of yeast cells during fermentation (Dombek & Ingram 1986c; Pamment *et al.* 1990; Birch & Walker 1996). Despite this, the roles of magnesium ions in industrial fermentations have not been fully appreciated (Walker *et al.* 1996). The element is required for the function of over 300 enzymes and is involved in a structural capacity for the survival of yeast cells. Yeasts, therefore, possess an absolute requirement for magnesium. In contrast, calcium, although essential for cell growth, is known to have only a few specific biochemical functions in eukaryotes and many cells actively secrete this element (Helin & Slaughter 1977; Silver 1977), however the ion is required in some form for biological function. Calcium growth requirements, therefore, seem less important (Dedyukhina & Eroshin 1991; Youatt 1993) and calcium may in fact act antagonistically with magnesium, inhibiting many essential biochemical functions of yeast. Most industrial media (malt wort excepted) have an incorrect balance of magnesium and calcium, such that the relative concentration ratios favour calcium rather than magnesium, a situation generally not conducive to fermentation (Walker 1994). The ionic deficiencies which occur in the majority of natural sources of carbohydrates used as alcoholic fermentation feedstocks, are amplified by the complications of chelating, sequestering and adsorbing materials present, which act to reduce the effective available ionic concentration (Lie *et al.* 1975; Jones & Greenfield 1984). In addition, despite an absolute requirement for magnesium, not all the magnesium from the growth medium is absorbed by yeast cells. Much of the initial binding of metal ions is to non-specific groups in the cell wall and these may have no physiological function (Walker 1978). Even at the onset of magnesium limitation, although an increase in the proportion absorbed does occur with the approach of limitation (Saltukoglu & Slaughter 1983), much is bound to the cell wall and is not usefully used by the cell. This situation is influenced not only by the intrinsic characteristics of the magnesium pump within the cell, but also the chelating power of the medium, hence medium composition plays a large part in determining the level at which magnesium becomes a limitation on growth.

Nevertheless, yeasts are regarded as having a much higher affinity for magnesium than calcium (Fuhrmann & Rothstein 1968).

Cells growing in Mg^{2+}-supplemented media show an enhanced rate of glucose consumption and consequent ethanol production (Dombek & Ingram 1986c; Walker *et al.* 1995; Walker *et al.* 1996). Such influences of Mg^{2+} ions may be expressed at the level of glycolytic enzyme activation and cell membrane stabilisation. Thus, maintenance of high Mg:Ca concentration ratios may serve to alleviate biological antagonism of essential Mg^{2+}-dependent functions by Ca^{2+} (Walker *et al.* 1995). The presence of excess Ca^{2+} ions and low Mg:Ca concentration ratios appear to be counter-productive in terms of ethanol production by yeast. It would appear, therefore, that straightforward addition of Mg^{2+} (in the form of magnesium salts) may be all that is required to ameliorate the inhibitory effects of excess Ca^{2+} commonly encountered in fermentation media (Wolniewicz *et al.* 1988; Walker *et al.* 1996), and by enhancing the bioavailability of Mg^{2+} corresponding enhancement of yeast fermentation performance would be achieved. However, to be able to fully understand the influences and effects of these important ions on yeast, one must consider the antagonism between Mg^{2+} and Ca^{2+} and the effects this has on yeast growth and metabolism under natural growth and fermentative conditions.

3.1.3 RESEARCH OBJECTIVE

A definitive study into the influence of specific metal ions in controlling growth and metabolism of wine yeast strains has yet to be undertaken. The aim of this research work was to carry out preliminary investigations into the effects of magnesium and calcium on the growth and physiology of a selection of *Saccharomyces* and non-*Saccharomyces* wine strains, in both complex and semi-synthetic media, and to observe the effects of altering the media concentrations of these ions on cellular ion homeostasis.

3.2 EXPERIMENTAL APPROACH

3.2.1 SEMI-SYNTHETIC MEDIA STUDIES

CULTURES AND INOCULUM PREPARATION

Saccharomyces cerevisiae strains; DCL 'M', L-2226 and DBVPG2168 and *Torulaspora delbrueckii* strain DBVPG6168, were used in this study. Sources of strains used are as described in Chapter 2. Inocula were prepared in PYN media and incubated orbitally at 25^{o}C/48hr. 10ml volumes of these inocula were centrifuged (2500 G/5min), the media removed and pellets washed three times in dH_2O. Cell pellets were then resuspended in 5ml of the respective experimental media and added to the corresponding experimental flask to give a 3% v/v inoculum level.

EXPERIMENTAL CONDITIONS

500ml conical flasks containing 300ml media were prepared for the experimental fermentations. Semi-synthetic base medium of PYN, with an elevated glucose level of 16% w/v and without magnesium and calcium additions, was prepared in 10 flasks. Magnesium and calcium additions were made as described in Table 3.1, all additions were made aseptically from sterile stock solutions of the two cations, following sterilisation of the base medium at 121^{o}C/15min. Ion free medium was sterilised following addition of 15g Chelex® 100 Resin (Sodium form; 100-200 Mesh: Biorad Laboratories, CA, USA) an analytical grade chelating resin, to the base medium, PYN. This mixture was stirred for approx. 1 hr. and then the solution filtered into a clean, deionised 500ml flask and sterilised at 121^{o}C/15min. Following this treatment, this medium was assumed to be deionised. In all the other media, trace levels of Mg and Ca may be present in the components of PYN and the ratios calculated were made in addition to these actual initial levels.

Table 3.1 Magnesium and calcium additions to semi-synthetic PYN Media.

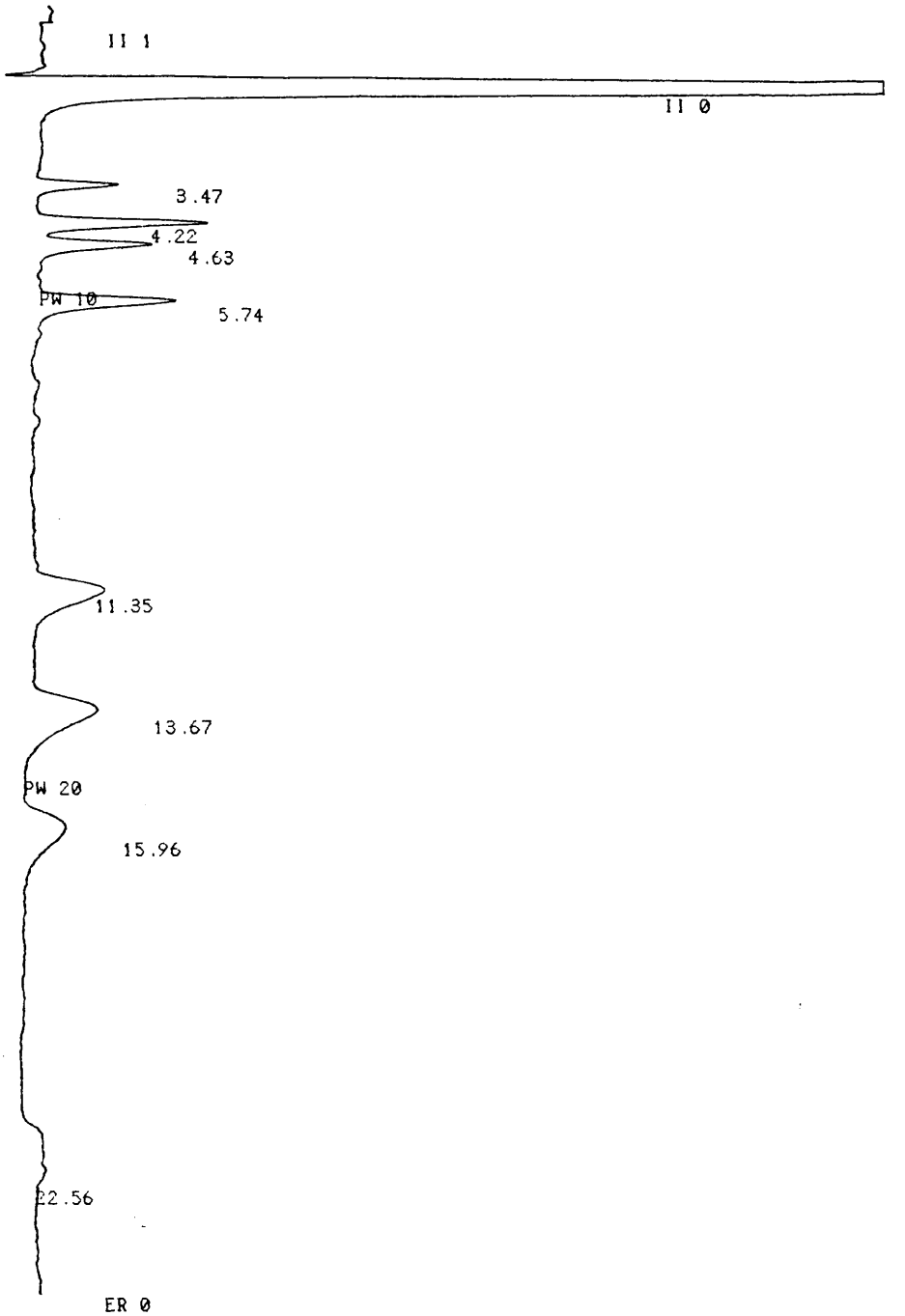
Flask	Molar Ratio Mg:Ca	Mg:Ca Actual Conc. Added	mg/L Ratio Mg:Ca
A	1000:1	100mM : 0.1mM	489.5:1
B	100:1	10mM : 0.1mM	58.84:1
C	13.6:1	29.3mM : 2.15mM	4.85:1
D	10:1	1mM : 0.1mM	6.86:1
E	1.36:1	2.93mM : 2.15mM	0.54:1
F	1:1	0.1mM : 0.1mM	1.18:1
G	0:0	No added Mg/Ca	1.92:1
H	0.90:1 (Wine)	2.38mM : 2.65mM	0.45:1
I	1:7.75	2.93mM : 21.5mM	0.094:1 or 1:10.65
J	1:10	0.1mM : 1mM	0.075:1 or 1:13.29
K	1:100	0.1mM : 10mM	0.025:1 or 1:39.82
L	1:1000	0.1mM : 100mM	0.003:1 or 1:333.6
M	Ion Free	Chelex® 100	0.71:1 or 1:1.41

Experimental flasks were incubated at 25°C in an orbital incubator (~150rpm) and growth allowed to proceed under conditions of self-induced anaerobiosis. Fermentations were allowed to proceed until ≤3% sugar was achieved, approx. 144hr. Samples were taken at 3 hourly intervals for the first and third 12 hr periods and after 30hr samples were taken twice daily until the end of fermentation.

Samples were analysed for biomass (dry weight), total sugar (enzymatic kits), ethanol production (GC or enzymatic kits: Flasks C,E,I) and extracellular and intracellular magnesium and calcium, by the methods described in Chapter 2. Analysis of magnesium and calcium by AAS was employed with all intracellular and extracellular samples of the semi-synthetic media studies. Free cation levels were determined for the strain *Sacch. cerevisiae* DCL 'M', by HPLC: Ion chromatography. Analysis of final sugar profiles of selected samples of experiments using the strains *Sacch. cerevisiae* L-2226 and DBVPG2168, was carried out by HPLC following the methods described in Chapter 2.

ION CHROMATOGRAPHY

Free cation levels were determined from extracellular samples taken at various points throughout the course of the fermentations. Levels of free magnesium and calcium were assessed by HPLC using an IC-Pak™ M/D Cation Column (Waters, USA) for the analysis of mono- and divalent cations. Sample filtrates were loaded onto the column (3.9mm x 150mm) by an autosampler (HPLC 360: Kontron Instruments) to maintain a high degree of accuracy between injections and run at a flow rate of 1ml/min (IsoChrom LC Pump: Spectra Physics) using 3mM Nitric Acid containing 0.1mM EDTA, as the running buffer. Detection from the HPLC cation system was by a conductivity detector (ConductoMonitor III: Milton Roy) and peaks were recorded on an integrator (Chromjet Integrator: Spectra Physics). A single level calibration of external standards was used to calibrate the system. Standards were prepared in running buffer (0.1mM EDTA in 3mM Nitric Acid) at the following concentrations: lithium 0.25ppm; sodium 1ppm; ammonium 3ppm; potassium 2ppm; magnesium 3ppm; calcium 3ppm; barium 5ppm and strontium 5ppm (Figure 3.2). Identification of sample components was based on retention times. Identified peaks in the sample were compared with the respective peak in the calibration sample. Concentrations of metal ions (cations) in the analysis sample were calculated as a ratio comparison to the external standard (known concentration). Peak threshold was set at 250, calculated based on the noise level of the detector used and a 10% window was allowed for retention times. Free magnesium and calcium levels were recorded as ppm.



Cation Method 29-04-96 15:38:08 CH= "A" PS= 1.

FILE 2. METHOD 5. RUN 3 INDEX 1 CALIB

NAME	ppm	RT	AREA BC	RF
Lithium	0.25	3.47	12574 01	50296.
Sodium	1.	4.22	30411 02	30411.
Ammonium	1.	4.63	22326 03	22326.
Potassium	3.	5.74	32049 01	10683.
Magnesium	2.	11.35	35538 01	17769.
Calcium	3.	13.67	44727 01	14909.
Strontium	5.	15.96	30702 01	6140.4
Barium	5.	22.56	18657 01	3731.4
TOTALS	20.25		226984	

Figure 3.2 Cationic HPLC Calibration Profile: Mono- & divalent metal ions.

3.2.2 WINE MUST STUDIES

CULTURES AND INOCULUM PREPARATION

Sacch. cerevisiae (DBVPG2168 and L-2226) and *T. delbrueckii* (DBVPG6168), all wine yeast strains, were maintained on Yeast Extract Agar (Oxoid) at 25^{o}C and stored at 4^{o}C until required for fermentations. Inoculum preparation was carried out for shake flask experiments, by transferring a loopful of culture to 30ml sterile grape must, bioreactor studies required a 60ml pre-culture to be prepared. These pre-cultures were incubated at 25^{o}C/48hr in a shaking incubator. Inoculation of the flasks with 10ml of the respective pre-cultures, or in the case of the bioreactor studies 60ml of culture was used, to give a 3% (v/v) inoculum level in the experimental broths.

EXPERIMENTAL CONDITIONS

White grape must (Region: Deruta (PG), Umbria. Italy.; Grape Cultivar: *Trebbiano toscano*) was used for the yeast fermentations. Magnesium and calcium contents of the must (specific gravity 1060^{o}P), were determined and unsupplemented grape must and grape must supplemented with 7.5g/l $MgSO_4.7H_2O$ (Carlo Erba Analyticals), to result in an approximate ten-fold increase in Mg content of must, were prepared for the bioreactor studies. Unsupplemented white grape must (0.5:1 Mg:Ca mg/L ratio) and grape musts supplemented to give approximately 10-fold increases in either magnesium or calcium respectively, were prepared (Table 3.2). Sterilisation of these media was carried out in situ at 105^{o}C/15min, or at 100^{o}C/15min for the shake flasks.

Small scale batch fermentations were carried out, using 300ml volumes in 500ml conical flasks, fitted with water-trapped exit ports for the escape of CO_2. Flasks were incubated at 25^{o}C in an orbital incubator (~150rpm) and growth allowed to proceed under conditions of self-induced anaerobiosis. Fermentations were stopped at a total sugar concentration of

<1%, analysed by Fehlings Test (approx. 4-6 days). Batch fermentations in 2 litre volumes were carried out with *Sacch. cerevisiae* and *T. delbrueckii* in both media, giving a total of four fermentations. The fermenter (Bioindustrie Mantovane: BM3000: Plate 3.1) was fully automated, with agitation being maintained at approximately 300rpm and temperature 25^{o}C. Prior to inoculation, sterilisation was carried out in-situ, then the media was sparged to 100% O_2 and growth was allowed to proceed to optimum anaerobiosis for the particular yeast in question. Fermentations were stopped at a total sugar concentration of <1%, analysed by a Fehlings test.

Table 3.2 Magnesium and calcium additions to white grape must.

Code	Base Media	Added Mg (g/L)	Approx. Mg Conc. (mg/L)	Added Ca (g/L)	Approx. Ca Conc. (mg/L)	Actual Final Ratio Mg:Ca
M1	Must	0.00	55	0.00	100	0.48:1
M2	Must	6.49	500	0.00	100	4.78:1
M3	Must	0.00	55	3.29	1000	0.07:1
FM1	Must	0.00	55	0.00	100	0.56:1
FM2	Must	7.50	500	0.00	100	6.66:1

Note: Magnesium and calcium additions (g/l) were made using $MgSO_4.7H_2O$ and $CaSO_4.2H_2O$ respectively. All increases were approximately 10-fold, however when original media levels of magnesium and calcium are taken into account the actual ratio values are slightly different.

Samples were taken approx. every 3hrs for the first 9hrs and then samples were removed at various intervals throughout the course of the experiment until the end of fermentation. Samples were analysed for biomass (dry weight), ethanol (enzymatic kit), total sugar (enzymatic kits) and extra- and intra-cellular magnesium and calcium by the methods described in Chapter 2. Determination of magnesium and calcium by AAS was employed in the analysis of intracellular samples of the fermentations, whereas extracellular samples were analysed using bioanalytical kits. Analysis of final sugar profiles of the yeast *Sacch. cerevisiae* L-2226 was carried out by HPLC, following the method described in Chapter 2.

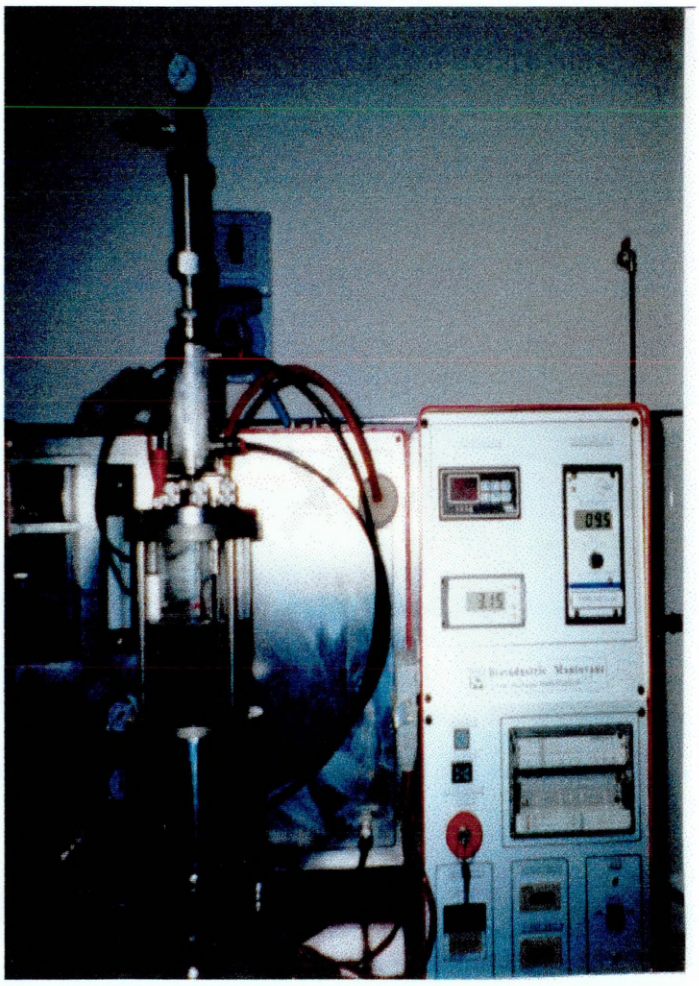


Plate 3.1 2L batch fermenter: Bioindustrie Mantovane BM3000

3.2.3 PRE-CONDITIONING OF WINE YEASTS

CULTURES AND INOCULUM PREPARATION

A culture of *Sacch. cerevisiae* DBVPG2168 was maintained on Malt Extract Agar and inoculum prepared in complete modified minimal media (CMMM). 1ml of this pre-culture was added to the experimental culture to give a 1% inoculum level in the final medium.

EXPERIMENTAL CONDITIONS

Pre-Conditioning

100ml volumes of modified minimal media in 250ml conical flasks were prepared, without magnesium. To this base medium magnesium was added to give final magnesium levels of; 0.05g/L, 0.1g/L, 0.2g/L, 0.4g/L and 0.8g/L Mg. The culture was inoculated into 0.05g/L Mg CMMM and grown up for 24hr, following this period a 1% inoculum was transferred into 0.1g/L Mg CMMM and so on every 24hr until 0.8g/L Mg pre-culturing was achieved. A control flask of 0.05g/L Mg CMMM was treated in the same manner, always transferring into 0.05g/L Mg CMMM. Cell numbers and intracellular magnesium levels were recorded for the course of pre-conditioning of cells over the five day period.

Fermentation

Fermentations were then carried out in base medium: 0.05g/L Mg CMMM. 1ml cell pellets of the control and the pre-conditioned cultures were used to inoculate the two fermentation flasks. Cell pellets were washed three times in deionised water (18 Ω) prior to resuspension in the experimental medium.

Samples were analysed for cell number (Coulter Counter), % Viability (haemocytometer), ethanol (GC) and glucose (HPLC) at 0, 6, 9, 24, 48 and 72hrs. Methods for these analyses are fully described in Chapter 2.

3.3 RESULTS AND DISCUSSION

3.3.1 SEMI-SYNTHETIC MEDIA STUDIES

Inorganic nutrition is of fundamental importance in yeasts in terms of growth and physiology, a factor often overlooked when considering the nutritional constituents of a particular fermentation medium. An imbalance in ionic nutrition is reflected in complex, and often subtle, alterations of metabolic patterns and growth characteristics (Jones & Greenfield 1984). Magnesium and calcium are known to directly influence yeast fermentative metabolism and the correct balance and availability of these cations are vital for a successful fermentation. Maintenance of high Mg:Ca concentration ratios may serve to alleviate biological antagonism of essential Mg^{2+}-dependent functions by Ca^{2+} (Walker *et al.* 1995).

GROWTH AND FERMENTATION PARAMETERS

Typical fermentation progress in semi-synthetic media, with adjusted Mg:Ca ratios to simulate levels in grape must (Figs. 3.3-3.6) shows, for each strain, a similar trend of biomass and ethanol production, and sugar consumption. *Sacch. cerevisiae* L-2226 (Fig. 3.3) and DBVPG2168 (Fig. 3.4) exhibited higher levels of biomass and more rapid sugar consumption than that of DCL'M', a distillers yeast (Fig. 3.5). Ethanol levels across these three strains were however fairly uniform, with levels of around 40-60g/L being produced. *T. delbrueckii* DBVPG6168 (Fig. 3.6) exhibited reduced levels of both biomass and ethanol, and a much slower consumption of sugar, when compared with the *Saccharomyces* strains, suggesting *Saccharomyces* is the genus best suited to growth in such high sugar concentrations.

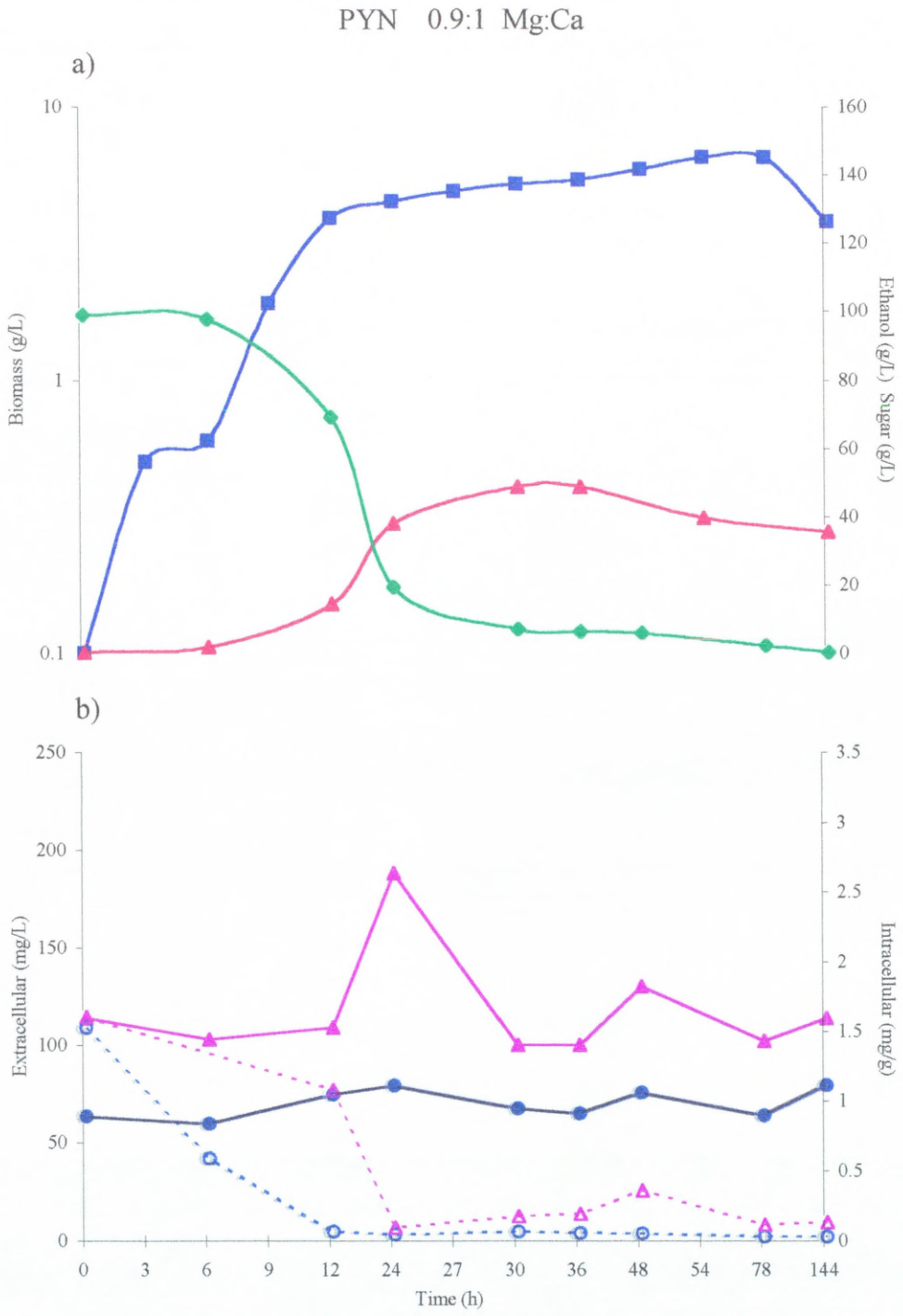


Figure 3.3 Typical fermentation progress of *Sacch. cerevisiae* L-2226 in semi-synthetic media; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -△- intracellular calcium.

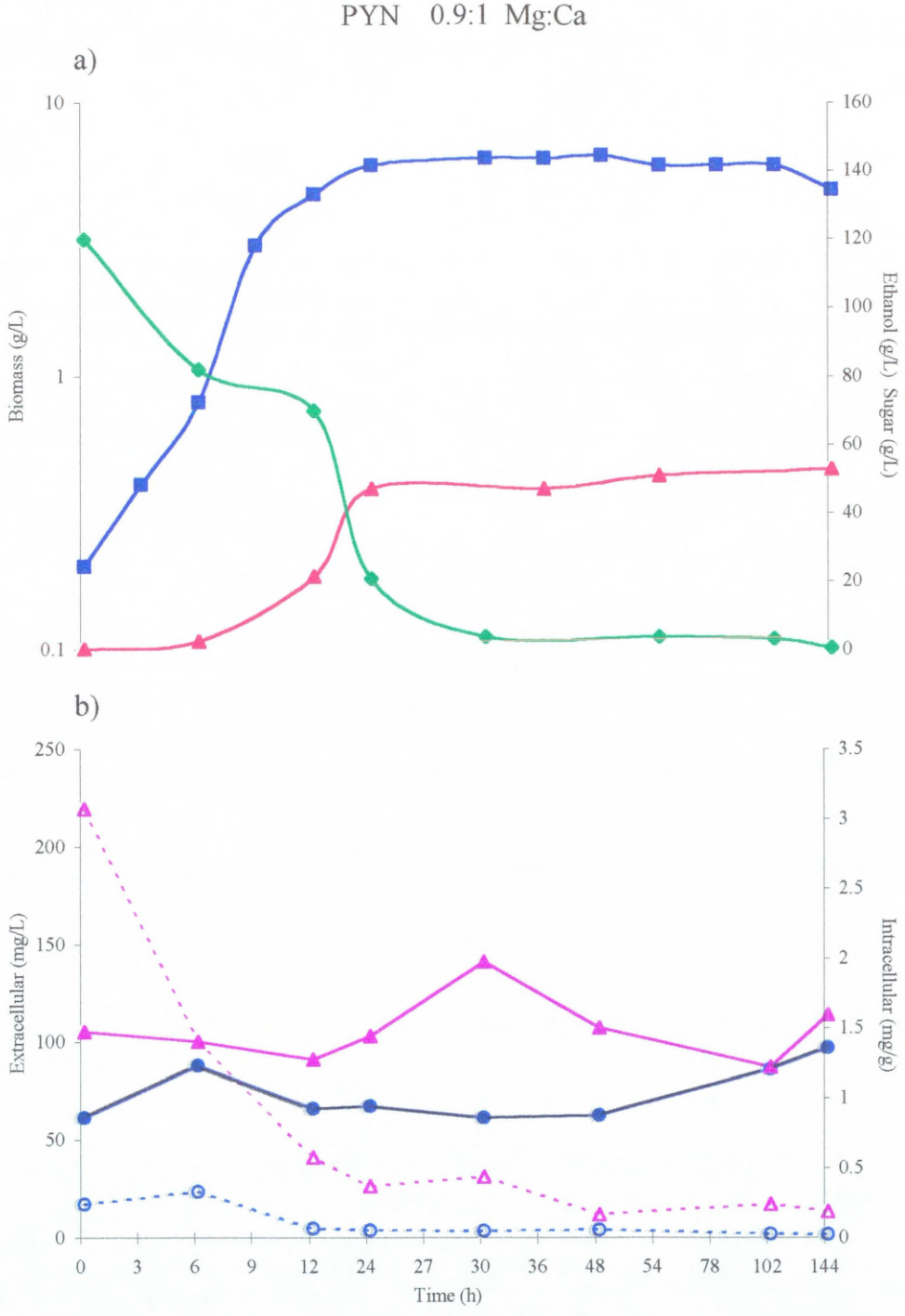


Figure 3.4 Typical fermentation progress of *Sacch. cerevisiae* DBV2168 in semi-synthetic media; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -Δ- intracellular calcium.

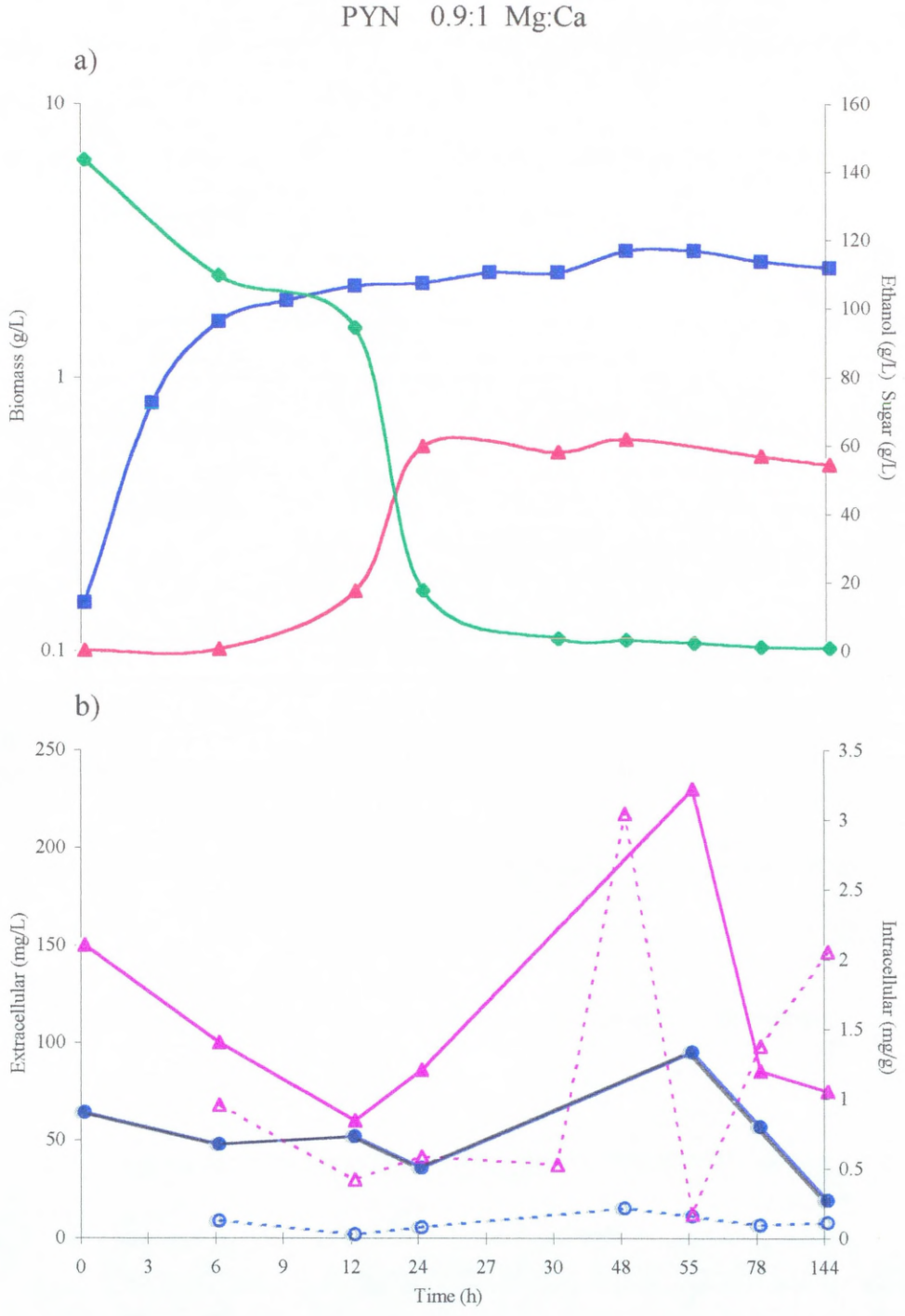


Figure 3.5 Typical fermentation progress of *Sacch. cerevisiae* DCL'M' in semi-synthetic media; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -Δ- intracellular calcium.

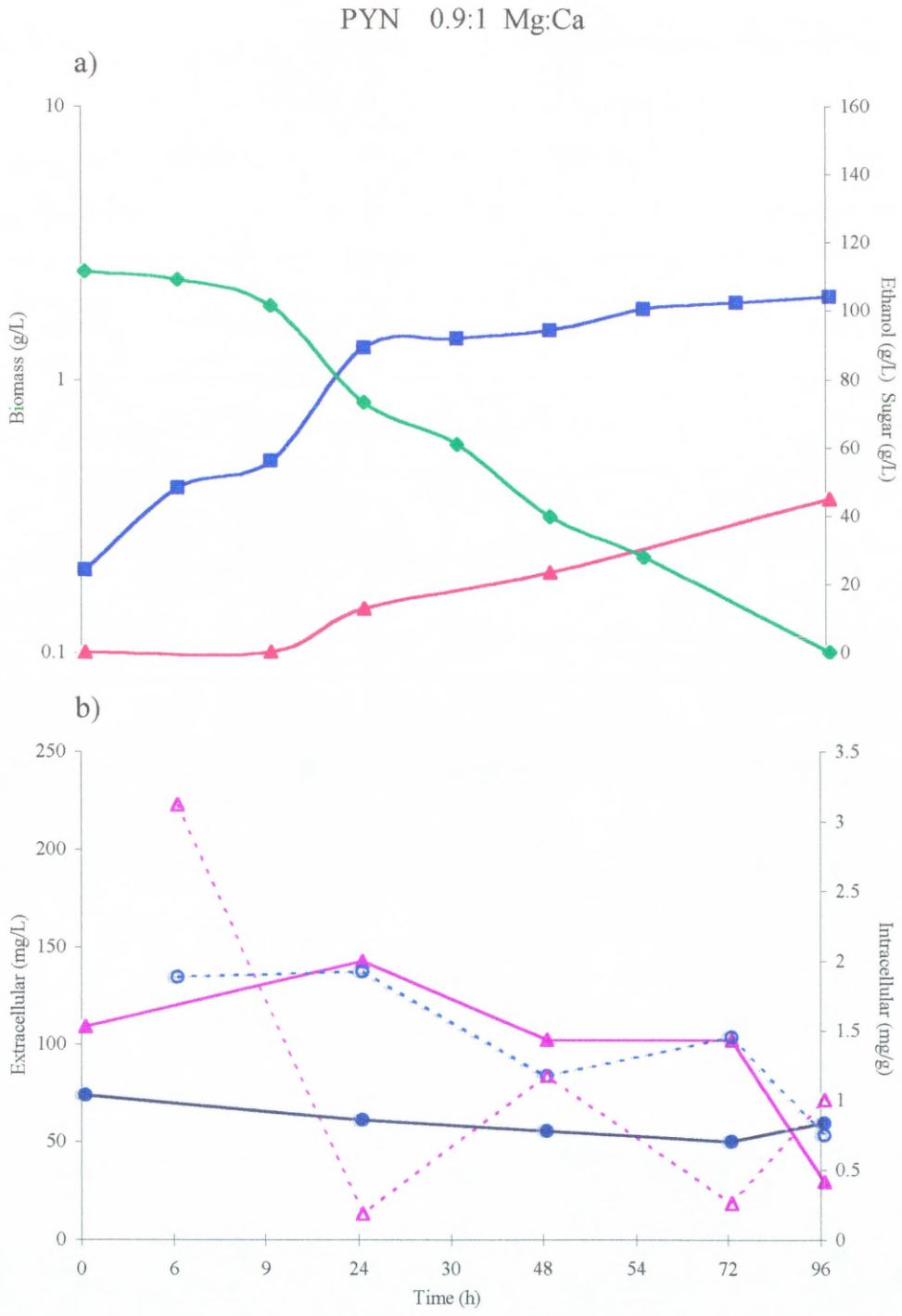


Figure 3.6 Typical fermentation progress of *Torulaspora delbrueckii* DBV6168 in semi-synthetic media; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -Δ- intracellular calcium.

In altering the Mg:Ca concentration ratios over a range from 1000:1 to 1:1000 Mg:Ca (molar range) one can observe (Tables 3.3-3.5) that increased levels of magnesium, or a Mg:Ca ratio equivalent to that of grape must, produces the best results in terms of yeast growth and ethanol production. The Mg:Ca ratios giving best fermentation performance for *Sacch. cerevisiae* DCL'M' (Table 3.3) were; 0.9:1 (approximation to levels in white grape must) resulting in biomass productivity of $0.0174gl^{-1}.h^{-1}$ and ethanol productivity of $0.377gl^{-1}.h^{-1}$; 100:1 and 1000:1, each with $0.016gl^{-1}.h^{-1}$ productivity of biomass and ethanol productivity levels of 0.405 and $0.386gl^{-1}.h^{-1}$ respectively. For this strain it can be seen that high levels of calcium are not conducive to fermentation, with the 1:1000 Mg:Ca concentration ratio only yielding $0.309gg^{-1}$ ethanol at 144h. Levels of residual sugar were also high indicating a much slower rate of sugar consumption. Flasks of 1:1 and 0:0 Mg:Ca concentration ratios did not favour growth either due to the fact that these media would not have contained suitable quantities of essential metal ions required for optimum growth and fermentation.

Sacch. cerevisiae L-2226 (Table 3.4) follows a similar trend with the higher ratios of magnesium resulting in high biomass and increased ethanol yields. Levels of magnesium up to 100mM (1000:1 Mg:Ca concentration ratio) gave reasonable yields of biomass and ethanol, although sugar consumption was not as efficient as the increased Mg ratios up to this level, indicating the initiation of slight inhibition due simply to an excess of this essential ion. Ionic constituents essential for growth and cellular functioning, at excessive levels, become toxic, inhibiting growth and fermentation characteristics e.g. sugar consumption and ethanol production. Mg:Ca ratio mimicking grape must, as with strain DCL'M', produced good biomass yield at $0.024gg^{-1}$ and ethanol productivity of $0.246gl^{-1}.h^{-1}$. Increases in the levels of calcium resulted in a reduction of growth yields and levels of ethanol produced. A similar trend was observed with *Sacch. cerevisiae* DBVPG2168 (Table 3.5) with the exception of, on elevation of calcium levels to 1:10 and 1:100 ratios an increase in biomass yield and consequently productivity was observed, as were ethanol yields. An explanation for this may be that this yeast strain is more suited to a higher level of calcium (having been isolated from grape must (Martini *et al.* 1978)) therefore,

Table 3.3 Fermentation parameters of *Sacch. cerevisiae* DCL'M' during fermentation in semi-synthetic media with altered Mg:Ca ratios.

	PYN Mg:Ca#	Biomass (g/L)			Ethanol (%)			Sugar Utilised (g/L)			Fermentation Progress			Final Fermentation Parameters (@144h)				
														Y(x)	P(x)	Y(etoh)	P(etoh)	Res. Sugar
	Time	B	M	E	B	M	E	B	M	E	B	M	E	($g.g^{-1}$)	($g.l^{-1}.h^{-1}$)	($g.g^{-1}$)	($g.l^{-1}.h^{-1}$)	($g.l^{-1}$)
A	1000:1	1.6	2.9	2.3	0.10	7.10	7.04	57.53	157.66	159.71	0.359	0.985	0.998	0.0144	0.0160	0.348	0.386	0.293
B	100:1	1.0	2.8	2.3	0.08	7.70	7.39	36.94	156.78	159.12	0.231	0.980	0.994	0.0144	0.0160	0.367	0.405	0.879
C	13.6:1*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
D	10:1	1.4	2.8	2.1	0.10	7.30	7.09	69.76	154.73	158.9	0.436	0.967	0.993	0.0132	0.0146	0.352	0.389	1.10
E	1.36:1*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
F	1:1	1.4	3.0	2.0	0.10	7.43	7.38	19.95	157.36	158.32	0.125	0.984	0.990	0.0126	0.0139	0.368	0.405	1.68
G	0:0	1.0	1.9	2.0	0.10	4.46	6.22	32.29	127.18	159.63	0.202	0.795	0.998	0.0125	0.0139	0.308	0.341	0.366
H	0.90:1	1.6	2.9	2.5	0.05	7.80	6.87	50.42	157.07	159.34	0.315	0.982	0.996	0.0157	0.0174	0.301	0.377	0.66
I	1:7.27*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
J	1:10	1.2	2.65	2.2	0.10	7.43	6.87	19.95	157.07	160	0.125	0.982	1.00	0.0138	0.0153	0.339	0.377	0.00
K	1:100	1.4	2.7	2.2	0.10	6.79	6.92	48.07	158.54	159.12	0.300	0.991	0.994	0.0138	0.0153	0.344	0.380	0.879
L	1:1000	1.4	2.4	1.9	0.00	5.37	6.22	21.74	125.55	150.84	0.136	0.778	0.943	0.0126	0.0132	0.309	0.341	9.16
M	Ion Free	0.9	1.7	2.4	0.00	0.64	2.25	28.74	37.82	87.63	0.180	0.236	0.548	0.0274	0.0167	0.203	0.123	72.37

Note: B = Beginning 6h; M = Middle 48h; E = End 144h (*96h)
molar ratio NT = Not Tested

Table 3.4 Fermentation parameters of *Sacch. cerevisiae* L-2226 during fermentation in semi-synthetic media with altered Mg:Ca ratios.

	PYN Mg:Ca#	Biomass (g/L)			Ethanol (%)			Sugar Utilised (g/L)			Fermentation Progress			Final Fermentation Parameters (@144h)				
	Time	B	M	E	B	M	E	B	M	E	B	M	E	Y(x) ($g.g^{-1}$)	P(x) ($g.l^{-1}.h^{-1}$)	Y(etoh) ($g.g^{-1}$)	P(etoh) ($g.l^{-1}.h^{-1}$)	Res. Sugar ($g.l^{-1}$)
A	1000:1	1.5	5.1	4.1	0.20	6.52	4.85	76.93	157.08	157.59	0.481	0.982	0.985	0.026	0.028	0.243	0.266	2.41
B	100:1	0.8	5.8	2.6	0.27	5.61	5.06	94.48	154.74	159.85	0.590	0.967	0.999	0.016	0.018	0.250	0.278	0.15
C	13.6:1*	0.8	4.5	6.4	0.96	5.16	6.21	30.14	98.38	120.00	0.251	0.820	1.0	0.053	0.067	0.409	0.511	0.00
D	10:1	1.5	5.6	3.2	0.15	5.77	5.14	62.09	157.37	158.54	0.388	0.984	0.991	0.020	0.022	0.256	0.282	1.46
E	1.36:1*	0.2	4.1	4.4	0.83	4.73	5.22	23.33	85.72	120.00	0.193	0.714	1.0	0.037	0.046	0.344	0.430	0.00
F	1:1	1.8	6.2	3.0	0.17	5.21	4.62	74.01	157.95	159.85	0.462	0.987	0.999	0.019	0.021	0.228	0.253	0.15
G	0:0	0.6	3.4	3.9	0.16	3.04	4.95	87.46	132.80	159.93	0.547	0.830	0.999	0.024	0.027	0.244	0.271	0.07
H	0.90:1	0.6	4.9	3.8	0.21	6.16	4.48	62.30	154.30	159.83	0.389	0.964	0.999	0.024	0.026	0.221	0.246	0.18
I	1:7.27*	1.0	3.4	4.2	0.76	5.03	5.7	19.78	99.26	120.00	0.165	0.827	1.0	0.035	0.044	0.375	0.469	0.00
J	1:10	1.0	5.4	3.6	0.18	6.21	5.22	55.87	157.15	159.84	0.349	0.982	0.999	0.022	0.025	0.258	0.286	0.16
K	1:100	1.6	5.5	1.8	0.18	5.16	4.31	82.78	156.64	159.84	0.517	0.979	0.999	0.011	0.012	0.213	0.236	0.16
L	1:1000	0.4	2.4	3.0	0.07	4.00	5.69	47.68	153.27	159.89	0.298	0.958	0.999	0.019	0.021	0.281	0.312	0.11
M	Ion Free	0.1	0.6	1.0	0.07	0.26	0.95	52.36	87.03	100.70	0.327	0.544	0.629	0.010	0.007	0.074	0.052	59.30

Note: B = Beginning 6h; M = Middle 48h; E = End 144h (*96h)
molar ratio

Table 3.5 Fermentation parameters of *Sacch. cerevisiae* DBV2168 during fermentation in semi-synthetic media with altered Mg:Ca ratios.

	PYN Mg:Ca#	Biomass (g/L)			Ethanol (%)			Sugar Utilised (g/L)			Fermentation Progress			Final Fermentation Parameters (@144h)				
	Time	B	M	E	B	M	E	B	M	E	B	M	E	Y(x) ($g.g^{-1}$)	P(x) ($g.l^{-1}.h^{-1}$)	Y(etoh) ($g.g^{-1}$)	P(etoh) ($g.l^{-1}.h^{-1}$)	Res. Sugar ($g.l^{-1}$)
A	1000:1	0.9	5.0	3.9	0.76	6.81	6.45	19.60	156.34	159.77	0.122	0.977	0.998	0.024	0.027	0.319	0.354	0.23
B	100:1	1.1	6.8	4.9	0.23	8.10	6.52	10.75	157.81	159.82	0.086	0.986	0.999	0.031	0.034	0.322	0.358	0.18
C	13.6:1*	0.5	1.6	1.7	0.91	6.58	6.11	31.18	119.70	120.00	0.260	0.998	1.0	0.014	0.018	0.402	0.503	0.00
D	10:1	1.0	6.4	5.0	0.30	6.82	7.48	13.75	157.81	159.82	0.086	0.986	0.999	0.032	0.035	0.370	0.410	0.18
E	1.36:1*	0.6	1.6	1.7	0.98	6.01	5.84	19.26	119.14	120.00	0.160	0.993	1.0	0.014	0.018	0.384	0.481	0.00
F	1:1	1.6	5.0	4.2	0.29	5.81	6.81	16.20	157.10	159.80	0.101	0.982	0.999	0.027	0.030	0.337	0.374	0.20
G	0:0	0.8	5.4	5.0	0.54	3.80	5.79	19.60	132.21	159.77	0.122	0.826	0.998	0.031	0.035	0.286	0.318	0.23
H	0.90:1	0.8	6.4	4.8	0.28	5.94	6.67	78.10	156.34	159.71	0.488	0.977	0.998	0.030	0.033	0.330	0.366	0.29
I	1:7.27*	0.3	1.7	1.7	0.96	5.78	5.91	34.81	119.57	120.00	0.290	0.996	1.0	0.014	0.018	0.389	0.486	0.00
J	1:10	1.1	5.6	5.5	0.27	7.64	6.49	48.85	158.54	159.82	0.305	0.991	0.999	0.034	0.038	0.321	0.356	0.18
K	1:100	0.6	5.6	6.0	0.26	7.52	6.97	16.20	158.46	159.71	0.101	0.990	0.998	0.038	0.042	0.345	0.382	0.23
L	1:1000	1.6	5.3	4.1	-	5.32	6.28	19.60	155.62	159.80	0.122	0.973	0.999	0.026	0.028	0.310	0.344	0.20
M	Ion Free	0.8	1.2	1.9	0.26	0.50	1.16	31.30	102.96	157.30	0.196	0.643	0.983	0.012	0.013	0.058	0.064	2.70

Note: B = Beginning 6h; M = Middle 48h; E = End 144h (*96h)
molar ratio

on elevation of levels to 1mM and 10mM a beneficial effect is observed. Alterations of the cellular balance of other ions may have some involvement with this, but without free intracellular ion studies this must only be speculation.

In ion free media all three strains produce a similar result. Residual sugar levels are exceptionally high after 144h, with the exception of DBVPG2168 and in all cases ethanol productivity is very low; 0.052-0.123$gl^{-1}.h^{-1}$. Biomass productivity's for the two wine strains L-2226 and DBVPG2168 were 0.007 and 0.013$gl^{-1}.h^{-1}$ respectively, however, DCL'M' seemed more able to grow in this reduced ionic environment, showing a biomass productivity of 0.0167$gl^{-1}.h^{-1}$ (Tables 3.3-3.5). These results simply serve to substantiate the claim that magnesium and calcium are essential ions for yeast growth and productivity. Pictorial representation of these trends at 24, 48 and 144h respectively, can be seen in Figures 3.7a-3.9a. From these graphs it can be seen more clearly those ratios of Mg:Ca which are not conducive fermentation, with the ratios 0:0, 1:1, 1:1000 Mg:Ca and Ion free, being the most prominent at 24h (Fig 3.7a) and throughout fermentation (Fig 3.8a: 48h & Fig 3.9a: 144h). Biomass levels, although decreasing slightly around 144h, are noticeably higher in strains L-2226 and DBVPG2168 suggesting that these strains are more adapted to growing in conditions of a semi-synthetic wine. Biomass levels at the peak of fermentation; 48h (Fig 3.8a) noticeably remain highest at the ratios 100:1, 10:1 and 0.90:1 (Figs 3.8 & 3.9) as has been observed when looking at the final fermentation parameters (Tables 3.3-3.5).

A closer look at growth characteristics and yeast fermentation characteristics (Tables 3.6-3.8) reveals that doubling times improve on increase of Mg:Ca ratios in favour of magnesium, up to a ratio of 100:1 Mg:Ca generally, although some strains do differ. DBVPG2168 (Table 3.7) exhibited a reduction in doubling time of 3.21h from 100:1 to 1000:1 Mg:Ca media, whereas with the other strains tested an increase in doubling times were noted. Generally, elevation of magnesium level up to 100:1 Mg:Ca concentration ratio involved a decrease in doubling times of around 20%, 10:1 ratio, or 38% 100:1 ratio for strain L-2226 (Table 3.6) and around 8% on increase to 10:1 Mg:Ca ratio for strain

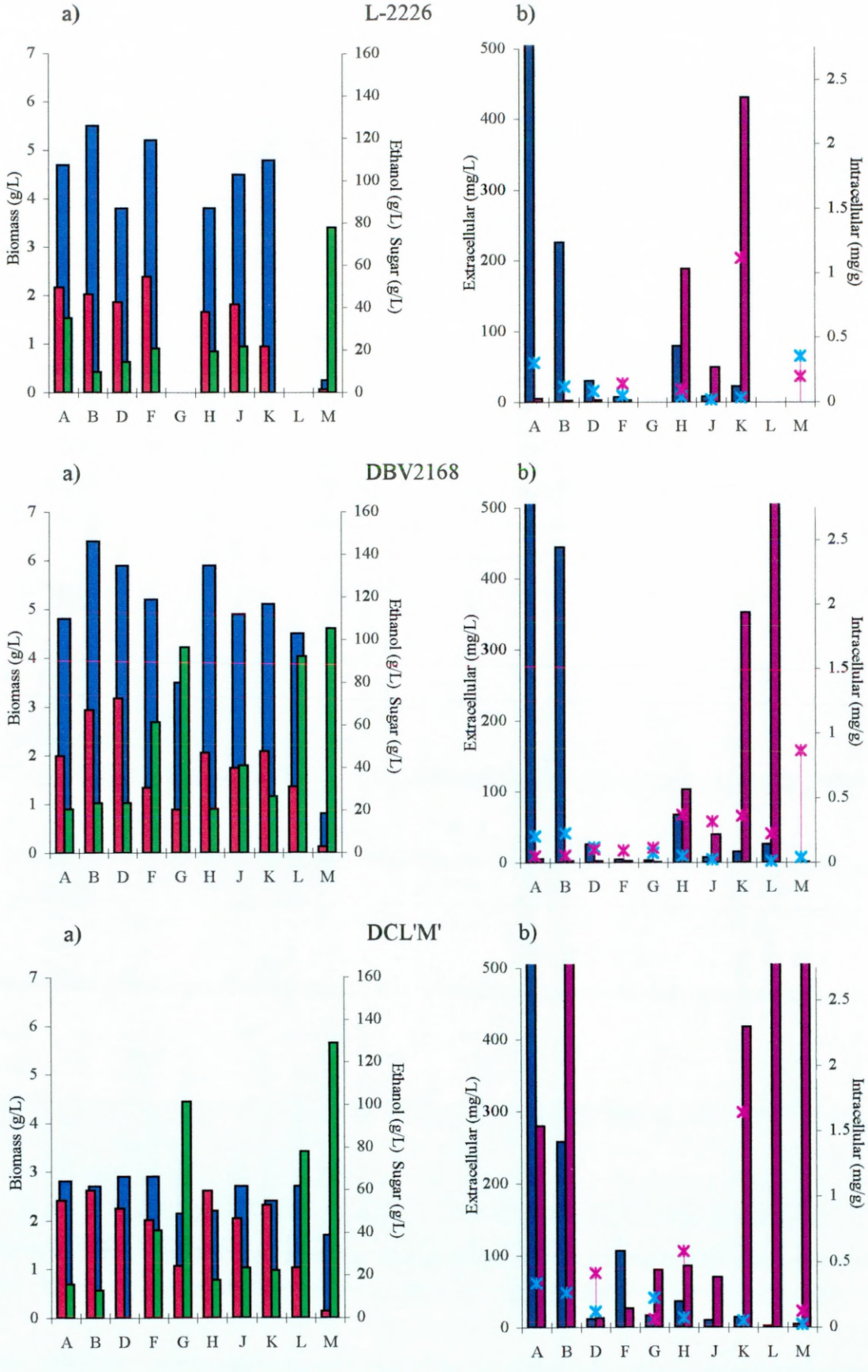


Figure 3.7 Fermentation and metal ion parameters after 24h in semi-synthetic media with altered Mg:Ca ratios; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: PYN media with altered molar Mg:Ca ratios; A 1000:1, B 100:1, D 10:1, F 1:1, G 0:0, H 0.9:1, J 1:10, K 1:100, L 1:1000, M Ion Free.

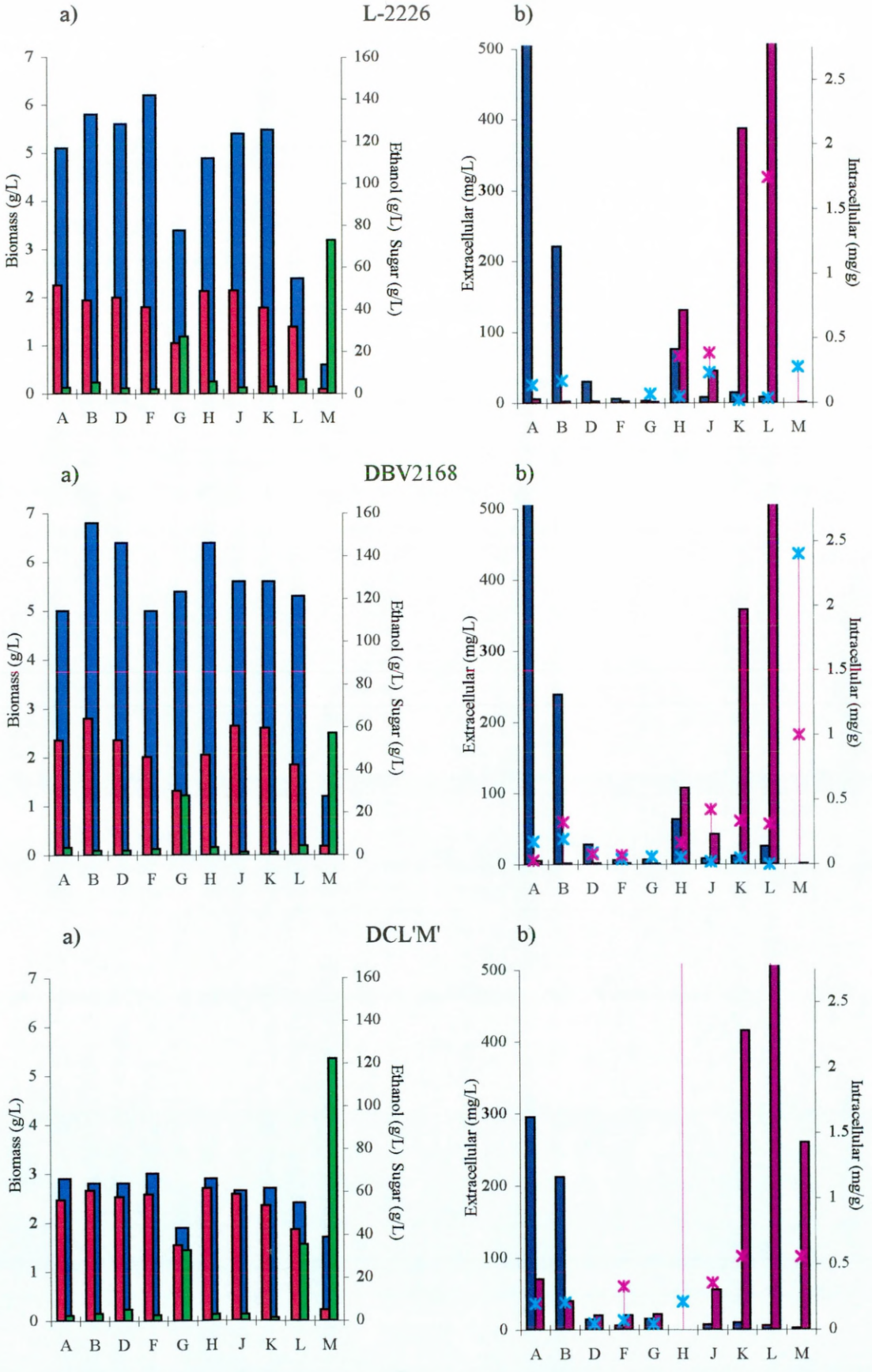


Figure 3.8 Fermentation and metal ion parameters after 48h in semi-synthetic media with altered Mg:Ca ratios; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: PYN media with altered molar Mg:Ca ratios; A 1000:1, B 100:1, D 10:1, F 1:1, G 0:0, H 0.9:1, J 1:10, K 1:100, L 1:1000, M Ion Free.

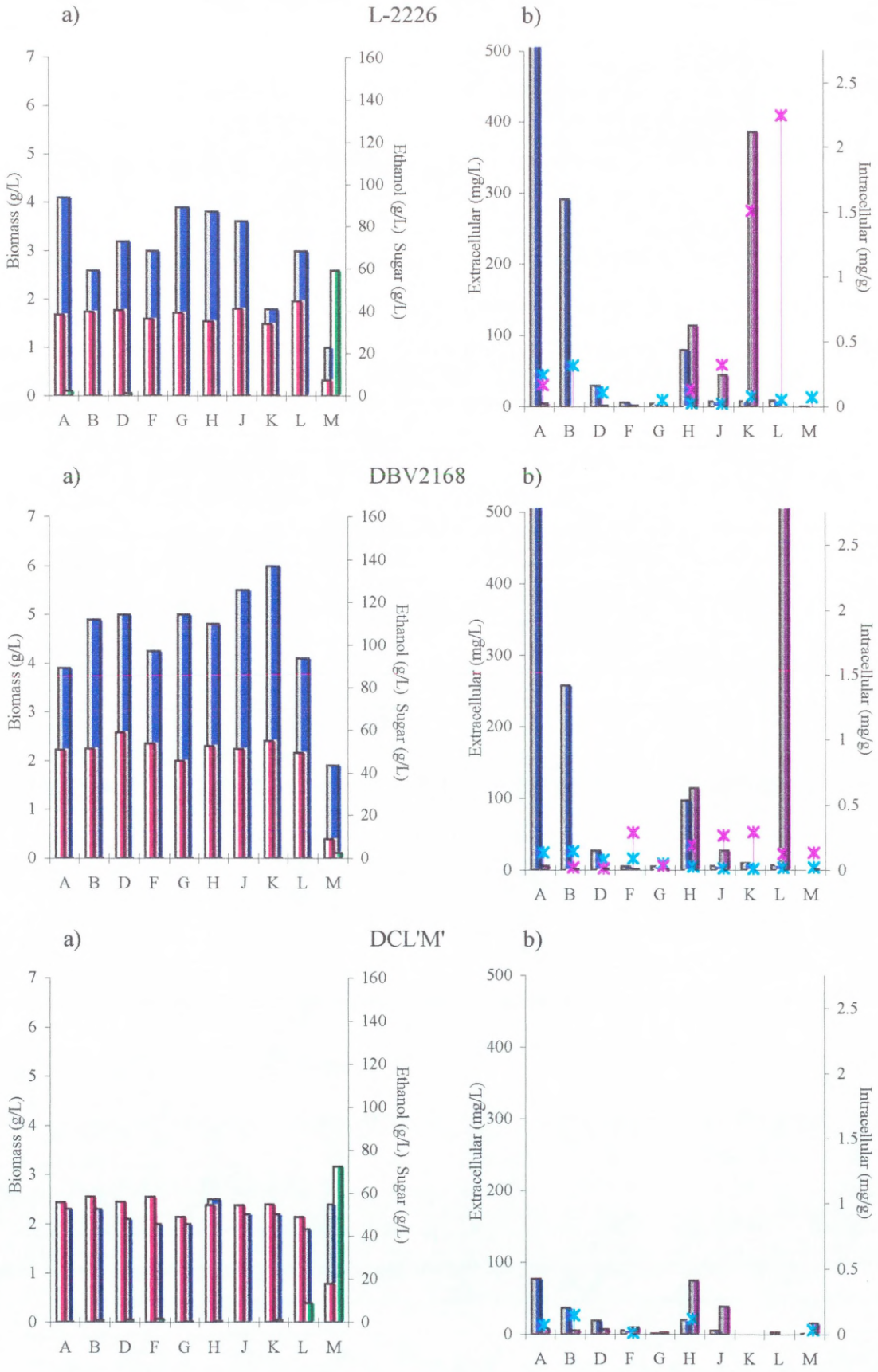


Figure 3.9 Fermentation and metal ion parameters after 144h in semi-synthetic media with altered Mg:Ca ratios; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: PYN media with altered molar Mg:Ca ratios; A 1000:1, B 100:1, D 10:1, F 1:1, G 0:0, H 0.9:1, J 1:10, K 1:100, L 1:1000, M Ion Free.

Table 3.6 Influence of Mg:Ca ratio on growth and fermentation characteristics of *Sacch. cerevisiae* L-2226.

					Yeast Growth Characteristics (@144h)			Yeast Fermentation Characteristics (@144h)			
	Media (molar)	Mg (mg/L)	Ca (mg/L)	Mg:Ca (mg/L)	Doubling Time (Td) (h)	μ (h^{-1})	Molar Growth Yield (Ym) ($g.M^{-1}$)	Ethanol Produced (g/L)	Y(etoh) ($g.g^{-1}$)	Q(L) ($g.g^{-1}DW.h^{-1}$)	Residual Sugar (g/L)
A	1000:1	2230	5.11	436.4:1	23.90	0.029	4.69	38.32	0.243	0.267	2.41
B	100:1	250.3	4.12	60.75:1	15.40	0.045	2.93	39.97	0.250	0.427	0.15
C	13.6:1*	680	116	5.86:1	19.80	0.035	6.96	49.06	0.409	0.195	0.00
D	10:1	27.9	4.32	6.5:1	19.25	0.036	3.64	40.61	0.256	0.344	1.46
E	1.36:1*	77	116	0.66:1	23.10	0.030	6.44	41.24	0.344	0.284	0.00
F	1:1	5.1	4.66	1.09:1	15.75	0.044	3.38	36.50	0.228	0.370	0.15
G	0:0	3.25	2.39	1.35:1	33.01	0.021	4.39	39.10	0.244	0.285	0.07
H	0.90:1	67.1	100	0.67:1	24.76	0.028	4.28	35.40	0.221	0.292	0.18
I	1:7.27*	77	564	0.14:1	34.66	0.020	6.38	45.03	0.375	0.298	0.00
J	1:10	5.3	54.0	1:10	31.51	0.022	4.06	41.24	0.258	0.308	0.16
K	1:100	17.0	500	1:29.4	23.90	0.029	2.03	34.05	0.213	0.617	0.16
L	1:1000	13.2	3700	1:280.3	21.66	0.032	3.38	44.95	0.281	0.370	0.11
M	Ion Free	0.429	0.66	0.65:1	46.21	0.015	1.79	7.50	0.074	0.699	59.30

* @96h

Table 3.7 Influence of Mg:Ca ratio on growth and fermentation characteristics of *Sacch. cerevisiae* DBV2168.

	Media	Mg	Ca	Mg:Ca	Yeast Growth Characteristics (@144h)			Yeast Fermentation Characteristics (@144h)			
					Doubling Time (Td)	μ	Molar Growth Yield (Ym)	Ethanol Produced	Y(etoh)	Q(L)	Residual Sugar
	(molar)	(mg/L)	(mg/L)	(mg/L)	(h)	(h^{-1})	($g.M^{-1}$)	(g/L)	($g.g^{-1}$)	($g.g^{-1}DW.h^{-1}$)	(g/L)
A	1000:1	2180	3.9	559:1	25.67	0.027	4.40	50.96	0.319	0.284	0.23
B	100:1	274	4.79	57.2:1	28.88	0.024	5.52	51.51	0.322	0.226	0.18
C	13.6:1*	447.8	116.4	3.85:1	24.75	0.028	2.12	48.27	0.402	0.735	0.00
D	10:1	29.0	3.97	7.3:1	19.25	0.036	5.64	59.09	0.370	0.222	0.18
E	1.36:1*	74.27	165.1	0.45:1	31.95	0.022	2.08	46.14	0.384	0.735	0.00
F	1:1	6.6	5.29	1.25:1	20.39	0.034	4.79	53.80	0.337	0.261	0.20
G	0:0	3.1	0.92	3.37:1	25.67	0.027	5.64	45.74	0.286	0.222	0.23
H	0.90:1	78.2	134	0.58:1	21.00	0.033	5.42	52.69	0.330	0.231	0.29
I	1:7.27*	101.22	1333.8	0.08:1	19.18	0.036	2.51	46.69	0.389	0.735	0.00
J	1:10	2.9	55	1:19	34.66	0.020	6.20	51.27	0.321	0.202	0.18
K	1:100	5.0	376	1:75.2	36.48	0.019	6.77	55.06	0.345	0.185	0.23
L	1:1000	8.8	3640	1:413.6	49.51	0.014	4.62	49.61	0.310	0.271	0.20
M	Ion Free	0.396	0.5	0.79:1	86.64	0.008	2.18	9.16	0.058	0.575	2.70

* @96h

Table 3.8 Influence of Mg:Ca ratio on growth and fermentation characteristics of *Sacch. cerevisiae* DCL'M'.

	Media	Mg	Ca	Mg:Ca	Yeast Growth Characteristics (@144h)			Yeast Fermentation Characteristics (@144h)			
					Doubling Time (Td)	μ	Molar Growth Yield (Ym)	Ethanol Produced	Y(etoh)	Q(L)	Residual Sugar
	(molar)	(mg/L)	(mg/L)	(mg/L)	(h)	(h^{-1})	($g.M^{-1}$)	(g/L)	($g.g^{-1}$)	($g.g^{-1}DW.h^{-1}$)	(g/L)
A	1000:1	578	140	4.13:1	34.66	0.020	2.59	56.62	0.348	0.482	0.293
B	100:1	193.2	20	9.66:1	23.90	0.029	2.60	58.38	0.367	0.480	0.879
C	13.6:1*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
D	10:1	24	52	0.46:1	19.80	0.035	2.38	56.01	0.352	0.525	1.10
E	1.36:1*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
F	1:1	8	44	0.18:1	46.21	0.015	2.28	58.30	0.368	0.550	1.68
G	0:0	6	42	0.14:1	99.02	0.007	2.56	49.14	0.308	0.554	0.366
H	0.90:1	66	136	0.48:1	49.51	0.014	2.83	77.97	0.301	0.443	0.66
I	1:7.27*	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
J	1:10	16	82	1:5.12	18.73	0.037	2.48	54.27	0.339	0.505	0.00
K	1:100	22	408	1:18.5	22.36	0.031	2.49	54.67	0.344	0.502	0.879
L	1:1000	4.4	4380	1:996	23.10	0.030	2.27	49.14	0.309	0.551	9.16
M	Ion Free	0.4	0.0	-	43.32	0.016	4.93	17.78	0.203	0.254	72.37

* @96h NT = Not Tested

DBVPG2168 (Table 3.7) using simulated wine (0.90:1 ratio) as a control. Ethanol yields and rates of sugar consumption also improved on these alterations of ionic ratios (Tables 3.6 & 3.7). Increasing calcium levels for strains L-2226 and DBVPG2168 (Tables 3.6 & 3.7) generally reduced the final levels of ethanol produced and in the case of L-2226, reduced molar growth yields. Growth yields for DCL'M' (Table 3.8) were, as have been noted previously, low in all media, however, the detrimental effect of calcium and beneficial effect of magnesium can still be witnessed. Also, in comparing oenological characters of the three *Sacch. cerevisiae* strains DCL'M', L-2226 and DBVPG2168 in semi-synthetic media (Table 3.9) at varying ratio extremes, it can be seen that increasing magnesium levels generally benefits all aspects of yeast fermentation; growth rates, ethanol capacity and final ethanol yields, when compared to results of fermentations with elevated calcium levels.

Organic Profiles of Wine

Although semi-synthetic media would never be used for the production of wine, one is still interested in the range and levels of flavour compounds produced by the yeast. Ethanol is not the sole product of fermentation of sugars, other secondary products for example, organic compounds such as organic acids and other carbohydrates, along with the levels of residual sugars, combine to produce the signature of the wine. The levels of each of these components contributing to the flavour and quality of the resultant wine. High levels of organic acids would make the wine tart and 'metally', whereas low levels of residual sugar result in a dry wine. All of these characters must, therefore, be finely balanced to produce a good wine and this depends not only on the grape chosen but also on the yeast involved.

Final fermentation profiles of semi-synthetic wine with altered Mg:Ca ratios, over the range 1000:1 to 1:1000, were assessed for the wine strains *Sacch. cerevisiae* L-2226 and DBVPG2168. Figure 3.10 shows a typical HPLC profile of a high magnesium and high calcium semi-synthetic wine produced by L-2226. The differences in profiles of these two media are quite noticeable in comparing traces (Fig. 3.10a & b). Fermentation in high magnesium media produces higher levels of citric acid, succinic acid and ethanol and a

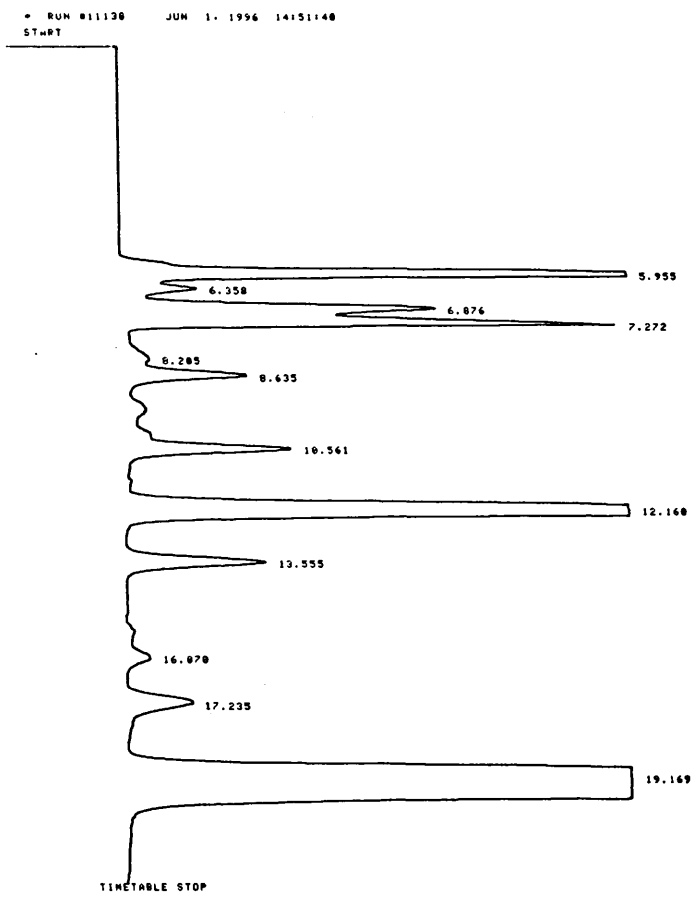
Table 3.9 Oenological characters of *Sacch. cerevisiae* strains DCL'M', L-2226 and DBV2168.

Media (molar ratio)	Strain	Ethanol Capacity (@ 48h) (g/L)	Fermentation Rate (@ 48h)	Doubling Time (Td) (h)	Specific Growth Rate (μ) (h^{-1})	Final Cell Density (@ 144h) (g/L)	Final Ethanol Yield (@ 144h) ($g.g^{-1}$)
100:1 Mg:Ca	*Sacch. cerevisiae* DCL'M'	60.83	0.980	23.90	0.029	2.3	0.367
	Sacch. cerevisiae L-2226	44.33	0.967	15.40	0.045	2.6	0.250
	Sacch. cerevisiae DBV2168	63.99	0.986	28.88	0.024	4.9	0.322
Wine Mg:Ca	*Sacch. cerevisiae* DCL'M'	61.62	0.982	49.51	0.014	2.5	0.301
(0.90:1)	*Sacch. cerevisiae* L-2226	48.70	0.964	24.76	0.028	3.8	0.221
	Sacch. cerevisiae DBV2168	46.91	0.977	21.00	0.033	4.8	0.330
1:100 Mg:Ca	*Sacch. cerevisiae* DCL'M'	53.64	0.991	22.36	0.031	2.2	0.344
	Sacch. cerevisiae L-2226	40.76	0.979	23.90	0.036	1.8	0.213
	Sacch. cerevisiae DBV2168	59.41	0.990	36.48	0.019	6.0	0.345

reduction in levels of acetic acid and acetaldehyde, a pattern also observed with DBVPG2168 (Fig. 3.11).

In order to observe if the alteration of Mg:Ca ratio had a significant effect on the final organic profile of the semi-synthetic wine, samples of strains L-2226 and DBVPG2168 were analysed. Tables 3.10 & 3.11 record the amounts of a selection of important organic components of wine for the full range of media and Figures 3.12 & 3.13 illustrate the trend in comparing; 100:1 Mg:Ca PYN, 0.90:1 Mg:Ca PYN (simulated grape must) and 1:100 Mg:Ca PYN. For *Sacch. cerevisiae* L-2226, levels of glycogen and trehalose, two storage carbohydrates were observed to increase on elevation of magnesium levels, as were levels of citric acid, succinic acid and glycerol. Levels of acetic acid and acetaldehyde were much reduced in comparison to "wine" levels. On the other hand, the increase of calcium levels to 10mM produced the reverse effect, with levels of acetic acid and acetaldehyde being much higher than in Mg-supplemented cultures and levels of the other compounds being reduced. A similar trend was observed with DBVPG2168 (Table 3.11 & Fig. 3.13) except that trehalose levels were found to increase on supplementation of calcium (a more expected result than that of L-2226) and the levels of acetic acid and acetaldehyde in the simulated wine (0.90:1) were much lower than levels observed with L-2226. In closer inspection of the full range of Mg:Ca ratios, it can be seen that the trend illustrated in Figures 3.12 & 3.13 was characteristic of the alterations (Tables 3.10 & 3.11). Elevated levels of calcium in the fermentation media resulted in high levels of acetic acid and acetaldehyde, lower levels of succinic acid and citric acid, although to a lesser degree with the latter, and generally higher levels of residual sugars, resulting in a tart/acidic, unbalanced wine. Elevation of magnesium, on the other hand, produced a well-balanced wine with levels of individual products altering dependent on the ratio involved. In all cases, however, levels of acetic acid and acetaldehyde were much reduced; 3-7 fold less dependent on the ratio of Mg:Ca (Table 3.10 & 3.11). Increases in acetic acid levels may also be the result of bioconversion of ethanol corresponding to a decrease in ethanol levels at the end of fermentation.

a)



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SUGAR/ETHANOL ANALYSIS

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/	NAME
5.955	PV	3037752	.169	299788	1R	2.980	GLYCOGEN
6.076	VV	1239272	.282	73269	2R	.622	TREHALOSE
7.272	VV	1579744	.239	110857	3R	1.123	CITRIC ACID
8.285	VV	164595	.453	6856	5R	.058	GLUCOSE
8.635	VV	512256	.297	28711	6R	.257	FRUCTOSE
10.561	VB	751582	.326	38384	7R	1.265	SUCCINIC ACID
12.160	VB	5038626	.288	346776	9R	4.612	GLYCEROL
13.555	BV	594995	.383	32764	10R	.795	ACETIC ACID
16.070	VP	150361	.419	5976	11R	.466	ACETALDEHYDE
19.169	ISBP	37034432	.517	1194770	12R	62.887	ETHANOL

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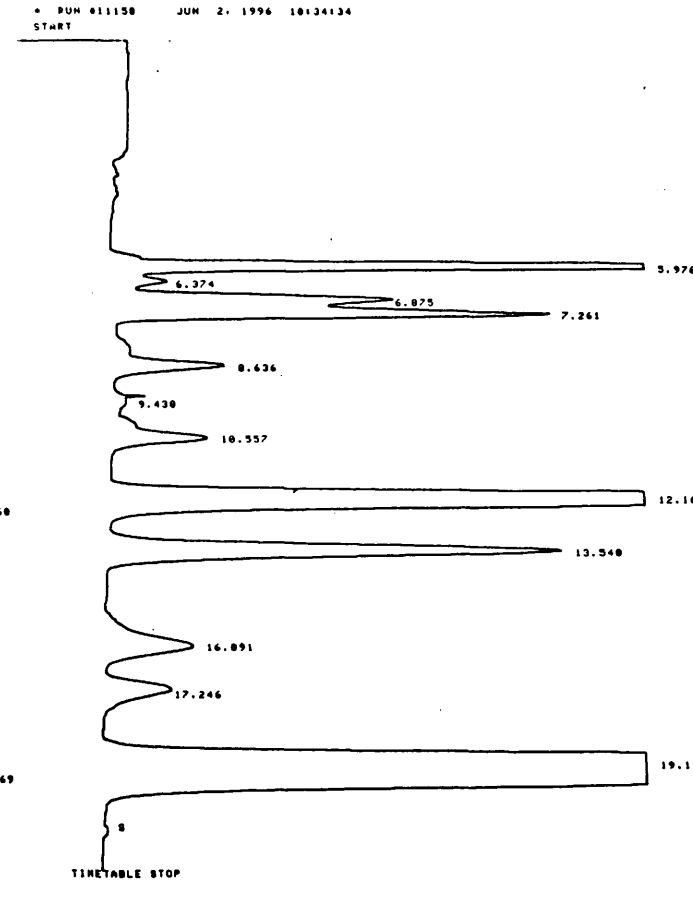
MUL FACTOR=1.0000E+00

b)



RUN# 11158 JUN 2, 1996 10:34:34

SUGAR/ETHANOL ANALYSIS

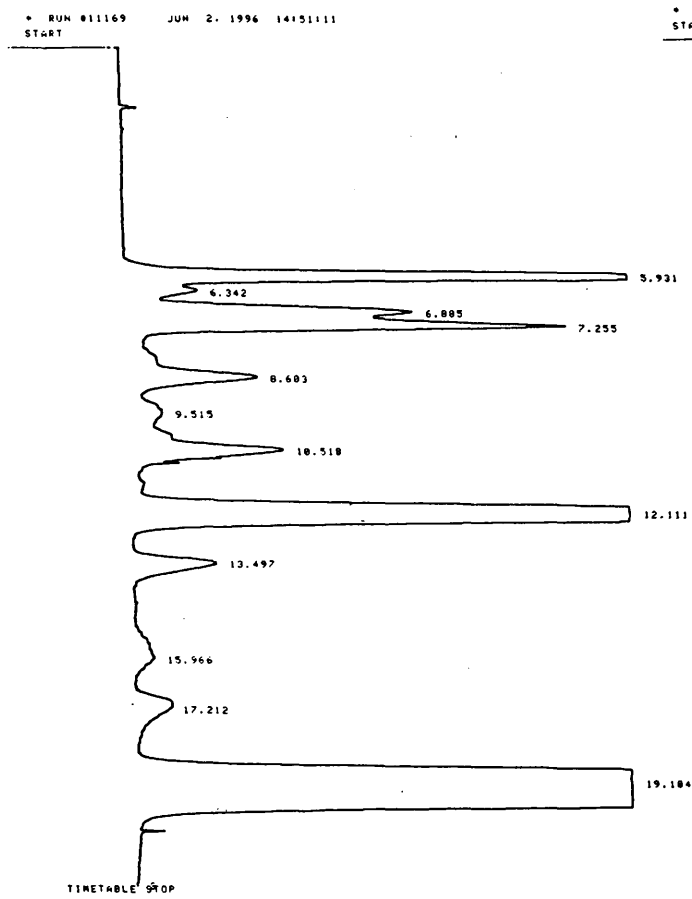
ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/	NAME
5.976	PV	2811077	.181	259053	1R	2.522	GLYCOGEN
6.875	VV	1135987	.291	64903	2R	.542	TREHALOSE
7.261	VV	1668294	.274	101345	3R	1.038	CITRIC ACID
8.636	VV	669137	.421	26518	6R	.237	FRUCTOSE
10.557	VV	598035	.436	22563	7R	.733	SUCCINIC ACID
12.160	VB	6580755	.354	310297	9R	4.133	GLYCEROL
13.548	BP	2382152	.360	104161	10R	2.485	ACETIC ACID
16.891	PV	703970	.627	28856	11R	1.668	ACETALDEHYDE
19.198	BPB	34001688	.548	1036412	12R	53.793	ETHANOL

TOTAL HEIGHT=1981584
MUL FACTOR=1.0000E+00

Figure 3.10 Typical final (144h) fermentation products profile of *Sacch. cerevisiae* L-2226 fermentation in semi-synthetic media. a) 100:1 Mg:Ca ratio PYN b) 1:100 Mg:Ca ratio PYN media.

a)



RUN# 11169 JUN 2. 1996 14:51:11

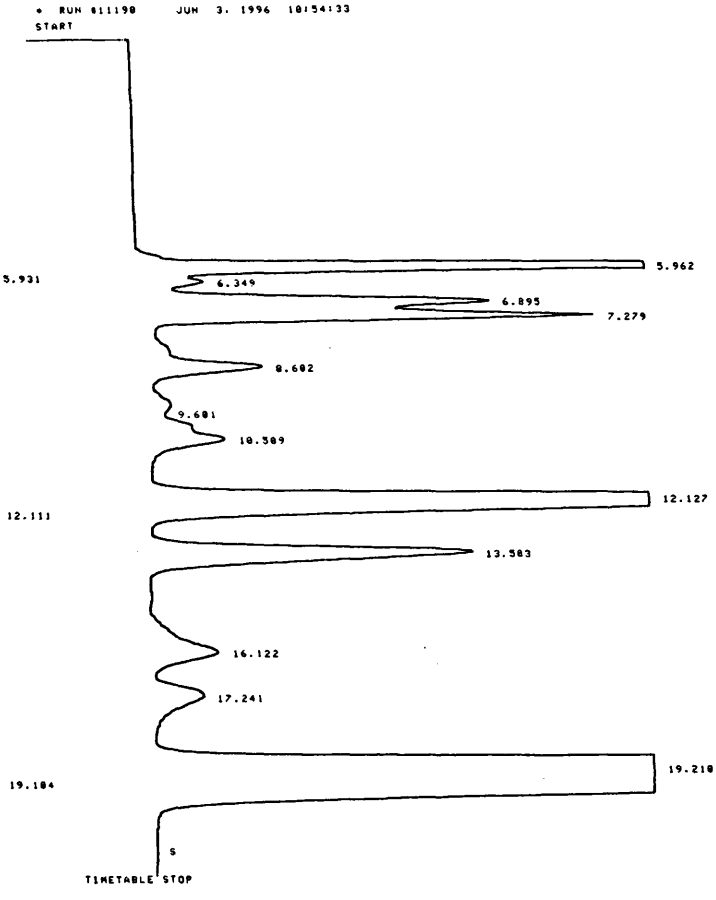
SUGAR/ETHANOL ANALYSIS

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/	NAME
5.931	BV	2911482	.215	225467	1R	2.144	GLYCOGEN
6.985	VV	1203816	.299	67092	2R	.562	TREHALOSE
7.255	VV	1868708	.382	103114	3R	1.055	CITRIC ACID
8.603	VV	998078	.531	31125	6R	.270	FRUCTOSE
10.518	VV	1162085	.523	37013	7R	1.222	SUCCINIC ACID
12.111	VV	6721114	.384	291824	9R	3.898	GLYCEROL
13.497	VV	577907	.459	20965	10R	.488	ACETIC ACID
15.966	VV	375876	1.817	6161	11R	.488	ACETALDEHYDE
19.184	SHB	38719872	.661	976376	12R	50.679	ETHANOL

TOTAL HEIGHT=1795569
MUL FACTOR=1.0000E+00

b)



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SUGAR/ETHANOL ANALYSIS

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/	NAME
5.962	PV	2843418	.284	232487	1R	2.223	GLYCOGEN
6.895	VV	1523179	.312	81372	2R	.708	TREHALOSE
7.279	VV	1759362	.278	105354	3R	1.077	CITRIC ACID
8.602	VV	868360	.511	28314	6R	.253	FRUCTOSE
10.509	VV	771614	.677	18993	7R	.694	SUCCINIC ACID
12.127	VV	6191709	.393	262708	9R	3.508	GLYCEROL
13.583	VV	1857647	.413	75013	10R	1.807	ACETIC ACID
16.122	PV	630686	.684	15573	11R	1.267	ACETALDEHYDE
19.218	SHB	37049920	.648	965348	12R	50.107	ETHANOL

Figure 3.11 Typical final (144h) fermentation products profile of *Sacch. cerevisiae* DBV2168 fermentation in semi-synthetic media. a) 100:1 Mg:Ca ratio PYN b) 1:100 Mg:Ca ratio PYN media.

Table 3.10 Analytical characters of semi-synthetic wine (*Sacch. cerevisiae* L-2226) with a range of altered Mg:Ca ratios.

Compound (mg/ml)	A 1000:1	B 100:1	*C 13.6:1	D 10:1	*E 1.36:1	F 1:1	G 0:0	H 0.90:1	*I 1:7.27	J 1:10	K 1:100	L 1:1000	M Ion Free
Glycogen	13.51	2.974	ND	1.060	ND	0.871	0.568	1.936	1.605	1.182	2.355	8.620	0.453
Trehalose	0.863	1.111	1.190	0.624	1.130	0.610	0.629	0.582	1.470	0.602	0.528	0.798	0.578
Citric Acid	1.142	1.154	1.360	1.112	0.844	1.123	1.124	1.126	0.994	1.165	1.002	1.106	1.142
Tartaric Acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucose	0.128	0.510	0.040	ND	ND	0.045	0.105	ND	0.042	ND	0.035	0.322	11.641
Fructose	0.268	0.258	0.088	0.246	0.089	0.282	0.228	0.284	0.063	0.326	0.223	ND	5.042
Succinic Acid	1.144	1.262	1.227	1.176	1.073	0.806	0.922	0.962	1.116	0.992	0.636	0.948	0.310
Lactic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glycerol	4.546	4.610	3.568	4.430	3.296	4.255	5.006	4.154	2.716	4.822	3.762	5.577	1.358
Acetic Acid	0.660	0.792	0.401	1.482	0.50	3.394	1.254	2.654	1.559	3.929	2.279	1.926	0.244
Acetaldehyde	0.458	0.466	0.663	0.568	0.806	1.586	8.818	1.018	4.126	1.727	1.796	3.403	1.422
Ethanol	64.10	66.502	60.986	60.579	59.581	64.241	62.62	63.714	66.468	67.091	53.101	45.811	8.114

@ 144h except * @ 98h
ND = Not Detected

Table 3.11 Analytical characters of semi-synthetic wine (*Sacch. cerevisiae* DBV2168) with a range of altered Mg:Ca ratios.

Compound (mg/ml)	A 1000:1	B 100:1	D 10:1	F 1:1	G 0:0	H 0.90:1	J 1:10	K 1:100	L 1:1000	M Ion Free
Glycogen	6.190	2.066	0.607	0.588	0.446	1.480	0.757	2.220	12.290	0.578
Trehalose	0.618	0.556	0.618	0.748	0.583	0.629	0.580	0.689	0.717	0.759
Citric Acid	1.114	1.042	1.016	1.068	1.050	1.041	1.052	1.076	1.209	1.070
Tartaric Acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glucose	0.126	ND	ND	0.084	0.112	ND	ND	ND	0.232	15.994
Fructose	0.334	0.272	0.275	0.293	0.272	0.272	0.232	0.254	0.444	2.388
Succinic Acid	1.330	1.192	1.034	0.902	0.950	1.080	0.533	0.614	0.842	ND
Lactic acid	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Glycerol	4.058	3.868	3.592	2.766	3.157	3.780	2.820	3.743	4.898	0.912
Acetic Acid	0.616	0.459	0.404	0.470	0.384	0.558	1.210	2.031	2.568	0.178
Acetaldehyde	0.836	0.434	0.382	1.121	1.964	0.501	2.632	1.336	2.852	1.676
Ethanol	53.561	53.510	45.846	58.021	54.389	56.606	55.201	54.422	48.204	8.647

@ 144h
ND = Not Detected

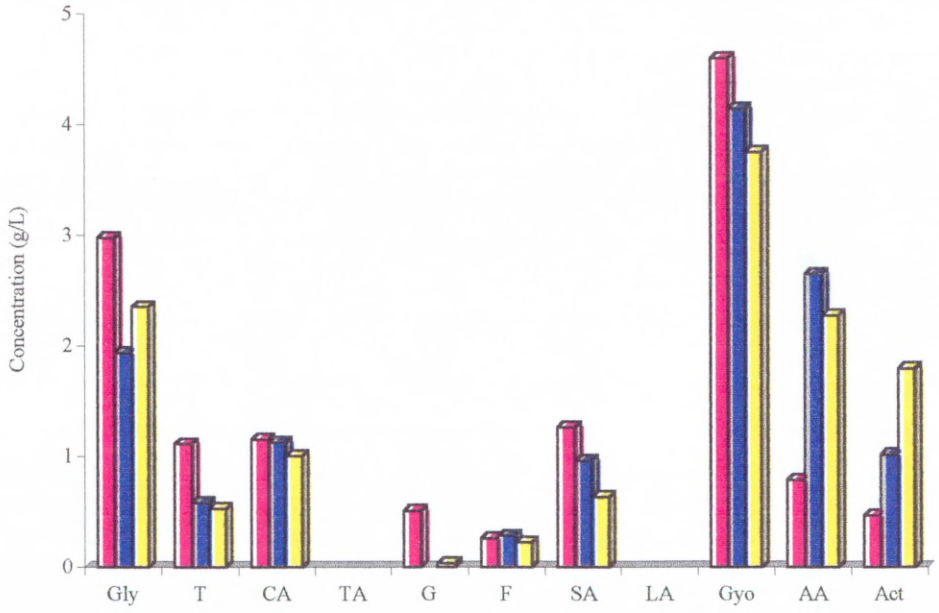


Figure 3.12 Comparison of effect of Mg:Ca ratio on final fermentation profiles of semi-synthetic wine (*Sacch. cerevisiae* L-2226); Gly-glycogen, T-trehalose, CA-citric acid, TA-tartaric acid, G-glucose, F-fructose, SA-succinic acid, LA-lactic acid, Gyo-glycerol, AA-acetic acid, Act-acetaldehyde. Media: ■ 100:1 Mg:Ca PYN, ■ 0.90:1 Mg:Ca PYN, ■ 1:100 Mg:Ca PYN.

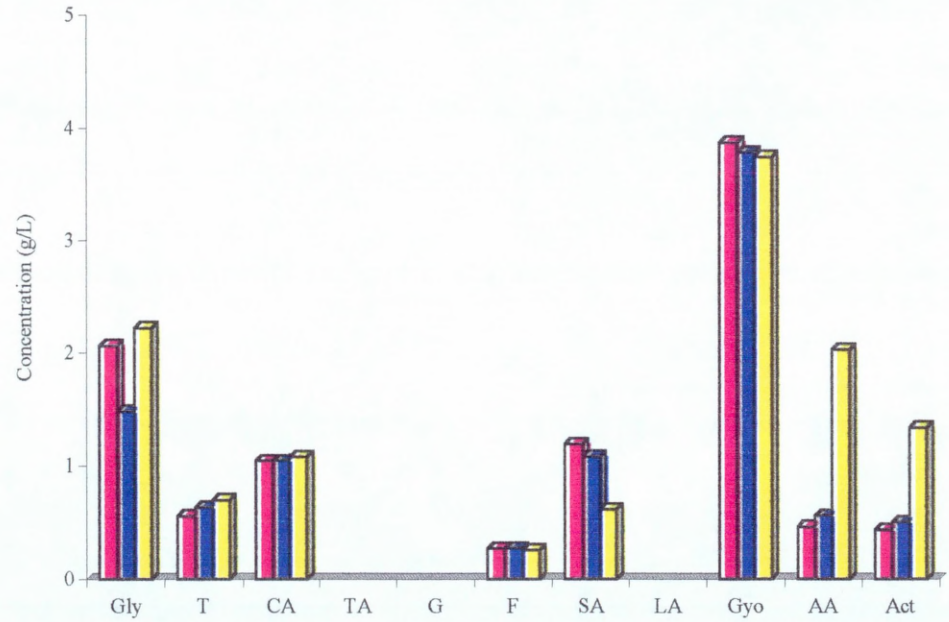


Figure 3.13 Comparison of effect of Mg:Ca ratio on final fermentation profiles of semi-synthetic wine (*Sacch. cerevisiae* DBV2168); Gly-glycogen, T-trehalose, CA-citric acid, TA-tartaric acid, G-glucose, F-fructose, SA-succinic acid, LA-lactic acid, Gyo-glycerol, AA-acetic acid, Act-acetaldehyde. Media: ■ 100:1 Mg:Ca PYN, ■ 0.90:1 Mg:Ca PYN, ■ 1:100 Mg:Ca PYN.

Maintenance of Mg:Ca levels around those simulating grape must or media with elevated magnesium levels will, therefore, not only produce high levels of ethanol, but will also produce a more palatable wine. The results illustrate the influence yeast behaviour during fermentation has on the resultant "wine" produced. Alteration of the inorganic nutrition to favour yeast growth and improve performance will also produce a more balanced range of flavour compounds due simply to the biochemistry of the yeast involved. Obviously the quality of grape must would play a large role in actual wine production, but this serves to illustrate the role of yeast physiology and basic ionic nutrition in influencing the taste of the final wine.

METAL ION PARAMETERS

Cellular uptake patterns of metal ions vary from strain to strain. Generally, extracellular magnesium levels remain fairly constant throughout the course of fermentation in semi-synthetic must, although a slight increase in levels may occur towards the end of fermentation corresponding to a decrease in intracellular levels at this juncture. This point is illustrated for a *Saccharomyces* strain in Figure 3.5 around 55h and to a lesser degree in *Torulaspora* at 72h (Fig. 3.6). Cells will either display one or two peaks in extracellular calcium levels during fermentation in semi-synthetic media. A peak in extracellular levels around 24-30h is illustrated with strains L-2226 (Fig 3.3), DBVPG2168 (Fig. 3.4) and *T. delbrueckii* (Fig. 3.6). Increases in levels around 55-72h may also occur with some strains (Figs. 3.3, 3.5 & 3.6) and this is usually followed by a sharp decline towards the end of fermentation unless cellular autolysis occurs then a rise in extracellular ionic levels obviously ensues (Figs 3.3 & 3.4).

Intracellular magnesium levels, typically, remain fairly constant during fermentation with no major influxes apart from a steady decrease if initial levels of this ion are high (Fig 3.3 & 3.6). Intracellular calcium levels on the other hand, tend to portray a mirror image of the events of extracellular calcium levels. *Sacch. cerevisiae* DCL'M' (Fig. 3.5) exhibits an

increase in intracellular levels of calcium during the early stages of stationary phase, followed by a sharp decline corresponding to the increase in extracellular levels observed, subsequently an uptake of calcium follows. This release and then uptake of calcium may be due to the levels of stress these cells are under at this stage of stationary phase, biochemical changes occurring in the cells result in an expulsion of intracellular calcium followed by a requirement at a later stage of cellular physiology. Other strains of *Sacch. cerevisiae* do not display this dramatic rise in intracellular calcium levels and both L-2226 and DBVPG2168, after a decrease in intracellular levels during logarithmic growth phase maintain a fairly constant cellular level with only slight increases during stationary phase (Figs. 3.3 & 3.4). Intracellular calcium levels in *T. delbrueckii* (Fig. 3.6) fluctuate quite dramatically with an initial decline in levels during logarithmic growth then a rise when cells enter stationary phase of growth, followed by a decrease (and corresponding increase in extracellular levels) around 72h. These trends are not surprising since calcium is known to act in an antagonistic manner towards magnesium, it follows that during logarithmic growth cells would actively extrude calcium in order to maximise magnesium uptake and utilisation for cellular activities, growth and cell division. The reason that there is no perceived increase in magnesium levels extracellularly during fermentation is that the ion is utilised as quickly as it is taken up and cells will only absorb (actively transport) magnesium to fulfil their requirement for the ion, irrespective of whether there are excess levels of magnesium in the fermentation medium.

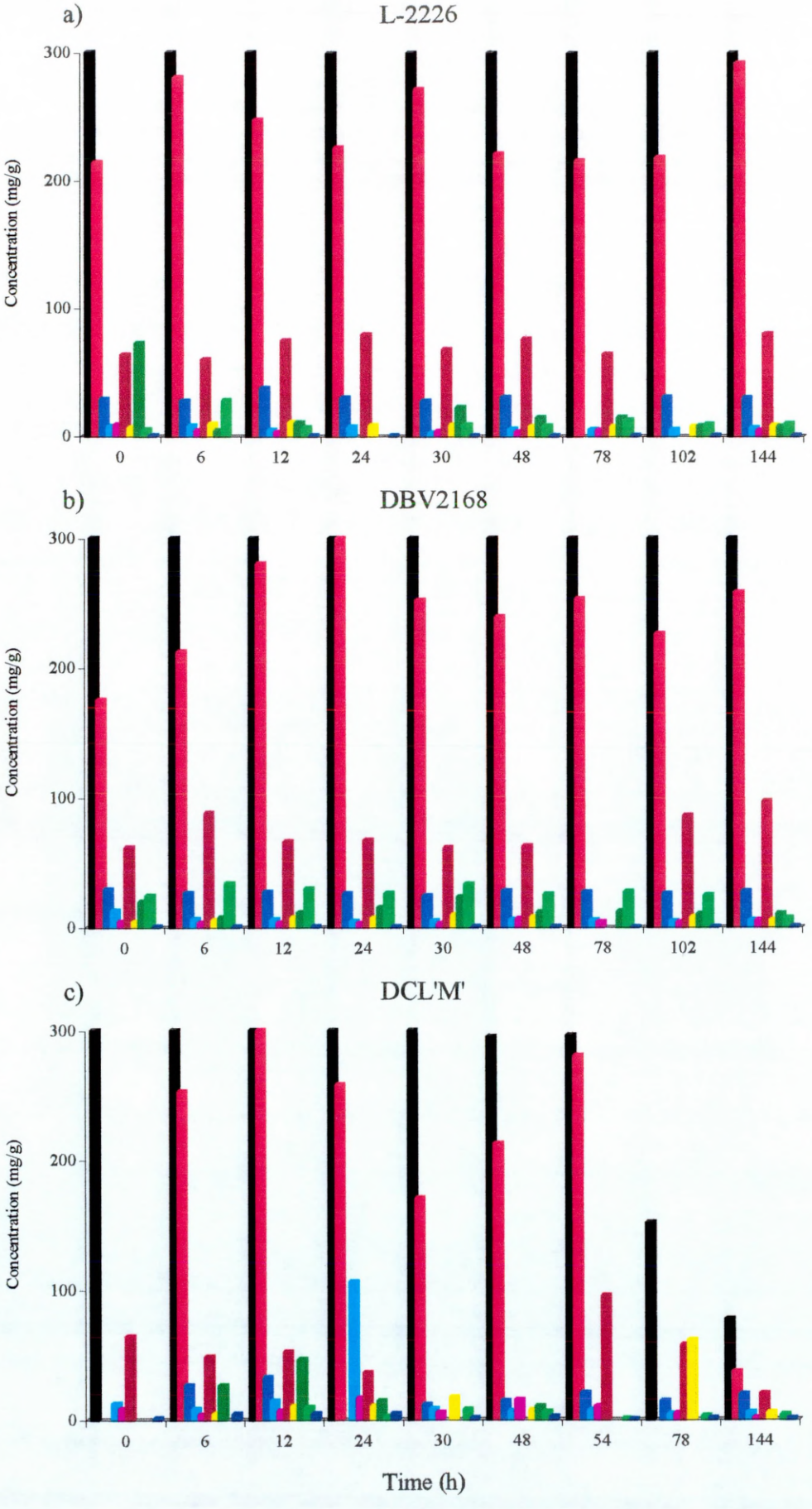
The influence of altering Mg:Ca ratios of semi-synthetic media on cellular ion homeostasis during fermentation was assessed. After 24h (Fig. 3.7b) extracellular levels of magnesium remained high in 1000:1 and 100:1 Mg:Ca ratio flasks and likewise for calcium levels in 1:100 and 1:1000 Mg:Ca ratio flasks. Intracellular levels of magnesium remained fairly low at less than 0.5mg/gDW, however the trend can be seen for all three strains that levels are higher in elevated ratios: 1000:1; 100:1; 10:1 and then remain very low in all other media. Intracellular calcium levels generally follow the same pattern in reverse, 1:1000; 1:100 and 1:10 flasks exhibit highest levels of intracellular calcium (1-1.5mg/g) with levels being very low under elevated magnesium conditions. The trend observed in ion free media varies with

strain. *Sacch. cerevisiae* L-2226 and DBVPG2168 exhibit undetectable levels of extracellular magnesium or calcium, but contain intracellular levels of magnesium between 0.1-0.5mg/g and calcium between 0.3-1mg/g, strain DCL'M' however, contains low levels of intracellular ions and very high levels of extracellular calcium (>500mg/L). Leakage of intracellular calcium ions appears to occur with this strain, thus elevating the extracellular concentrations. Figure 3.8b shows that for strains L-2226 and DBVPG2168, ion levels both intracellularly and extracellularly have not changed a great deal by 48h except for a slight increase in intracellular calcium levels of yeast fermenting in ion free media and in simulated wine. In addition, DBVPG2168 exhibits an elevation of intracellular magnesium levels in ion free media. A dramatic increase to 3.043mg/g intracellular calcium occurs with DCL'M' grown in simulated wine and again an increase in intracellular calcium occurs in cells grown in ion free media, with a corresponding drop in extracellular levels and extracellular levels of magnesium in the two highest magnesium ratios drop to a levels of around 300 and 200 mg/L respectively after 48h fermentation of this strain. The scenario at 144h for strains L-2226 and DBVPG2168 (Fig. 3.9) is very similar to that at 48h with a few exceptions, intracellular levels of magnesium and calcium drop by the end of fermentation in simulated wine for both strains, elevating extracellular levels slightly and being indicative of some cellular autolysis occurring at 144h. DCL'M' shows very reduced levels both intra- and extracellularly of both ions at 144h and little explanation can be offered for this observation.

In assessing the pattern of cellular ion homeostasis throughout fermentation in semi-synthetic media, it can be seen again that the pattern of extracellular magnesium for strains L-2226 and DBVPG2168 (Fig. 3.14a & b) are similar, with high levels being maintained in ratios 1000:1 and 100:1 Mg:Ca. Levels in all other media follow a typical pattern of elevation and reduction corresponding to particular stages of yeast growth and physiology. Extracellular magnesium levels in strain DCL'M' (Fig. 3.14c) follow a different pattern to the "wine strains" during fermentation. Levels in the two highest magnesium concentration ratios remain high with some fluctuations until 54h of fermentation, then a dramatic drop is witnessed through to the end of fermentation. Extracellular levels in simulated must start

off at a reasonable level then drop off around 30-48h (exponential growth) and then rise again towards the end of fermentation. An increase in levels towards the end of fermentation is also observed with 1:10 Mg:Ca cultures. The low levels of extracellular magnesium observed in media with elevated levels of calcium may be explained by the fact that in the presence of calcium yeast cells do not release magnesium. Calcium binds phospholipids and stops leakage of Mg^{2+}, K^{+} and PO_4^{-} (F. Mochaba: personal communication). When cells get ready to ferment sugar, there is a change in membrane fluidity and leakage of these ions causing a physiological response of salts. If calcium binds phospholipids and prevents this physiological response then obviously fermentation will be detrimentally affected, as has been observed with previous results.

On observation of intracellular magnesium levels (Fig. 3.14d-f) it can be seen for DCL'M' (Fig. 3.14f) that they remain fairly low <0.5mg/g throughout fermentation, but this may correspond to the reduced growth of this strain in the range of media. More interesting results can be seen with the two "wine strains" (Fig. 3.14d & e). L-2226 shows initial high levels of magnesium in most media, however, these levels gradually decrease by about 12-24h of fermentation as the magnesium is utilised by the cell in the processes of cell division (Walker 1986) and other physiological activities essential to growth and cell survival. Levels remain quite low through the latter stages of fermentation with any magnesium taken into the cell being used for enzyme activation and other activities essential for fermentation and ethanol production. DBVPG2168 (Fig. 3.14e) shows a similar pattern in the latter stages of fermentation with the exception of a peak at 48h in cultures in ion free media. Due to the slower growth rate of cultures in this medium resultant of the lack of essential ions (not only Mg^{2+} and Ca^{2+}) this peak may illustrate an attempt by the cell to uptake sufficient levels of any available ion for cellular activities and cell division. Initial levels of magnesium in this strain are slightly elevated (although not to the same degree as in L-2226) and again these levels decrease around the early stages of logarithmic growth of the cells. Levels were observed to be highest in cultures grown in 10:1, 1:100 and Ion free media, suggesting that magnesium is taken up initially to counteract ionic imbalances in the cells attempting to grow in these media. Cultures with elevated magnesium do not contain


a)
L-2226
b)
DBV2168
c)
DCL'M'
Concentration (mg/g)
300
200
100
0
0
6
12
24
30
48
54
78
102
144
Time (h)

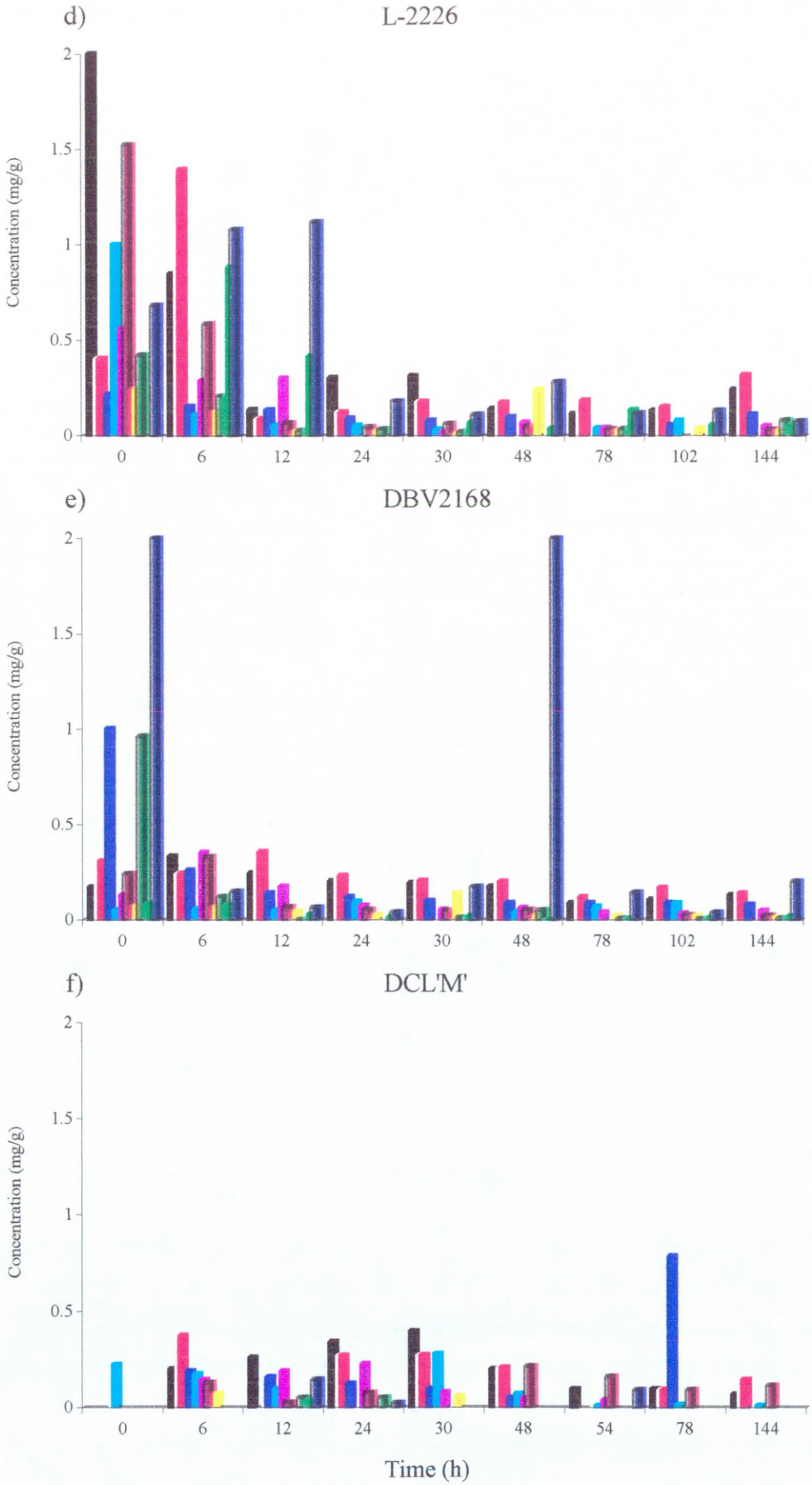


Figure 3.14 Effect of Mg:Ca ratio on magnesium parameters during the course of fermentation in semi-synthetic media; a)-c) extracellular magnesium, d)-f) intracellular magnesium. Media: A ■, B ■, D ■, F ■, G ■, H ■, J ■, K ■, L ■, M ■.

high intracellular levels of magnesium simply because yeast cells will only take up enough magnesium to utilise for specific functions irrespective of the supply of excess ions in the fermentation medium.

Although these results of intra- and extra-cellular ion levels for magnesium and calcium can give us some insight into the events of fermentation and the effect of altering the ionic nutrition of the yeasts themselves, the analysis by AAS reflects only the total cell contents (or total media contents in the case of extracellular analysis) of these metals and gives no information as to their distribution intracellularly or from cell to cell, nor about their existence as free ions or bound complexes. In an attempt to assess the actual availability of metal ions in semi-synthetic media with altered Mg:Ca ratios, extracellular free cation levels were followed through the course of fermentation. Analysis was carried out by ion chromatography however, as can be seen from a typical example of results in Table 3.12, some problems were encountered with this analysis due to damage to the HPLC column. It was accepted therefore that analysis by AAS, although being an assessment of total (available and unavailable) ionic levels, was a more accurate analysis of both extra- and intra-cellular ion levels for the purpose of this work. The assumption must be made that due to the presence of chelating compounds, more particularly in complex media, only a percentage of ionic supplements made will actually be available to the cells as free cations. The level of chelation of ions will vary dependent on the media itself and a range of variables affecting its composition but from the results it can be seen that supplementation of media with magnesium was beneficial to cell growth and physiology.

Table 3.12 Extracellular free cation levels during fermentation of *Sacch. cerevisiae* DCL'M' in semi-synthetic media with altered Mg:Ca ratios.

Time	1000:1		100:1		10:1		1:1		0:0		0.90:1		1:10		1:100		1:1000		Ion Free	
(h)	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca	Mg	Ca
Media	-	-	0.12	-	-	-	-	-	-	0.92	383	1821	0.19	3.21	147	66.8	0.11	-	0.12	-
0	-	3.76	-	-	-	1.06	-	9.14	-	-	0.08	1.70	0.13	-	89.2	-	0.15	-	0.12	-
6	0.04	-	-	1.24	-	-	-	-	-	-	0.69	-	17.6	5.82	0.09	-	43.9	14.0	0.08	1.42
12	-	-	-	-	-	-	-	-	-	-	-	-	0.24	-	0.10	-	-	-	0.11	152
24	-	-	-	-	-	1.84	-	0.44	-	-	139	399	0.12	-	0.13	-	0.14	-	0.1	20.8
30	-	-	-	1.10	-	-	-	0.80	-	-	0.08	-	0.13	1.06	0.14	-	0.09	-	0.15	-
48	0.05	-	-	0.85	-	-	-	-	-	-	0.14	-	0.08	-	0.10	-	0.16	-	0.11	34.5
55	0.04	7.60	-	-	-	6.98	-	2.37	-	315	0.11	-	0.08	-	0.13	-	0.06	29.1	0.09	-
78	-	9.22	-	0.99	0.50	19.2	0.04	-	0.10	5.34	0.10	3.02	178	93.6	0.18	0.99	0.17	0.83	0.13	-
144	-	3.01	-	2.42	-	-	-	-	0.06	-	0.12	-	-	-	0.11	2.34	0.11	-	0.08	1.15

units = mg/L

3.3.2 WINE MUST STUDIES

The nutritional complexity of grape must plays a large part in the resultant wine produced, in terms of growth of the yeast strain and therefore the sensory complexity of the wine. Ionic imbalances occur in most complex media. The scenario of supply and demand in media such as grape must and in fact, the applications of recommended concentrations of inorganic nutrients in an industrial context, is further complicated by the chelation of metal ions by organic components of the fermentation substrate, therefore limitation of inorganic nutrients may be more prevalent than is realised (Jones & Greenfield 1984). Most industrial media have the incorrect balance of magnesium and calcium such that the relative concentration ratios favour calcium rather than magnesium, which may not be conducive to fermentation (Walker 1994).

GROWTH AND FERMENTATION PARAMETERS

Typical fermentation progress of all three wine strains examined exhibit a fairly similar pattern of growth, ethanol production and sugar consumption in grape must (Figs 3.15-3.17), with *Sacch. cerevisiae* DBVPG2168 producing the highest quantities of ethanol; 81.4 g/L (Fig 3.16). *T. delbrueckii* DBVPG6168 resulted in the lowest biomass yield of the three strains grown in *Trebbiano toscano* must but production trends were comparable.

Alteration of Mg:Ca ratios in grape must by supplementation of the complex media with magnesium or calcium has a pronounced effect on growth and fermentation parameters of the various strains. Tables 3.13-3.15 show levels of biomass and ethanol production and sugar consumption at specific points throughout the course of the fermentation and the resultant fermentation progress at each stage. It can be seen from these tables that supplementation with magnesium is beneficial to the yeast cells in terms of improved fermentation parameters. Both yeast strains *Sacch. cerevisiae* DBVPG2168 and *T. delbrueckii* DBVPG6168 (Tables 3.14 & 3.15) exhibited increased fermentation progress

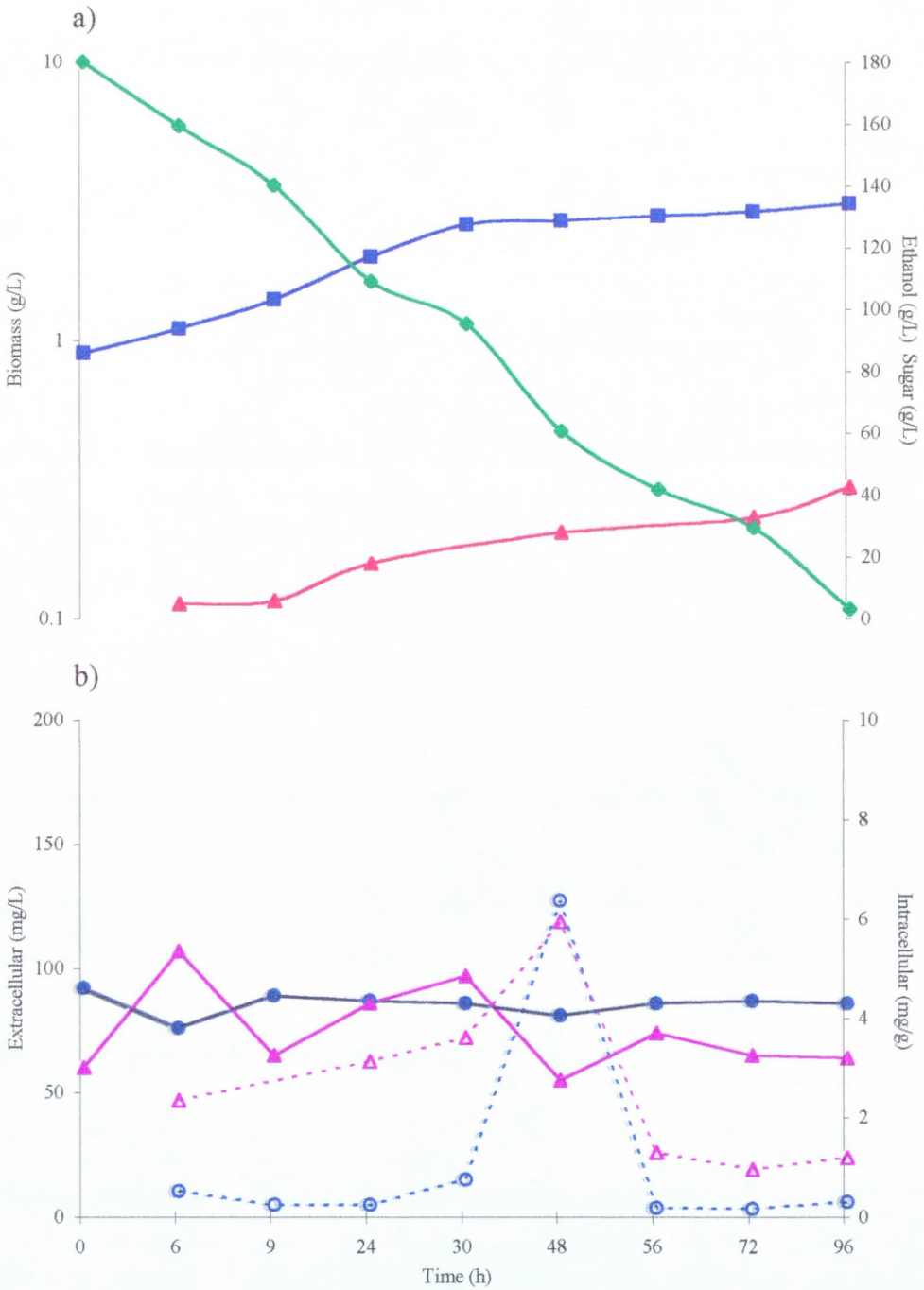


Figure 3.15 Typical fermentation progress of *Sacch. cerevisiae* L-2226 in white grape must; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -Δ- intracellular calcium.

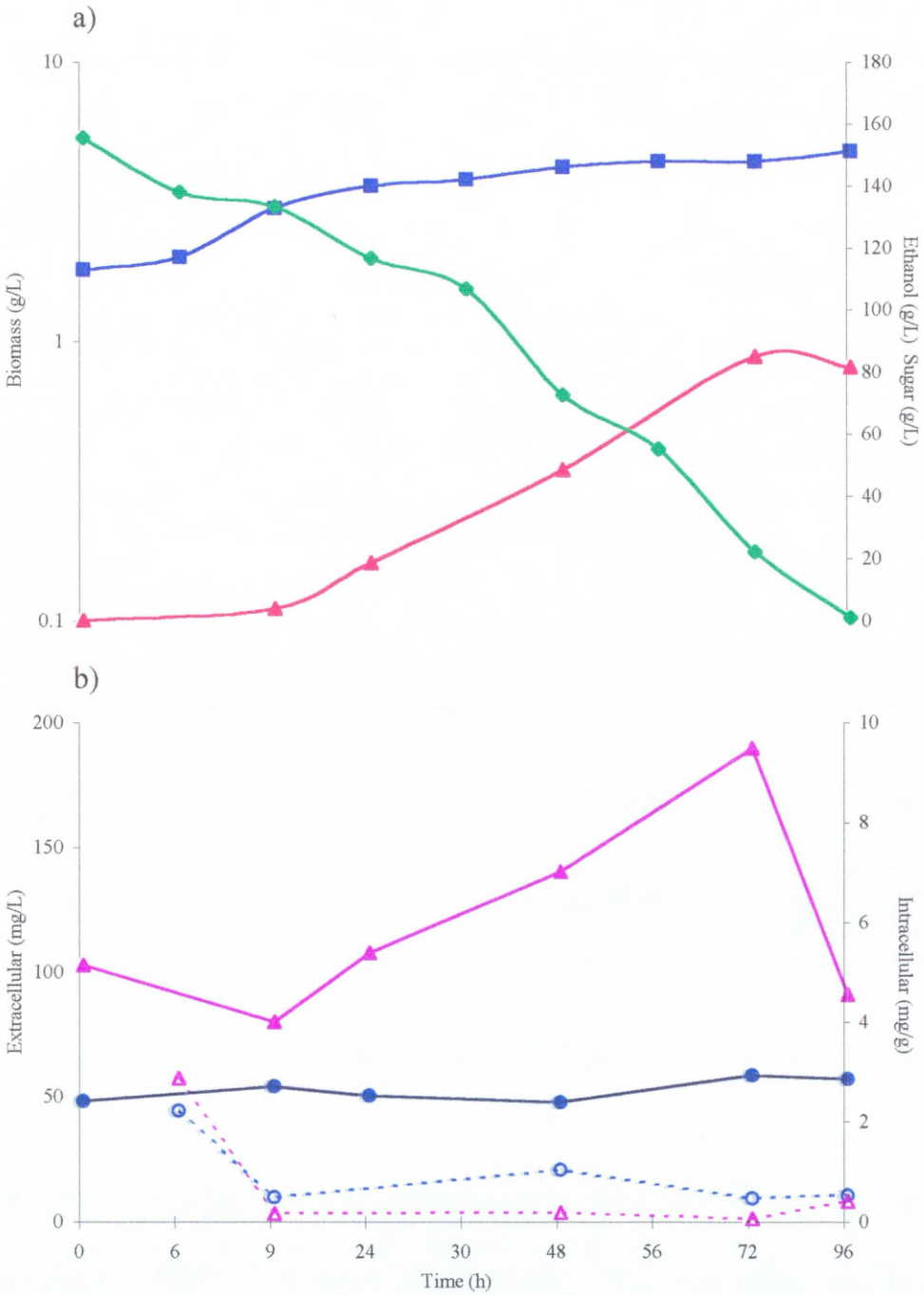


Figure 3.16 Typical fermentation progress of *Sacch. cerevisiae* DBV2168 in white grape must; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -Δ- intracellular calcium.

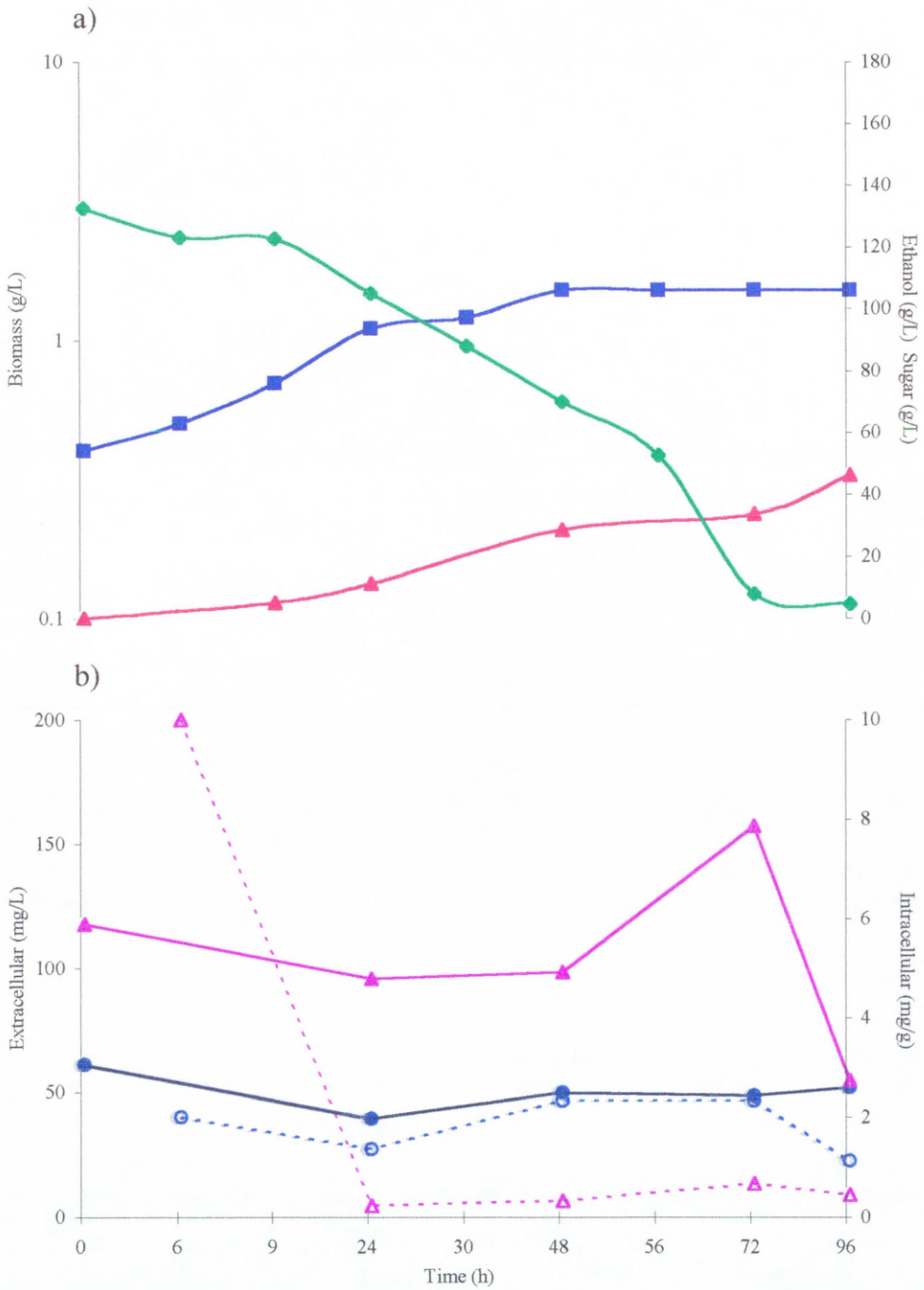


Figure 3.17 Typical fermentation progress of *Torulaspora delbrueckii* DBV6168 in white grape must; a) Fermentation parameters: ■ growth, ◆ sugar, ▲ ethanol, b) Metal ion parameters: ● extracellular magnesium, ▲ extracellular calcium, -○- intracellular magnesium, -Δ- intracellular calcium.

throughout the major stages of fermentation on magnesium supplementation. Biomass yield and productivity were increased on supplementation with magnesium and residual sugar levels at the end of fermentation (96h) were less for these two strains, a factor which is more pronounced with *Sacch. cerevisiae* DBVPG2168.

The pictorial display of fermentation parameters at 24h (Fig 3.18) shows little difference between control flasks (unsupplemented must) and those supplemented with magnesium (5:1 Mg:Ca) or calcium (0.05:1 Mg:Ca) for strain L-2226, sugar consumption, biomass and ethanol levels were all fairly constant. With strain DBVPG2168, Mg-supplemented media shows a slight increase in biomass levels and concurrent decrease in sugar levels. Biomass levels of *T. delbrueckii* throughout fermentation in all media were much reduced when compared to that of the two strains of *Sacch. cerevisiae*. After 48h (Fig. 3.19) the results were more significant in terms of biomass levels of DBVPG2168, again with Mg-supplemented must. Strain L-2226, however, seems to display an increase in biomass in Ca-supplemented media, yet ethanol levels in all *Saccharomyces* strains appear relatively equal, irrespective of the level of Mg:Ca ratio, *i.e.* high magnesium or high calcium. *T. delbrueckii* DBVPG6168, on the other hand, at 48h showed very little ethanol production and had high levels of sugar remaining. Figure 3.20 illustrates the situation of the three strains at the end of fermentation (96h) when, generally, the sugar source is exhausted. Contrary to the expected result with strain L-2226, biomass levels are highest in Ca-supplemented media (0.05:1 Mg:Ca). Ethanol levels are not remarkably different across the three media suggesting, for all strains examined, that chelation by organic materials in the must of ionic species reduced the effect of altering Mg:Ca ratios. A similar scenario is observed with *T. delbrueckii* DBVPG6168, where the Ca-supplemented media shows improved growth but reduced ethanol levels, when compared to Mg-supplemented must (4.10% v/v compared with 6.91% v/v: Table 3.15).

Overall for the three strains, *Saccharomyces* and non-*Saccharomyces*, although the actual ethanol yields of fermentation were quite similar, ethanol productivity was demonstrated to be highest in the control; unsupplemented must (Tables 3.13-3.15) thus suggesting that for

Table 3.13 Fermentation parameters of *Sacch. cerevisiae* L-2226 during fermentation in white grape must with altered Mg:Ca ratios.

	Must Mg:Ca[#]	Biomass (g/L)			Ethanol (%)			Sugar Utilised (g/L)			Fermentation Progress			Final Fermentation Parameters (@96h)				
	Time	B	M	E	B	M	E	B	M	E	B	M	E	Y(x) ($g.g^{-1}$)	P(x) ($g.l^{-1}.h^{-1}$)	Y(etoh) ($g.g^{-1}$)	P(etoh) ($g.l^{-1}.h^{-1}$)	Res. Sugar ($g.l^{-1}$)
M1	0.5:1	1.1	2.7	3.1	0.60	3.51	5.37	20.70	119.44	176.96	0.115	0.663	0.983	0.018	0.032	0.24	0.442	3.04
M2	5:1	0.1	2.6	4.3	0.39	3.72	4.73	28.40	101.09	145.29	0.178	0.632	0.908	0.029	0.045	0.26	0.389	14.71
M3	0.05:1	1.8	3.0	4.8	0.40	3.18	5.06	26.72	90.72	150.16	0.167	0.567	0.938	0.032	0.050	0.27	0.416	9.84

Note: B = Beginning 6h; M = Middle 48h; E = End 96h
[#] mg/L ratio

Table 3.14 Fermentation parameters of *Sacch. cerevisiae* DBV2168 during fermentation in white grape must with altered Mg:Ca ratios.

	Must Mg:Ca[#]	Biomass (g/L)			Ethanol (%)			Sugar Utilised (g/L)			Fermentation Progress			Final Fermentation Parameters (@96h)				
	Time	B	M	E	B	M	E	B	M	E	B	M	E	Y(x) ($g.g^{-1}$)	P(x) ($g.l^{-1}.h^{-1}$)	Y(etoh) ($g.g^{-1}$)	P(etoh) ($g.l^{-1}.h^{-1}$)	Res. Sugar ($g.l^{-1}$)
M1	0.5:1	2.0	4.2	4.8	0.49	6.13	10.3	22.03	87.56	159.09	0.138	0.547	0.994	0.030	0.050	0.512	0.849	0.91
M2	5:1	2.4	5.0	6.0	0.50	6.58	9.5	20.31	99.61	159.92	0.127	0.622	0.999	0.038	0.062	0.469	0.782	0.08
M3	0.05:1	2.6	4.6	5.2	0.62	6.86	9.0	12.33	88.87	158.20	0.077	0.555	0.989	0.033	0.054	0.449	0.741	1.80
FM1	0.5:1	0.5	2.1	2.2	0.21	2.87	5.15	19.8	64.71	99.86	0.124	0.404	0.624	0.022	0.023	0.408	0.424	60.14
FM2	5:1	0.3	2.2	2.6	0.53	4.99	7.28	20.48	84.27	149.40	0.128	0.527	0.934	0.017	0.027	0.385	0.599	10.60

Note: B = Beginning 6h; M = Middle 48h; E = End 96h
[#] mg/L ratio

Table 3.15 Fermentation parameters of *Torulaspora delbrueckii* DBV6168 during fermentation in white grape must with altered Mg:Ca ratios.

	Must Mg:Ca[#]	Biomass (g/L)			Ethanol (%)			Sugar Utilised (g/L)			Fermentation Progress			Final Fermentation Parameters (@96h)				
														Y(x)	P(x)	Y(etoh)	P(etoh)	Res. Sugar
	Time	B	M	E	B	M	E	B	M	E	B	M	E	($g.g^{-1}$)	($g.l^{-1}.h^{-1}$)	($g.g^{-1}$)	($g.l^{-1}.h^{-1}$)	($g.l^{-1}$)
M1	0.5:1	0.5	1.5	1.5	0.64	3.61	5.84	36.79	90.29	155.43	0.230	0.564	0.971	0.010	0.016	0.297	0.480	4.57
M2	5:1	0.2	1.2	1.2	0.52	3.46	5.06	53.92	101.04	155.60	0.337	0.632	0.972	0.008	0.012	0.257	0.416	4.40
M3	0.05:1	0.4	0.7	1.8	0.44	-	4.47	38.15	71.14	158.67	0.238	0.445	0.992	0.011	0.019	0.222	0.368	1.33
FM1	0.5:1	0.7	2.4	2.8	0.69	3.51	6.91	19.97	43.88	83.32	0.125	0.274	0.521	0.034	0.029	0.655	0.569	76.68
FM2	5:1	0.7	1.3	1.4	0.48	3.26	4.10	21.36	49.94	63.42	0.133	0.312	0.403	0.022	0.014	0.511	0.337	96.58

Note: B = Beginning 6h; M = Middle 48h; E = End 96h
[#] mg/L ratio

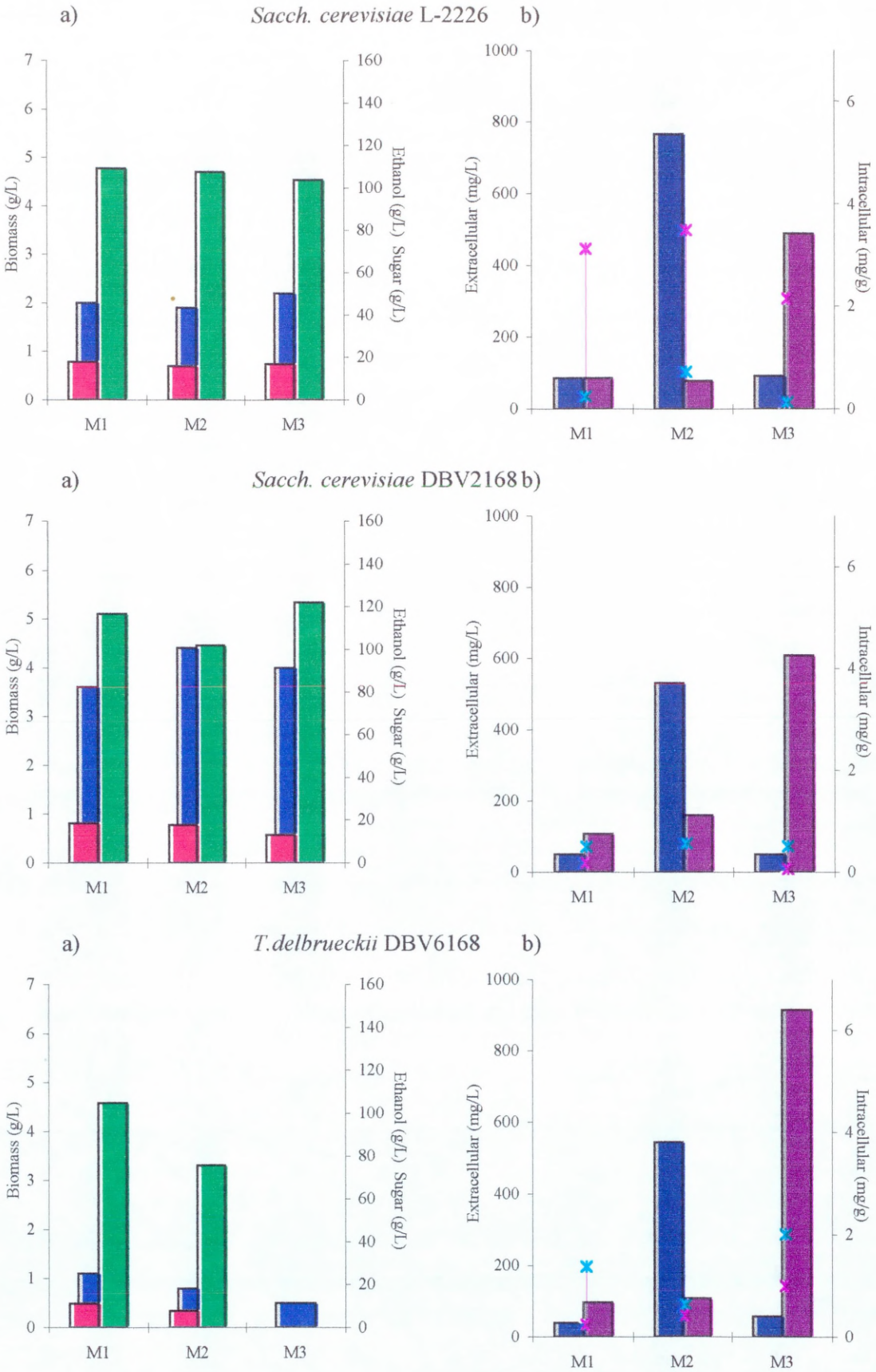


Figure 3.18 Fermentation and metal ion parameters after 24h in grape must with altered Mg:Ca ratios; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: White grape must with altered mg/L Mg:Ca ratios; M1 0.5:1, M2 5:1, M3 0.05:1.

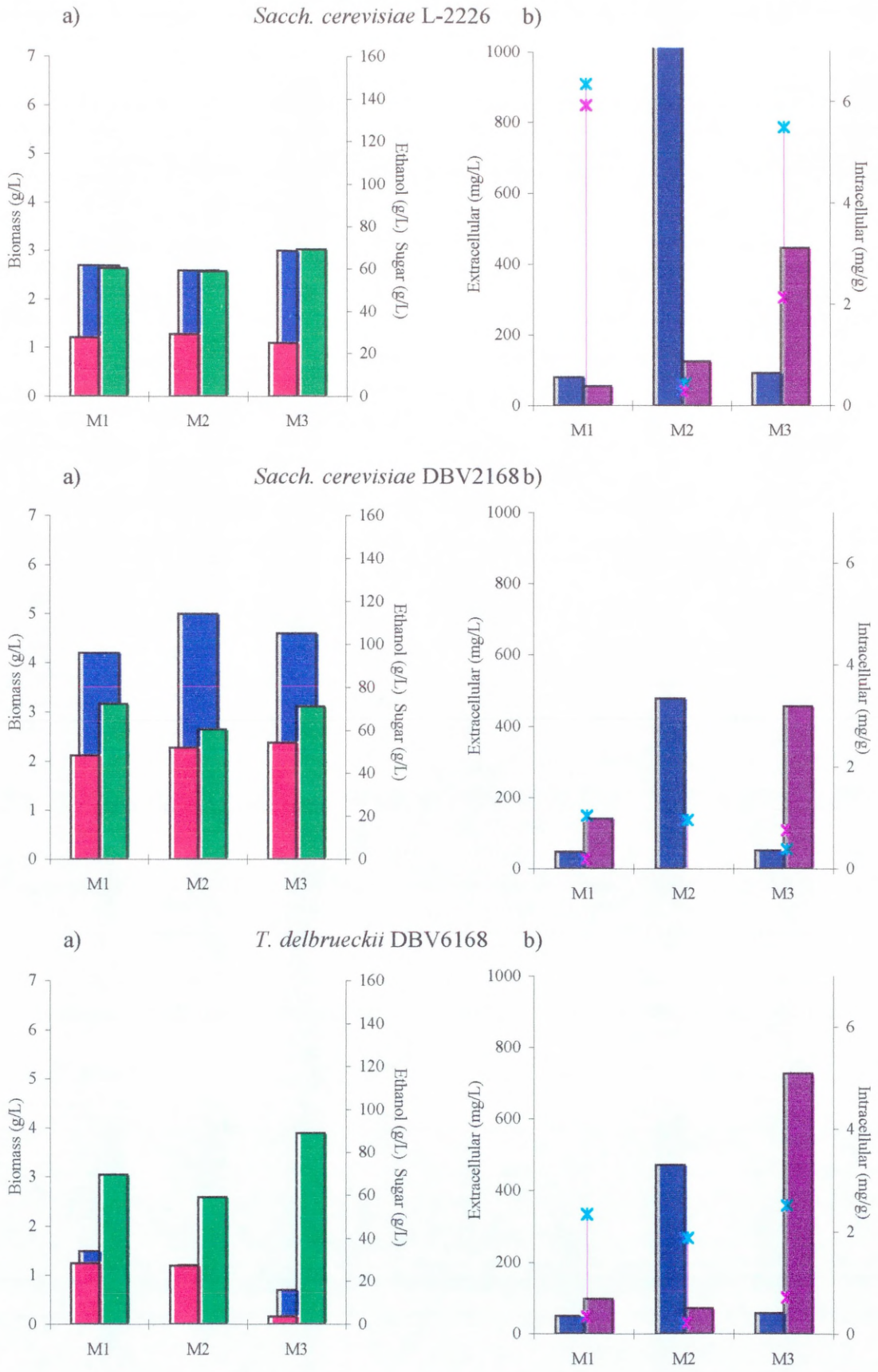


Figure 3.19 Fermentation and metal ion parameters after 48h in grape must with altered Mg:Ca ratios; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: White grape must with altered mg/L Mg:Ca ratios; M1 0.5:1, M2 5:1, M3 0.05:1.

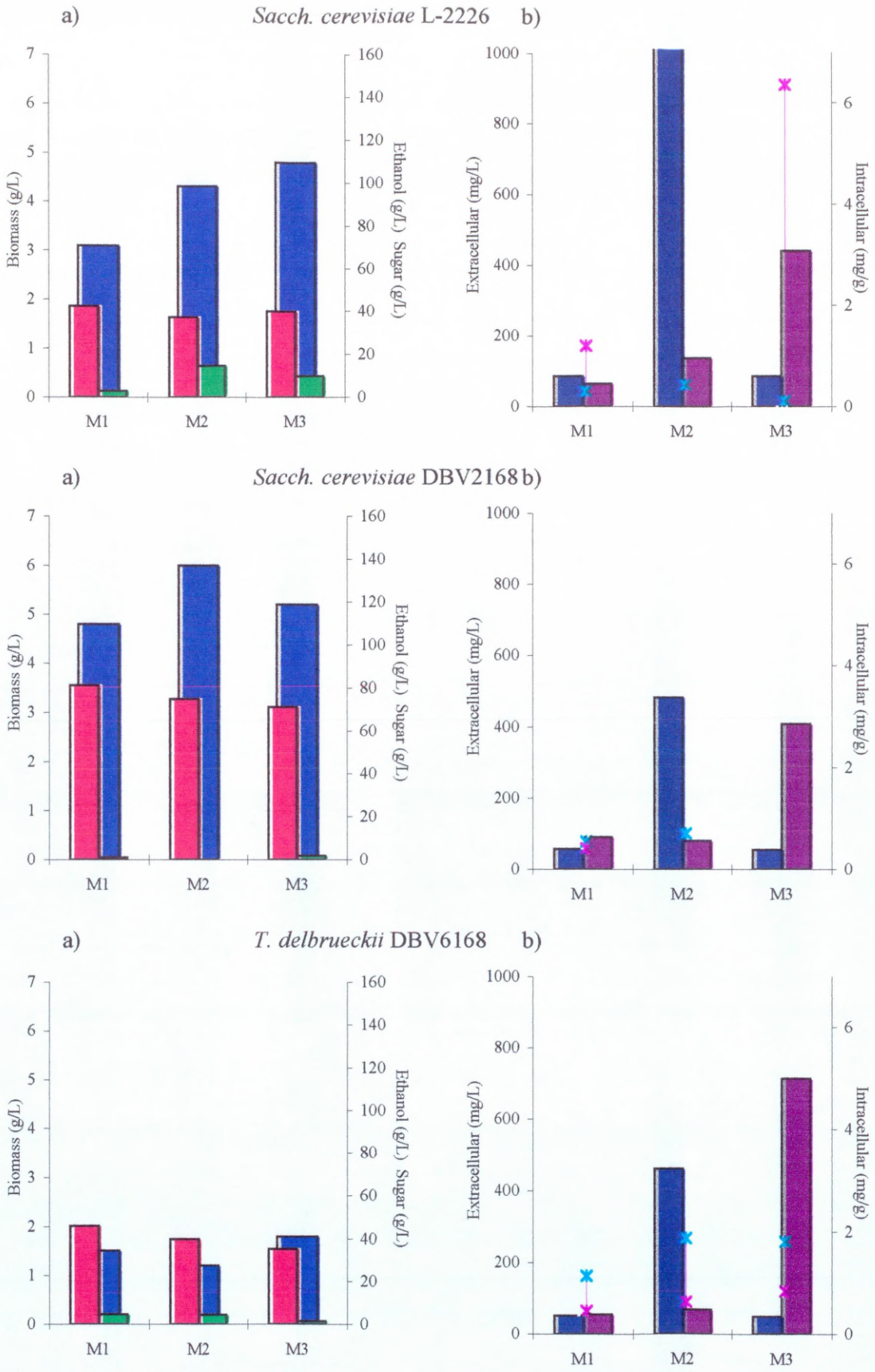


Figure 3.20 Fermentation and metal ion parameters after 96h in grape must with altered Mg:Ca ratios; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: White grape must with altered mg/L Mg:Ca ratios; M1 0.5:1, M2 5:1, M3 0.05:1.

increased levels of ethanol production, simple supplementation of media with $MgSO_4$ has little effect. The effect of altering the Mg:Ca ratios to favour magnesium is portrayed in terms of the increase in rates of production, improving fermentation times, increasing growth rates and enhancing the rate of ethanol production. This is demonstrated most clearly with the non-*Saccharomyces* wine strain *T. delbrueckii* DBVPG6168, showing a 39% decrease in doubling time on growth in media supplemented to a level of 5:1 Mg:Ca mg/L concentration ratio (Table 3.18). Supplementation of grape must with calcium resulted in a slight decrease in doubling time (8.2%) however this improvement was not found to be significant. Molar growth yields (Ym) for this yeast under Mg-supplemented conditions also illustrate the beneficial effects of magnesium. *Sacch. cerevisiae* strains L-2226 and DBVPG2168 (Tables 3.16 & 3.17) do not appear to exhibit the same response to an elevation of Mg:Ca concentration ratios in small scale batch fermentations (300ml shake flasks). In both cases control flasks (unsupplemented must) resulted in faster growth doubling times and higher ethanol yields, however molar growth yields display the fact that Mg-supplementation does inflict a positive effect on the growth of these yeast during fermentation in grape must. Growth and fermentation in media supplemented with calcium is inhibited in the case of strain DBVPG2168 (Table 3.17) with μmax dropping to 0.010 compared with control levels of 0.016, a 53% increase in doubling time of the yeast. Ethanol yields for this strain were also reduced on calcium supplementation and levels of residual sugar at the end of fermentation were found to be higher, indicating not only a slower fermentation but a reduced consumption of sugar by the yeast strain. L-2226 (Table 3.16) on the other hand seemed to improve its fermentation performance on supplementation with calcium; with a 25% improvement in yeast cell doubling time and a slightly improved molar growth yield over Mg-supplementation at $5.08gM^{-1}$, and a 60% improvement over the control (unsupplemented must). Levels of residual sugar however remained high (9.84g/L) and the rate of sugar consumption was reduced compared with both control and Mg-supplemented flasks plus levels of ethanol produced and ethanol yields were not improved, thus shedding some doubt on the validity of the perceived improvements observed with this strain.

Table 3.16 Influence of Mg:Ca ratio on growth and fermentation characteristics of *Sacch. cerevisiae* L-2226.

					Yeast Growth Characteristics (@96h)			Yeast Fermentation Characteristics (@96h)			
	Media (molar)	Mg (mg/L)	Ca (mg/L)	Mg:Ca (mg/L)	Doubling Time (Td) (h)	μ (h^{-1})	Molar Growth Yield (Ym) ($g.M^{-1}$)	Ethanol Produced (g/L)	Y(etoh) ($g.g^{-1}$)	$Q_{(L)}$ ($g.g^{-1}DW.h^{-1}$)	Residual Sugar (g/L)
M1	0.76:1	72	156	0.46:1	46.21	0.015	3.16	42.42	0.24	0.595	3.04
M2	8.46:1	800	156	5.13:1	69.31	0.010	4.69	37.37	0.26	0.352	14.71
M3	0.14:1	72	880	0.08:1	34.66	0.020	5.08	39.97	0.27	0.326	9.84

Table 3.17 Influence of Mg:Ca ratio on growth and fermentation characteristics of *Sacch. cerevisiae* DBV2168.

					Yeast Growth Characteristics (@96h)			Yeast Fermentation Characteristics (@96h)			
	Media (molar)	Mg (mg/L)	Ca (mg/L)	Mg:Ca (mg/L)	Doubling Time (Td) (h)	μ (h^{-1})	Molar Growth Yield (Ym) ($g.M^{-1}$)	Ethanol Produced (g/L)	Y(etoh) ($g.g^{-1}$)	Q(L) ($g.g^{-1}DW.h^{-1}$)	Residual Sugar (g/L)
M1	0.78:1	48.4	102.9	0.47:1	43.32	0.016	4.67	81.53	0.512	0.345	0.91
M2	6.37:1	467	120.9	3.86:1	52.51	0.013	5.48	75.05	0.469	0.278	0.08
M3	0.10:1	50.6	810.7	0.06:1	66.49	0.010	3.90	71.10	0.449	0.317	1.80
FM1	0.81:1	66.9	135.6	0.49:1	25.67	0.027	4.71	40.71	0.408	0.473	60.14
FM2	17.6:1	1217.4	114.2	10.7:1	21.18	0.033	4.03	57.51	0.385	0.598	10.60

Table 3.18 Influence of Mg:Ca ratio on growth and fermentation characteristics of *Torulaspora delbrueckii* DBV6168.

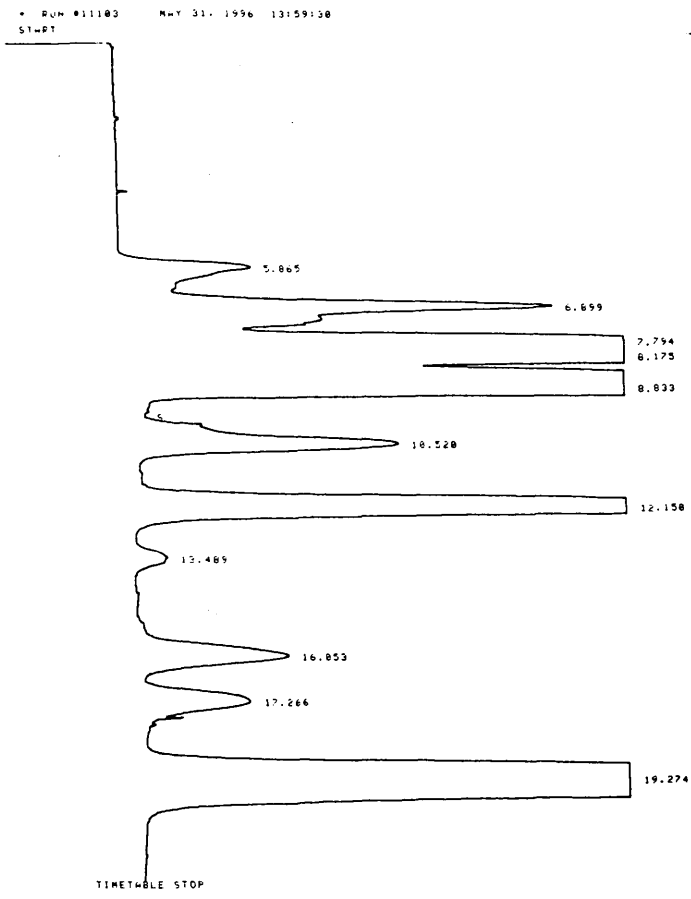
					Yeast Growth Characteristics (@96h)			Yeast Fermentation Characteristics (@96h)			
	Media (molar)	Mg (mg/L)	Ca (mg/L)	Mg:Ca (mg/L)	Doubling Time (Td) (h)	μ (h^{-1})	Molar Growth Yield (Ym) ($g.M^{-1}$)	Ethanol Produced (g/L)	Y(etoh) ($g.g^{-1}$)	Q(L) ($g.g^{-1}DW.h^{-1}$)	Residual Sugar (g/L)
M1	0.86:1	61.1	117.8	0.52:1	30.28	0.023	2.48	46.14	0.297	1.079	4.57
M2	8.82:1	528.7	98.9	5.34:1	18.57	0.037	2.98	39.97	0.257	1.35	4.40
M3	0.11:1	82.8	1242.2	0.07:1	32.78	0.021	1.92	35.31	0.222	0.918	1.33
FM1	1.10:1	60.2	89.8	0.67:1	47.87	0.014	6.82	54.59	0.655	0.310	76.68
FM2	5.92:1	535.0	149.0	3.59:1	63.95	0.011	3.36	32.39	0.511	0.472	96.58

Organic Profiles of Wine

In producing a fine wine the vintner is not solely looking for maximisation of the levels of ethanol produced, although this is an important consideration. The mixture of compounds present in the wine at the end of fermentation is also very important in terms of flavour compounds, organic acids and residual sugars, all of which affect the palate of the wine. Grape must and wine contain a variety of organic acids, concentrations varying greatly dependent on the condition and maturity of the grapes, as well as the biochemistry of fermentation (Radler 1992). Organic acids contribute significantly to the taste of the wine and succinate is the main carboxylic acid produced by yeast during wine fermentation (Thoukis *et al.* 1965). Acetate is the main product of oxidation of ethanol by acetic acid bacteria and wine is legally regarded as spoiled when its acetate content is higher than about 20meq/L. Acetate is also a normal by-product of alcoholic fermentation by yeasts, its formation by strains of *Sacch. cerevisiae* being affected by sugar concentration and pH (Shimazu & Watanabe 1981; Monk & Cowley 1984). Levels of acetaldehyde and acetic acid excreted during fermentation of grape juice differs dependent on strain (Millan & Ortega 1988).

Final fermentation profiles in grape must with altered Mg:Ca ratios were assessed for the wine strain *Sacch. cerevisiae* L-2226. Figure 3.21 shows a typical HPLC profile of the resultant wine, showing high levels of fructose, tartaric acid, succinic acid, glycerol and ethanol. In order to observe if the alteration of Mg:Ca ratio in grape must had a significant effect on the final organic profile of the wine, analysis was carried out on must of the ionic concentration ratios; 0.5:1, 5:1 and 0.05:1 Mg:Ca. Table 3.19 records the level of a selection of important organic compounds of grape must and wine and Figure 3.22 illustrates that on supplementation of grape must with magnesium, levels of glycogen, citric acid, fructose and glycerol are increased, whereas levels of trehalose, tartaric acid, acetic acid and acetaldehyde are reduced in the resultant wine, with the largest increase being in that of the storage carbohydrate glycogen. On elevation of calcium levels, citric acid, glucose, fructose, glycerol, acetic acid and acetaldehyde levels all increase and glycogen,

a)



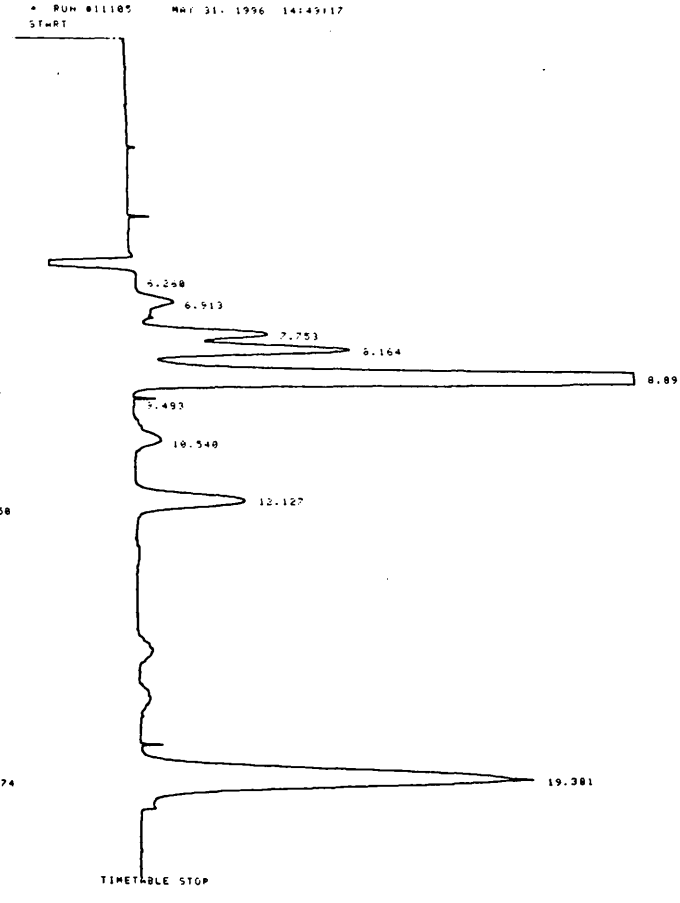
RUN# 11103 MAY 31, 1996 13:59:30

SUGAR/ETHANOL ANALYSIS

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/	NAME
5.865	PV	999987	.543	30686	1R	.167	GLYCOGEN
6.899	VV	3046461	.502	101106	2R	.898	TREHALOSE
7.794	VV	4539984	.274	276564	4R	2.512	TARTARIC ACID
8.175	VH	10415648	.325	534682	5R	4.565	GLUCOSE
8.833	SH6	51051808	.438	1948909	6R	17.928	FRUCTOSE
10.520	BP	1758071	.492	59571	7R	1.930	SUCCINIC ACID
12.150	PP	6241235	.455	228594	9R	3.859	GLYCEROL
13.489	PF	285815	.447	7667	10R	.176	ACETIC ACID
16.053	PV	1241472	.590	35072	11R	2.747	ACETALDEHYDE
19.274	VB	26341120	.596	792139	12F	41.123	ETHANOL

b)



RUN# 11105 MAY 31, 1996 14:49:17

SUGAR/ETHANOL ANALYSIS

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/	NAME
6.913	VV	1327708	.585	37858	2R	.312	TREHALOSE
7.753	VV	1248141	.375	55418	4R	.475	TARTARIC ACID
8.164	VV	1594819	.362	72862	5R	.619	GLUCOSE
8.891	VV	6441776	.362	296398	6R	2.779	FRUCTOSE
10.540	VV	1111104	1.017	18214	7R	.576	SUCCINIC ACID
12.127	VB	9e0298	.532	30186	9F	.393	GLYCEROL
19.381	FB	2866470	.521	91755	13F	4.734	ETHANOL

Figure 3.21 Typical final (96h) fermentation products profile of *Sacch. cerevisiae* L-2226 fermentation of white grape must. a) neat b) 1:10 dilution.

Table 3.19 Analytical characters of wine (*Sacch. cerevisiae* L-2226) with elevated magnesium and calcium levels.

Compound (mg/ml)	M1 Mg:Ca 0.5:1	M2 Mg:Ca 5:1	M3 Mg:Ca 0.05:1
Glycogen	0.167	4.059	-
Trehalose	2.267	0.889	1.019
Citric Acid	-	0.392	0.479
Tartaric Acid	3.781	2.951	2.756
Glucose	5.322	6.196	6.832
Fructose	24.23	24.98	25.31
Succinic Acid	1.960	1.954	1.924
Lactic acid	-	-	-
Glycerol	3.396	3.978	4.208
Acetic Acid	0.176	-	0.722
Acetaldehyde	3.858	2.803	7.632
Ethanol	42.33	46.48	47.37

@ 98h

trehalose and tartaric acid levels are observed to decrease. Levels of succinic acid remain constant irrespective of ionic changes to the medium. These alterations in the final fermentation profiles of the wine suggest that increasing the calcium content of grape must by 10-fold, results in a wine with albeit higher ethanol levels but a correspondingly more acidic content making it much less palatable. Supplementation of grape must with magnesium, however, results in increased ethanol levels and also despite fluctuations in levels of certain organic components of the final wine, a balance is maintained and levels of acetic acid and acetaldehyde are reduced, indicating that a more pleasant tasting wine is produced.

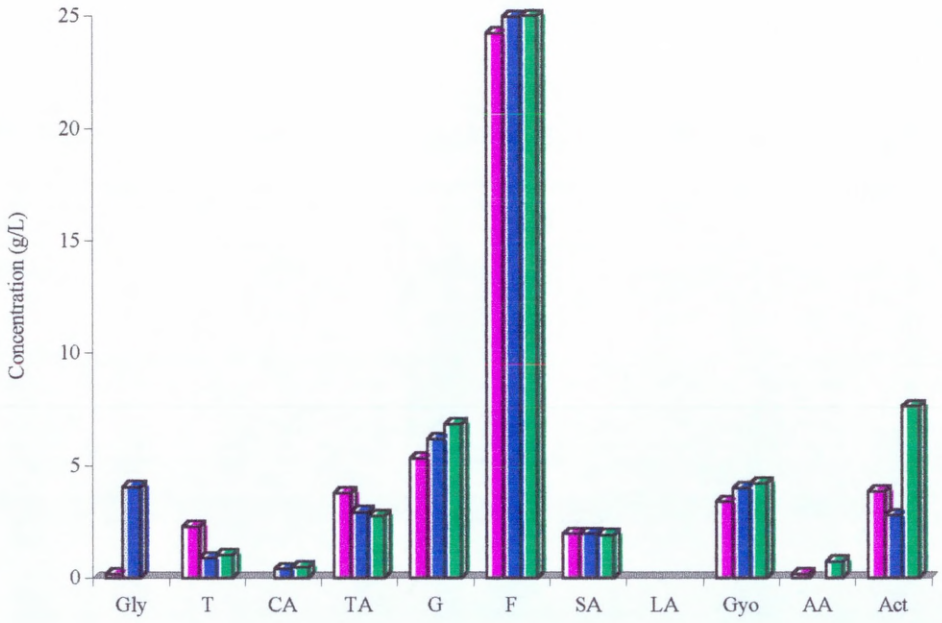


Figure 3.22 Comparison of effect of Mg:Ca ratio on final fermentation profiles of wine (*Sacch. cerevisiae* L-2226); Gly-glycogen, T-trehalose, CA-citric acid, TA-tartaric acid, G-glucose, F-fructose, SA-succinic acid, LA-lactic acid, Gyo-glycerol, AA-acetic acid, Act-acetaldehyde. Media: ■ 0.5:1 Mg:Ca Must, ■ 5:1 Mg:Ca Must, ■ 0.05:1 Mg:Ca Must.

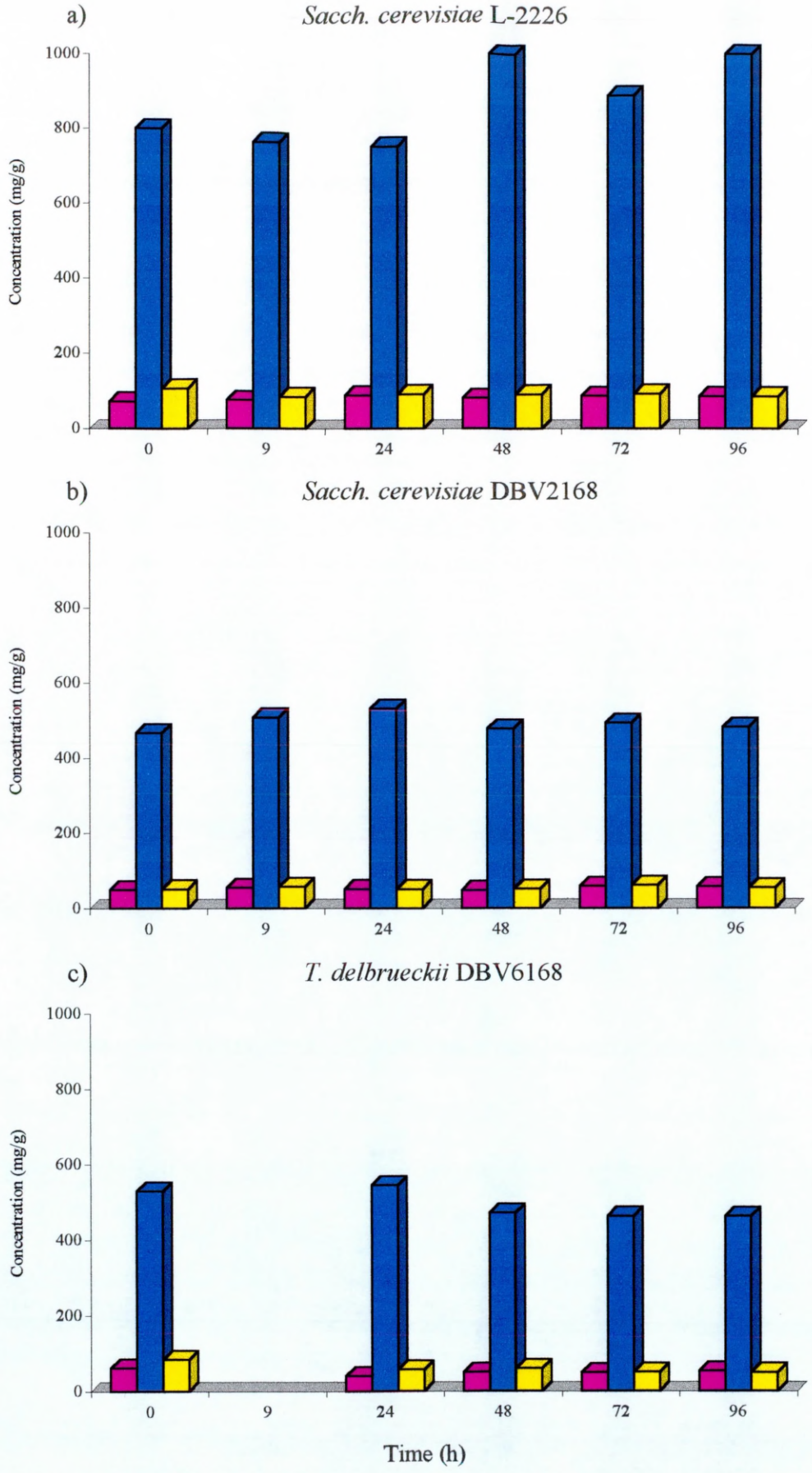
METAL ION PARAMETERS

Levels of essential ionic species fluctuate both extra- and intra-cellularly during fermentation and magnesium and calcium are no exception to this rule. Generally following a typical fermentation progress in grape must (Figs. 3.15-3.17) extracellular levels of magnesium remain relatively constant throughout the period of fermentation irrespective of yeast strain. Extracellular calcium levels fluctuate during fermentation with a characteristic drop in levels corresponding to the initiation of exponential growth of the yeast cells. After this, dependent on yeast strain, levels either fluctuate along a mean level (Fig. 3.15 *Sacch. cerevisiae* L-2226) or rise towards the end of fermentation peaking correspondingly with ethanol production and then dropping off sharply (Figs. 3.16 & 3.17, *Sacch. cerevisiae* DBVPG2168 & *T. delbrueckii* DBVPG6168). Patterns of intracellular levels of magnesium and calcium appear very much strain dependent, with L-2226 exhibiting fairly constant levels of both ionic species except for a peak in levels corresponding with the entry of cells into stationary phase of growth (Fig. 3.15) and intracellular calcium levels being higher than magnesium. The other two wine strains, demonstrated fairly constant intracellular levels of these two ions (Figs. 3.16 & 3.17) with magnesium levels being marginally higher than calcium, as is expected since the optimum cellular concentrations of these ions are; 4-17mmole/100gDW magnesium and only 0-1.5mmole/100gDW calcium. In both strains, a slight increase is noted at a point corresponding to the entry of cells into stationary phase, but this is much less pronounced than in L-2226.

The influence of altering Mg:Ca ratios on cellular ion homeostasis during fermentation was assessed. After 24h (Fig. 3.18) intracellular calcium levels were higher in strain L-2226 (2-4mg/g) compared to levels of Ca in DBVPG2168 and *T. delbrueckii* DBVPG6168 (<2mg/g), intracellular levels of magnesium and calcium were higher in Mg-supplemented media in both *Saccharomyces* strains, but the reverse was true for *T. delbrueckii* DBVPG6168. Surprisingly, intracellular calcium levels were not increased in Ca-supplemented media. After 48h (Fig 3.19) extracellular levels had not altered greatly except for an increase in extracellular magnesium levels (>1000mg/L) and a corresponding

decrease in intracellular magnesium levels in Mg-supplemented L-2226 cultures. Overall, intracellular levels in all three strains were higher than those at 24h, illustrating an uptake of these essential metal ions during exponential growth of the yeast. Cellular magnesium levels were not higher in Mg-supplemented media indicating that despite supplying an excess of an ion cells will only take up a specific amount corresponding to the needs of that cell. At the end of fermentation (96h: Fig. 3.20) extracellular ion levels were similar, if not identical, to those at 48h. Intracellular levels of magnesium were much reduced, however, an increase in intracellular levels was observed in supplemented cultures. A rise in intracellular calcium levels was also noted, in particular in L-2226 with Ca-supplemented cultures exhibiting the highest levels.

In assessing the pattern of cellular ion homeostasis throughout fermentation it can be seen that extracellular magnesium in unsupplemented must and must supplemented with calcium remains fairly constant during the course of fermentation in all three strains (Fig. 3.23a-c). In Mg-supplemented media an increase in extracellular levels is observed around 48h, corresponding with the end of exponential phase of growth. Intracellular levels (Fig. 3.23d-f) vary dependent on strain and essentially growth and fermentation behaviour. For *Sacch. cerevisiae* L-2226 (Fig. 3.23d) magnesium levels in unsupplemented and Ca-supplemented must peak at 48h, indication that between 24h and 48h a rapid uptake of magnesium occurs and during stationary phase this is excreted. Intracellular levels in yeast propagated in Mg-supplemented must start off containing high intracellular levels at the initiation of exponential phase which declines through the course of fermentation. *Sacch. cerevisiae* DBVPG2168 (Fig. 3.23e) maintains relatively low intracellular concentrations with a slight peaking in levels at 9h for Mg-supplemented cultures and 48h for unsupplemented and Ca-supplemented cultures. Intracellular levels in *T. delbrueckii* DBVPG6168 (Fig. 3.23f) rise to a peak at 72h (due to the slower growth rates of this yeast) for both unsupplemented and Mg-supplemented media, before levels fall off by 96h. In Ca-supplemented media the peak occurs at 48h followed by a sharp drop in intracellular magnesium concentration and a subsequent rise at the end of fermentation.


a)
Sacch. cerevisiae L-2226
b)
Sacch. cerevisiae DBV2168
c)
T. delbrueckii DBV6168
Concentration (mg/g)
1000
800
600
400
200
0
0
9
24
48
72
96
Time (h)

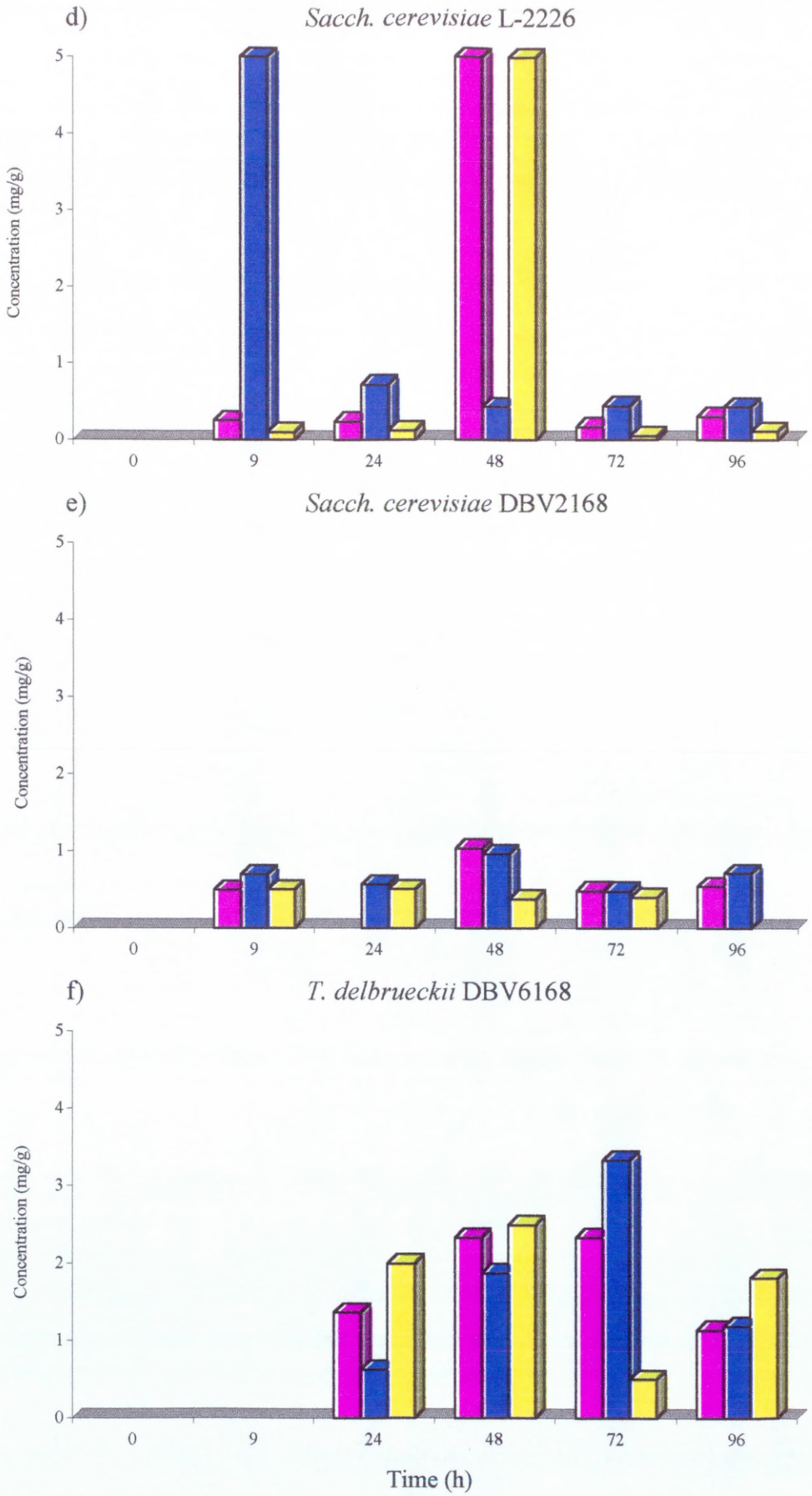


Figure 3.23 Effect of Mg:Ca ratio on magnesium parameters during the course of fermentation in white grape must; a)-c) extracellular magnesium, d)-f) intracellular magnesium. Media: M1 ■, M2 ■, M3 ■.

Categorisation of uptake patterns for different yeast strains can be assessed. From the observed results it is suggested that the magnesium supplied is utilised by cells during logarithmic growth and then a release of bound cellular magnesium occurs during stationary phase. Since magnesium is known to be required for growth and cell division (Walker 1986), uptake and utilisation of the magnesium required for cell growth and division occurs initially, however the rest of the supplemented magnesium is complexed by the must to varying degrees and is therefore unavailable to the cells. Calcium levels extracellularly at elevated magnesium concentration remain relatively unchanged with levels in the medium remaining constant with respect to initial levels, indicating that calcium has little effect on cell growth and under conditions of increased magnesium is not taken up by the cells to any great effect, since yeast cells have a higher affinity for magnesium than calcium (Saltukoglu & Slaughter 1983). In unsupplemented trials calcium levels also show little deviation from original levels, giving further support to the claim that calcium is not an essential element for yeast growth and in fact acts in an antagonistic manner towards magnesium.

COMPARISON OF FERMENTATION CHARACTERISTICS BETWEEN SHAKE FLASKS (300ML) AND BIOREACTORS (2L).

Small scale batch fermentations supply an important array of results to the microbiologist in terms of yeast behaviour under certain conditions, etc. Scale-up towards industrial size fermentations is required to see if observations made in these small scale experiments carry through to an industrial situation. Two of the strains analysed were selected for comparative studies, *Sacch. cerevisiae* DBVPG2168 and *T. delbrueckii* DBVPG6168 and figure 3.24 shows the fermentation and metal ion parameters after 24h. Mg:Ca concentration ratios were altered in both cases by the supplementation of grape must with a 10-fold addition to magnesium levels (5:1 Mg:Ca concentration ratio compared with 0.5:1 Mg:Ca). From the graphs it can be seen that after 24h (Fig 3.24) very little difference between unsupplemented and Mg-supplemented media can be seen, the only main difference being that for *Sacch. cerevisiae* DBVPG2168 biomass levels are higher in the shake flasks;

3.5-4.5g/L compared with 1-2g/L (bioreactors), whereas for *T. delbrueckii* DBVPG6168 biomass levels are higher in the bioreactor (1-2g/L compared with <1g/L). After 48h (Fig 3.25) the effect of altered metal ion ratio is more prominent in both vessel types and the effect of elevation of magnesium levels on *Sacch. cerevisiae* DBVPG2168 is beneficial in terms of improved fermentation parameters. This trend is carried through until the end of fermentation at 96h (Fig. 3.26) with doubling times decreasing by 17% in the bioreactor studies, however ethanol yields were slightly reduced in comparison to the control media (Table 3.17). For *T. delbrueckii* DBVPG6168 at 48h (Fig. 3.25) biomass levels seem somewhat retarded on the elevation of magnesium and the levels of ethanol produced are constant irrespective of media type or vessel size. At 96h (Fig. 3.26) the trend is similar except that ethanol levels are much higher in controls than in Mg-supplemented media (approx. 40% higher in bioreactor studies). The growth rate (μ) was also reduced and final biomass levels were observed to be quite low (Table 3.18). In both strains, the most outstanding feature is that the residual sugar levels in bioreactor studies are much higher at 96h, thus indicating that an increase in fermentation time ensues on scale-up an effect which has a greater importance for *T. delbrueckii* DBVPG6168 since the *Sacch. cerevisiae* wine strains are better adapted for fermentation under the conditions present and in bioreactor studies have a much faster doubling time.

In comparing the oenological characters of the three strains (Table 3.20) in shake flasks, *Sacch. cerevisiae* DBVPG2168 gave the best overall performance in all three media, in terms of increased ethanol yield, final cell density and fermentation rate. All strains examined performed well in unsupplemented grape must (0.5:1 Mg:Ca). On elevation of magnesium levels although final cell densities improved for the *Saccharomyces* strain DBVPG2168, doubling times increased resulting in a decrease in specific growth rate, although fermentation rates did not differ greatly. For *T. delbrueckii* DBVPG6168 the converse is true, final cell density was decreased, but growth rates improved and a 39% decrease in doubling time was observed. This suggests that although magnesium is beneficial to the cells, the full result of the addition is not fulfilled because when added to a complex medium such as grape must, although acting to balance the natural ionic imbalance

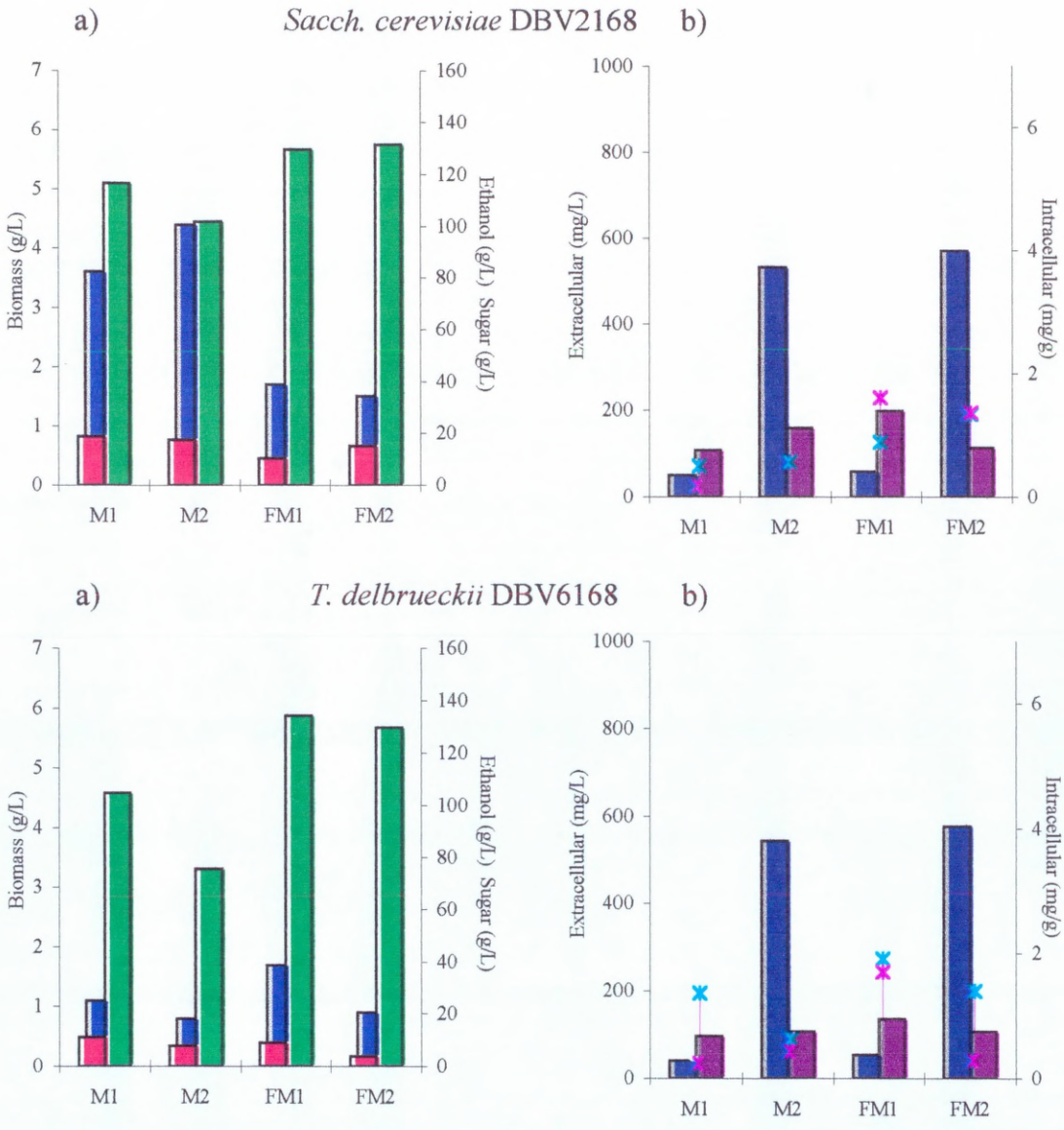


Figure 3.24 Comparison of fermentation and metal ion parameters between shake flasks (300ml) and bioreactor (2L) studies after 24h in grape must with altered magnesium levels; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: White grape must with altered mg/L Mg:Ca ratios; Shake Flasks (300ml): M1 0.5:1, M2 5:1, Bioreactors (2L): FM1 0.5:1, FM2 5:1.

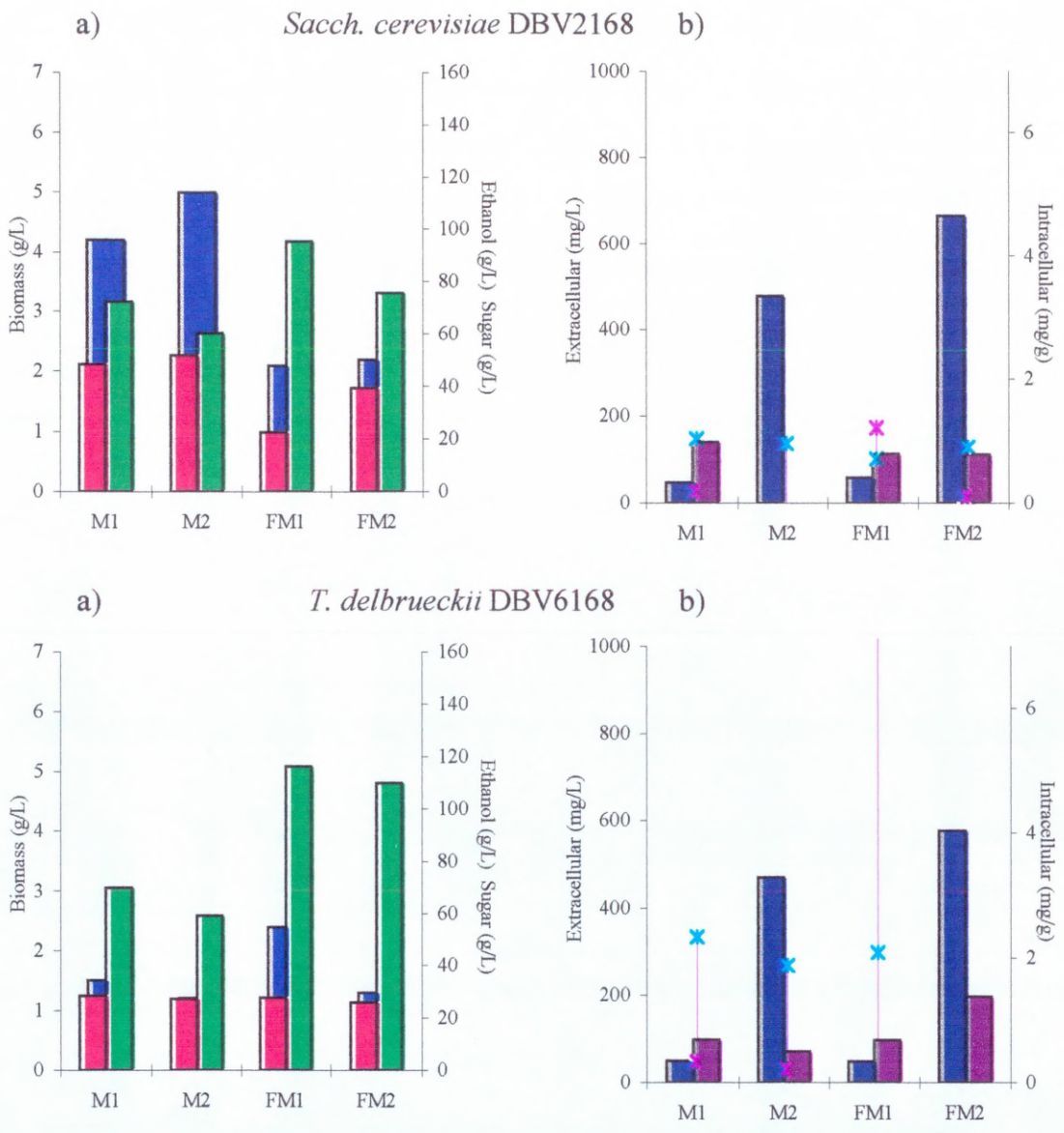


Figure 3.25 Comparison of fermentation and metal ion parameters between shake flasks (300ml) and bioreactor (2L) studies after 48h in grape must with altered magnesium levels; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: White grape must with altered mg/L Mg:Ca ratios; Shake Flasks (300ml): M1 0.5:1, M2 5:1, Bioreactors (2L): FM1 0.5:1, FM2 5:1.

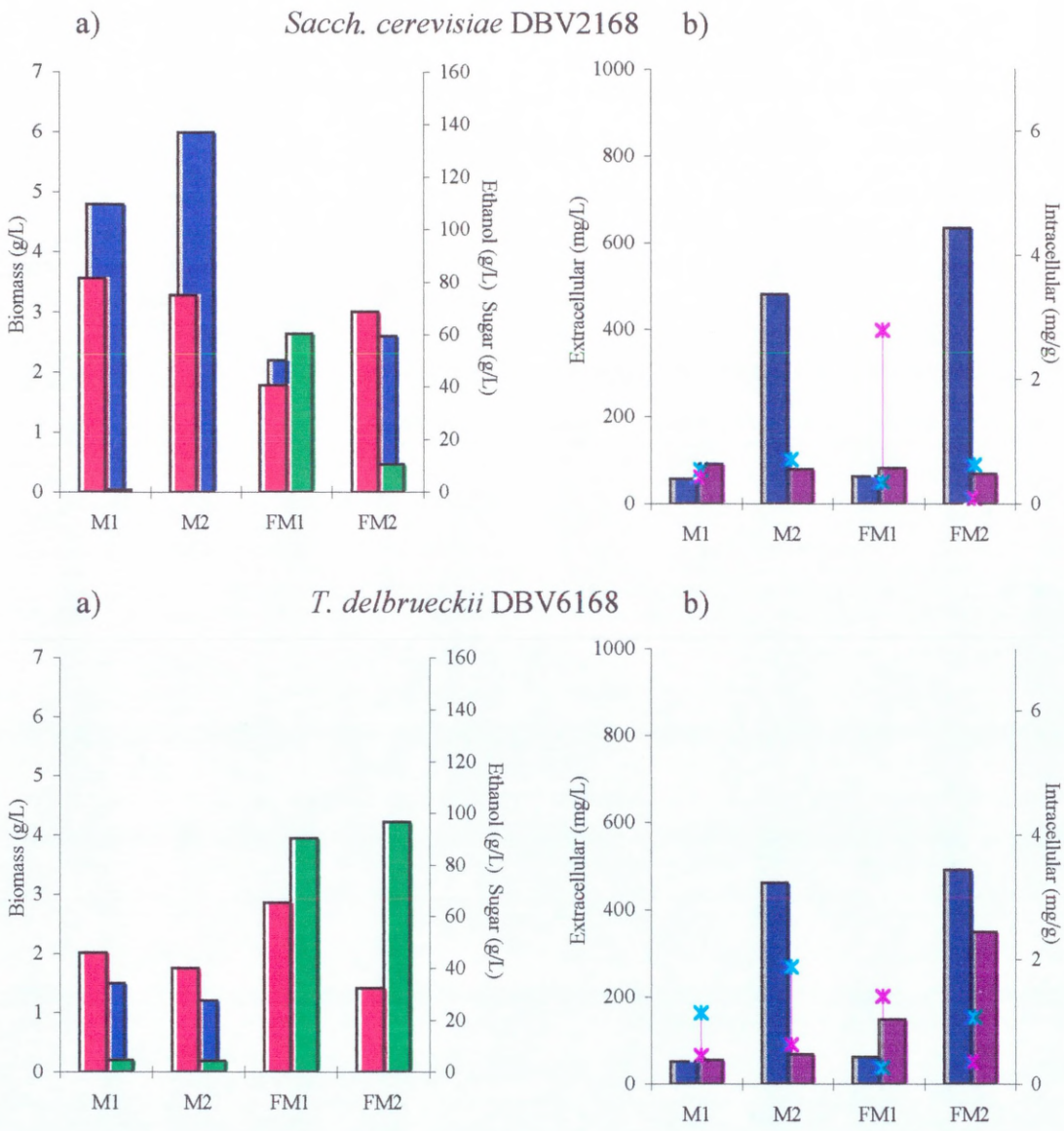


Figure 3.26 Comparison of fermentation and metal ion parameters between shake flasks (300ml) and bioreactor (2L) studies after 96h in grape must with altered magnesium levels; a) Fermentation parameters: ■ growth, ■ sugar, ■ ethanol, b) Metal ion parameters: ■ extracellular magnesium, ■ extracellular calcium, * intracellular magnesium, * intracellular calcium. Media: White grape must with altered mg/L Mg:Ca ratios; Shake Flasks (300ml): M1 0.5:1, M2 5:1, Bioreactors (2L): FM1 0.5:1, FM2 5:1.

towards calcium, some of this added magnesium may be complexed and chelated by organic materials in the medium, rendering it unavailable to the yeast cells.

Elevation of calcium levels in must caused improved final cell densities. *Sacch. cerevisiae* DBVPG2168 and *T. delbrueckii* DBVPG6168 displayed a reduction in growth rate and final ethanol yield with DBVPG2168 showing a marked (53%) increase in doubling time of the yeast. Fermentation rate was reduced for DBVPG2168 and showed slight improvement for *T. delbrueckii* DBVPG6168 but overall for this yeast biomass and ethanol production levels were quite low. In the bioreactor studies (Table 3.21) *Sacch. cerevisiae* DBVPG2168 performed much better than *T. delbrueckii* DBVPG6168 and overall the fermentation time in bioreactors was increased dramatically compared to shake flask experiments, a factor due simply to differences in O_2 transfer and vessel design. A marked improvement in both growth and fermentation parameters is seen with *Sacch. cerevisiae* DBVPG2168 on supplementation of must with a 10-fold increase in magnesium, final cell density and growth rates were improved as was ethanol yield and fermentation rate. *T. delbrueckii* DBVPG6168 on the other hand exhibited a much slower utilisation of the available sugars and this fermentation progressed for 240h, prior to the exhaustion of sugar reserves. The response to elevated magnesium is not as significant with *T. delbrueckii* DBVPG6168. Fermentation rates are improved, however both final ethanol and cell yields are much reduced and this is reflected in the reduction of growth rate of the cells, showing a slight inhibitory response to increased magnesium levels. These results indicate overall that scale-up and vessels design has no major effect on cellular biochemistry of these two important cations.

INFLUENCE OF MEDIA TYPE AND ALTERED MG:CA RATIO ON FERMENTATION PARAMETERS.

Obviously for industrial application, complex media is the medium of choice and the effects of altering the ionic nutrition of the yeast cells by altering Mg:Ca ratios have been examined and discussed earlier in this chapter, as have those of semi-synthetic (lab-based) media.

Table 3.20 Oenological characters of *Sacch. cerevisiae* L-2226 & DBV2168 and *Torulaspora delbrueckii* DBV6168, in shake flasks (300ml).

Media (mg/L ratio)	Strain	Ethanol Capacity (@ 96h) (g/L)	Fermentation Rate (@ 96h)	Doubling Time (Td) (h)	Specific Growth Rate (μ) (h^{-1})	Final Cell Density (@ 96h) (g/L)	Final Ethanol Yield (@ 96h) ($g.g^{-1}$)
0.5:1 Mg:Ca	*Sacch. cerevisiae* L-2226	42.42	0.983	46.21	0.015	3.1	0.240
	Sacch. cerevisiae DBV2168	81.53	0.994	43.32	0.016	4.8	0.512
	Torulaspora delbrueckii DBV6168	46.14	0.971	30.28	0.023	1.5	0.297
5:1 Mg:Ca	*Sacch. cerevisiae* L-2226	37.37	0.908	69.31	0.010	4.3	0.260
	Sacch. cerevisiae DBV2168	75.05	0.999	52.51	0.013	6.0	0.469
	Torulaspora delbrueckii DBV6168	39.97	0.972	18.57	0.037	1.2	0.257
0.05:1 Mg:Ca	*Sacch. cerevisiae* L-2226	39.97	0.938	34.66	0.020	4.8	0.270
	Sacch. cerevisiae DBV2168	71.10	0.989	66.49	0.010	5.2	0.449
	Torulaspora delbrueckii DBV6168	35.31	0.992	32.78	0.021	1.8	0.222

Table 3.21 Oenological characters of *Sacch. cerevisiae* DBV2168 and *Torulaspora delbrueckii* DBV6168, in small bioreactors (2L).

Media (mg/L ratio)	Strain	Ethanol Capacity (@ 150h) (g/L)	Fermentation Rate (@ 150h)	Doubling Time (Td) (h)	Specific Growth Rate (μ) (h^{-1})	Final Cell Density (@ 150h) (g/L)	Final Ethanol Yield (@ 150h) ($g.g^{-1}$)
0.5:1 Mg:Ca	*Sacch. cerevisiae* DBV2168	56.35	0.950	25.67	0.027	2.5	0.371
	Torulaspora delbrueckii DBV6168	@150h 54.62	@150h 0.462	47.87	0.014	@150h 2.8	@150h 0.739
		@240h 55.77	@240h 0.678			@240h 2.5	@240h 0.514
5:1 Mg:Ca	*Sacch. cerevisiae* DBV2168	69.44	0.999	21.18	0.033	2.8	0.434
	Torulaspora delbrueckii DBV6168	@150h 32.43	@150h 0.477	63.95	0.011	@150h 1.5	@150h 0.425
		@240h 48.43	@240h 0.637			@240h 1.4	@240h 0.412

Media type has a great effect on yeast physiology and a comparison of 10-fold increases in magnesium and calcium in both semi-synthetic (PYN) and complex (grape must) media, for the three wine strains *Sacch. cerevisiae* L-2226, DBVPG2168 and *T. delbrueckii* DBVPG6168, can thus be made (Tables 3.22-3.24).

Biomass yield and biomass productivity for *Sacch. cerevisiae* L-2226 (Table 3.22) were observed to be higher in semi-synthetic media studies and in both media types, elevation of magnesium lead to a corresponding increase in productivity. Ethanol yields were also higher in the semi-synthetic media studies, although actual productivity's of ethanol were not greatly different. Alteration of the Mg:Ca ratios in both media types resulted in increased conversion efficiencies with magnesium exhibiting a greater effect in semi-synthetic media for this strain. For *Sacch. cerevisiae* DBVPG2168, a strain isolated from winery environments (Martini *et al.* 1978), fermentation parameters were much improved within the complex media studies (Table 3.23) hardly surprising since this yeast's natural habitat is grape must. In semi-synthetic media this strain shows no difference in biomass productivity although the alteration of ionic ratios in favour of magnesium resulted in improved ethanol productivity and conversion efficiency. In must, growth is improved on ionic supplementation, to a greater extent with magnesium, although biomass productivity is also improved on supplementation with calcium. Ethanol yields are markedly higher in must compared with PYN media, but the addition of magnesium does not effect improvements in ethanol yields over the unsupplemented media. As have been observed previously, biomass yields for *T. delbrueckii* DBVPG6168 (Table 3.24) are fairly low and the alteration of Mg:Ca ratios produces little or no improvements in terms of growth characteristics. Ethanol yields were slightly improved in semi-synthetic media on Mg-supplementation and yields are observed to be higher in semi-synthetic media than in grape must. Again it is observed that unsupplemented grape must gives best yields in terms of both growth and ethanol levels, illustrating once more the difficulties in ionic supplementation of complex media.

Table 3.22 Influence of media type and Mg:Ca ratio on some parameters of fermentation of *Sacch. cerevisiae* L-2226.

Fermentation Parameters	PYN			Must		
Mg:Ca[#]	0.5:1	5:1	0.05:1	0.5:1	5:1	0.05:1
Fermentation time (h)	96	96	96	96	96	96
X_0 = Starting cell mass (g/L)	0.2	0.8	1.2	0.8	0.1	1.7
X_F = Final cell mass (g/L)	4.4	6.4	4.2	3.1	4.3	4.8
$Y_{(x)}$ = Biomass yield (g/g) @96h	0.037	0.053	0.035	0.018	0.029	0.032
$P_{(x)}$ = Biomass productivity (g/L/h)	0.046	0.067	0.044	0.032	0.045	0.050
S_0 = Starting sugar (g/L)	120	120	120	160	160	160
S_F = Final sugar (g/L)	0.0	0.0	0.0	3.04	14.71	9.84
S_U = Sugar utilised (g/L)	120	120	120	156.96	145.29	150.16
Q_L = Specific rate of sugar utilisation (g/L/h)	0.284	0.195	0.298	0.595	0.352	0.326
Eth_0 = Starting ethanol (g/L)	0.0	0.00	0.0	0.0	0.0	0.0
Eth_F = Final ethanol (g/L)	41.25	49.06	45.03	42.42	37.37	39.97
$Y_{(etoh)}$ = Ethanol yield (g/g)	0.344	0.409	0.375	0.240	0.26	0.27
$P_{(etoh)}$ = Ethanol productivity (g/L/h) @96h	0.430	0.511	0.469	0.442	0.389	0.416
C.E. = Conversion efficiency (%)	67.32	80.04	73.38	46.97	50.88	52.84

[#] mg/L ratio

Table 3.23 Influence of media type and Mg:Ca ratio on some parameters of fermentation of *Sacch. cerevisiae* DBV2168.

Fermentation Parameters	PYN			Must		
Mg:Ca[#]	0.5:1	5:1	0.05:1	0.5:1	5:1	0.05:1
Fermentation time (h)	96	96	96	96	96	96
X_0 = Starting cell mass (g/L)	0.4	0.2	0.2	1.8	2.0	2.4
X_F = Final cell mass (g/L)	1.7	1.7	1.7	4.8	6.0	5.2
$Y_{(x)}$ = Biomass yield (g/g) @96h	0.014	0.014	0.014	0.030	0.038	0.033
$P_{(x)}$ = Biomass productivity (g/L/h)	0.018	0.018	0.018	0.050	0.062	0.054
S_0 = Starting sugar (g/L)	120	120	120	160	160	160
S_F = Final sugar (g/L)	0.0	0.0	0.0	0.91	0.08	1.80
S_U = Sugar utilised (g/L)	120	120	120	159.09	159.92	158.20
Q_L = Specific rate of sugar utilisation (g/L/h)	0.735	0.735	0.735	0.345	0.278	0.317
Eth_0 = Starting ethanol (g/L)	0.0	0.0	0.0	0.0	0.0	0.0
Eth_F = Final ethanol (g/L)	46.14	48.27	46.69	81.53	75.05	71.10
$Y_{(etoh)}$ = Ethanol yield (g/g)	0.384	0.402	0.389	0.512	0.469	0.449
$P_{(etoh)}$ = Ethanol productivity (g/L/h) @96h	0.481	0.503	0.486	0.849	0.782	0.741
C.E. = Conversion efficiency (%)	75.15	78.67	76.12	100	91.8	87.9

[#] mg/L ratio

Table 3.24 Influence of media type and Mg:Ca ratio on some parameters of fermentation of *Torulaspora delbrueckii* DBV6168.

Fermentation Parameters	PYN			Must		
Mg:Ca[#]	0.5:1	5:1	0.05:1	0.5:1	5:1	0.05:1
Fermentation time (h)	96	96	96	96	96	96
X_0 = Starting cell mass (g/L)	0.2	0.3	0.3	0.4	0.1	0.2
X_F = Final cell mass (g/L)	2.0	2.0	1.7	1.5	1.2	1.8
$Y_{(x)}$ = Biomass yield (g/g) @96h	0.017	0.017	0.014	0.001	0.008	0.011
$P_{(x)}$ = Biomass productivity (g/L/h)	0.021	0.021	0.018	0.016	0.012	0.019
S_0 = Starting sugar (g/L)	120	120	120	160	160	160
S_F = Final sugar (g/L)	0.09	0.16	1.75	4.57	4.40	1.33
S_U = Sugar utilised (g/L)	119.91	119.84	118.25	155.43	155.60	158.67
Q_L = Specific rate of sugar utilisation (g/L/h)	0.624	0.624	0.724	1.079	1.35	0.918
Eth_0 = Starting ethanol (g/L)	0.0	0.0	0.0	0.0	0.0	0.0
Eth_F = Final ethanol (g/L)	44.84	46.69	41.55	46.14	39.97	35.31
$Y_{(etoh)}$ = Ethanol yield (g/g)	0.374	0.390	0.351	0.297	0.257	0.222
$P_{(etoh)}$ = Ethanol productivity (g/L/h) @96h	0.467	0.486	0.433	0.480	0.416	0.368
C.E. = Conversion efficiency (%)	73.23	76.24	68.77	58.12	50.29	43.44

[#] mg/L ratio

3.3.3 PRE-CONDITIONING OF WINE YEASTS

The optimisation of ion concentrations in complex industrial media can be difficult since the occurrence of chelation in both complex and laboratory media can be enigmatic. Complex media, such as grape must, contain a wide variety of chelating and adsorbing materials which serve to complex the ionic constituents present in the media, making them unavailable for yeast nutrition (Lie *et al.* 1975; Jones & Greenfield 1984). Laboratory media may suffer a similar problem, although not to the same extent, thus the expected ionic concentration and more importantly concentration ratios established, may be quite different from the actual amounts, since not all the ionic nutrient supplied in a particular media are freely available to the yeast cells. In an attempt to overcome this aspect of ionic nutrition, pre-conditioning the yeast cells with high levels of magnesium prior to exposure to the fermentation conditions was attempted. Based on the theory that if you could store up excessive levels of magnesium in the yeast cells initially the cells would be in good condition prior to fermentation, having sufficient magnesium for enzyme activation and enzyme protection/stabilisation and thence throughout fermentation the cells would have a supply of Mg^{2+} available to them to utilise, despite the media imbalances which may ensue.

From the results (Fig. 3.27) it can be seen that a four day pre-conditioning regime, doubling the magnesium concentration on each transfer, improved the fermentation performance of *Sacch. cerevisiae* DBVPG2168 in minimal media. In terms of yeast growth, although pre-conditioning the cells did not substantially improve the final cell number; 9.58×10^7 cell/ml compared with 8.26×10^7 cell/ml, the overall trend of biomass production was marginally improved over the control flask of 0.05g/L Mg concentration (Fig. 3.27a) a point more poignant when it is noticed that the starting cell numbers differs by approximately 3×10^5 cell/ml with the pre-conditioned culture initiating the fermentation with less biomass but a much shorter (if in existence at all) lag phase.

In terms of ethanol production and glucose consumption (Fig. 3.27b & c) the story is illustrated more prominently with ethanol concentration peaking at 7.09% v/v at 48h

followed by a slight decline in the pre-conditioned culture (0.8g/L Mg) whereas in the control flask the production of ethanol was much slower and peaked at 5.26% v/v at 72h (Fig. 3.27b). Sugar consumption in the pre-conditioned cultures also showed a more rapid progress with a sharp decline between 12 and 24h of fermentation and an exhaustion of the sugar reserves at 48h corresponding with the peak in ethanol production (Fig. 3.27c) thus explaining the decline in ethanol levels at 72h in pre-conditioned yeasts, as the ethanol would be being utilised as a secondary energy source by the remaining active yeasts. The control flask shows similar consumption of glucose up until 12h then, once the cells have entered the exponential phase fully, the consumption of sugar follows a more sedate pattern, reading zero levels by 72h (Fig. 3.27c) which again corresponds to the peak in ethanol production of the control yeasts being delayed until 72h in comparison to pre-conditioned cultures.

Overall these preliminary results show that pre-conditioning yeast cells with increased levels of magnesium has a beneficial effect on fermentation performance. The action of pre-conditioning cells from 0.05g/L to 0.8g/L Mg, a 16-fold increase in concentration of this essential metal ion, has been demonstrated to increase biomass production and dramatically increase the rate of ethanol production and glucose consumption accordingly. The implications being that in an industrial situation, by pre-conditioning inocula it may be possible to improve biomass and ethanol production and reduce fermentation time under conditions where it is too complicated or difficult to alter the ionic imbalances in the industrial media. Further to this, the use of pre-conditioning of inocula in addition to correcting the ionic imbalances of complex media may give dramatic improvements to the fermentation process, reducing fermentation time and allowing for better turn-over and faster through-put in industrial plants.

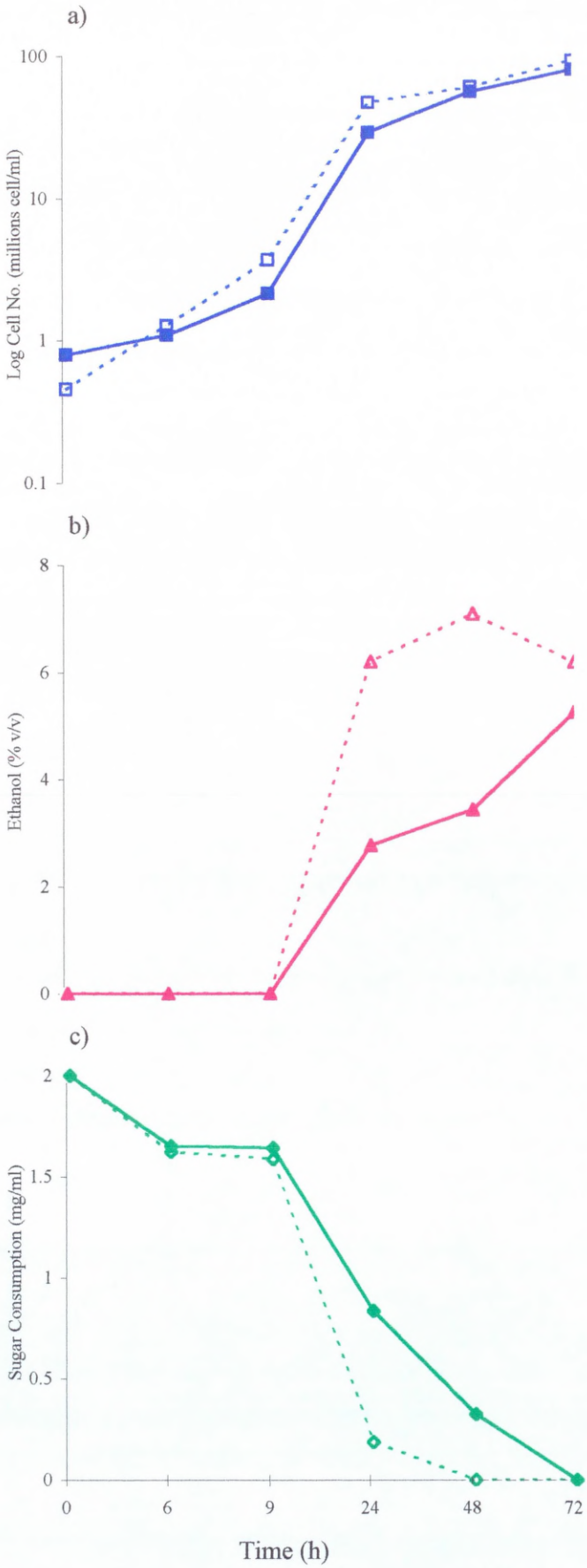


Figure 3.27 Effect of magnesium pre-conditioning of *Sacch. cerevisiae* DBV2168 on fermentation performance in minimal media: a) ■ growth, b) ▲ ethanol, c) ◆ sugar; ——— control, - - - - pre-conditioned.

3.4 CONCLUSIONS

Overall, comparing the wine strains *T. delbrueckii* DBVPG6168, *Sacch. cerevisiae* DBVPG2168 & L-2226, *Torulaspora* is observed to be unable to ferment as efficiently as the *Saccharomyces* strains. *Torulaspora* is one of three genera of relevant oenological interest along with *Saccharomyces* and *Zygosaccharomyces* (Zambonelli *et al.* 1989). Previously classified within the genera *Saccharomyces*, as either *Sacch. rosei* or *Sacch. delbrueckii* before being recognised as a single species; *Torulaspora delbrueckii* (Zambonelli *et al.* 1989). The organism is found in the natural habitat of fermenting must and can in some cases compete with *Sacch. cerevisiae* to practically take over the fermentation (Martini & Martini 1990). These results, however, show that the length of fermentation is much longer (at least 100h longer) therefore *T. delbrueckii* as a single, pure culture, could not be viably suggested for use in commercial wine production. The organism does however have uses in the wine industry in association with *Sacch. cerevisiae*, as its tolerance to ethanol and growth rates are higher than the apiculate yeasts (e.g. *Kloeckera apiculata*) which are also associated with the natural flora of grape must and it can be used in the deacidification of high malate musts (Martini & Martini 1990).

It can be concluded, that although the effects of increased metal ion content on must is not as relevant to cultures of *T. delbrueckii*, increasing magnesium levels in must can be beneficial for cultures of the commercial leader *Sacch. cerevisiae*. In contrast to the scenario presented by published data for malt wort, where levels of magnesium are higher than calcium, in grape must the ratio is found to be 2:1 in favour of calcium. This situation is found in most industrially important media, whereas laboratory culture media ratios favour magnesium over calcium (Walker 1994). The results of this work propose that high calcium ratios generally inhibit fermentation characteristics in both semi-synthetic and complex media, slowing fermentations and therefore questions the applicability of the nutritional make-up of industrial media. Results portrayed here illustrate, however, that growth rates, ethanol production and sugar consumption rates of most yeast strains are increased when magnesium levels are elevated in the growth medium, with the increase of

calcium levels tending to inhibit fermentation and also resulting in a more acidic wine by interfering with the cellular uptake of magnesium. Implications of this effect would be, for wine and related industries, that turn-over time of fermentations would be greatly reduced since individual fermentations would be quicker on supplementation with magnesium and that a better quality wine (balanced alteration of flavour compounds) would be produced.

CHAPTER 4

INVESTIGATION INTO THE ROLE OF METAL IONS AND OTHER FACTORS AFFECTING YEAST AGGLOMERATION

4.1 INTRODUCTION

Some strains of the yeast *Saccharomyces cerevisiae* have the tendency to form yeast aggregates. This agglomeration or 'grittiness' is described as: fresh or compressed yeast which only partially resuspend when mixed in water; most of the yeast remains as macroscopic clumps or aggregates of cells.

When dispersed in water, pressed baker's yeast is required to disperse rapidly and evenly to give a milky suspension. Occasionally this does not occur and the yeast suspension contains granular material, usually referred to by the baker as 'grit'. Grit formation is an idiosyncratic phenomenon which is detrimental to baker's yeast quality and therefore has great economic consequences for the manufacturer. Problems arise because the grit is visually obvious to the baker and it can impair adequate mixing into the bread dough, reducing baking performance by limiting its leavening ability; however if mixing is even, there is no adverse effect of grit on baking performance of the yeast. The main problem arises with customers who carry out a liquid pre-ferment process, currently used in approximately half of the baking plants in the USA. In this process, the yeast is suspended in a buffered sugar solution for 2-4 hours and the suspension is then added to the dough formulation. Problems occur with bakers who filter the pre-ferment prior to addition to the dough formulation, if the yeast is 'gritty' it rapidly blocks the filters and therefore not enough yeast biomass passes through to the dough formulation, due to the dissolved yeast being held back by the blocked filter. From this the baker observes two problems; baking performance is reduced and the grit is extensively visual to the baker.

This problem may not only be restricted to the baking industry, some evidence may also exist suggesting that some aerobically produced yeast for use in brewing, wine-making or distilling also exhibit the phenomenon of agglomeration, but the consequences for these industries, besides impaired mixing with wort or must are not so well defined.

4.1.1 EFFECT OF PRODUCTION METHODS AND MEDIA ON AGGLOMERATION

Manufacture of baker's yeast is a high volume-low profit process. It is therefore, important that the producer minimises costs when designing a medium in which to propagate the yeast. Almost all processes currently operated use sucrose as a source of carbon and energy, provided in the form of molasses. Molasses frequently contains compounds which inhibit yeast growth including particulate matter, hence it requires to be clarified to remove these impurities which would be absorbed by the yeast. Usually molasses (80-85°Brix) is diluted to 30-40°Brix to facilitate clarification. Molasses is of two basic types: beet and cane or 'blackstrap' and being a by-product its chemical composition is variable, which can have a profound effect on yeast production.

If a fermentation is initiated with a 'gritty' strain then the resultant yeast produced will also be 'gritty'. Grit is formed during the fermentation and growth of yeast on molasses. The 'gritty' or poor dissolving property is expressed depending on a number of factors including the environmental conditions. Tendency to form grit on resuspension is known to be strain dependent (Mann 1986) and to be affected by storage conditions (Veilleux 1995), as well as the ionic strength of the solvent in which it is suspended (Guinard & Lewis 1993). In a review of the knowledge of the subject, Mann (1986) reported that genetically all USA baker's strains produce grainy yeasts. However, work carried out recently by Dumont (1995) indicated that genetically, 'gritty' and non-'gritty' yeast strains are identical in their DNA profile by both polymerase chain reaction (PCR) and pulse field electrophoresis (PFE) techniques, suggesting factors other than the strict genetic make-up of the yeast are involved. Expression of the 'gritty' phenotype can be induced by environmental parameters, including fermenter design, fermentation media and environment *i.e.* process conditions, and yeast cell characteristics.

4.1.2 BIOCHEMICAL ASPECTS OF YEAST GROWTH AND AGGLOMERATION

Cellular ionic composition is highly dependent on the yeast species and the type of environment in which the yeast is grown. The nutritional requirements of yeast were one of the first properties of yeast biochemistry to be described (Rose 1977). Baker's yeast *Sacch. cerevisiae* may grow well on both simple and complex media, providing that basic requirements of vitamins and minerals are supplied. K^+, Zn^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and other metal ions are required in various concentrations for the function of vital metabolic enzymes (Dedyukhina & Eroshin 1991). An imbalance of these ionic species leads to complex and detrimental alterations of metabolic patterns and growth characteristics (Jones & Greenfield 1984)

Of the wide range of metal ions found to be involved in cell functions (Jones & Greenfield 1984), magnesium and calcium are considered to be major cations in cell metabolism (Brown 1966). Optimum growth requirements in *Sacch. cerevisiae* for calcium are less than 4.5mM (Jones & Gadd 1990) and for magnesium, between 2-4 mM (Diamond & Rose 1970). In industrial situations, however, the cellular composition of these ions varies significantly over a wide range of environmental changes. A further problem is the effect of ion-ion interactions and inter-relations, since with these the activity of one ion depends on the availability of another and therefore it is impossible to determine optimum levels. For example, increased magnesium concentration in the medium would result in an increased uptake of magnesium and potassium but a decrease in that of calcium (Borst-Pauwells 1981). Chandrasena *et al.* (1997) have recently studied metal ion interactions in yeast fermentation media and have developed statistical models to predict fermentation performance based on the levels of key ions such as K^+, Mg^{2+}, Ca^{2+} and Zn^{2+}.

The ionic composition of industrial media is varied. Most major industrial fermentation media, such as molasses, malt wort and wine must consists of complex plant materials with a correspondingly more complex ionic composition than defined media. Furthermore, the availability of cations for growth is limited, as there are a number of complexing

mechanisms occurring in the complex media (Lie *et al.* 1975) *i.e.* chelating, sequestering, absorbing or complexing with proteins, etc. in the medium. Magnesium and calcium ions have an affinity for the reactive groups $RCOO^-$, RHN^-, RS^- and H_2O therefore these ions are readily attracted to proteins, whether it be proteinaceous material in the media making the ion unavailable or binding to ion-specific protein binding sites on yeast cells.

Quantification of Mg^{2+} and Ca^{2+} in 'gritty' and non-'gritty' yeast has not previously been carried out, however there is a proven involvement in various cell-cell physical interactions and cell adhesion. Guinard and Lewis (1993) found that a positive correlation existed between yeast cell calcium and sedimentation of agglomerated cells, indicating that calcium ions play an important role in the 'gritty' phenomenon. Inorganic levels in molasses tend to favour that of calcium, with magnesium to calcium ratios in beet and cane molasses being 0.09:1 and 0.14:1 respectively, with levels of calcium in cane molasses being around seven times higher than those found in beet molasses (Walker *et al.* 1994). The involvement of magnesium in grit formation may be as an antagonist to calcium, a phenomenon observed in many biological situations. Ca^{2+} and Mg^{2+} have been known to act antagonistically with respect to inducing flocculation in brewer's yeast (Stratford & Brundish 1990). It has been hypothesised by Guinard and Lewis (1993), that calcium ions may act as cofactors in the activation of the binding capacity of proteinaceous components in 'gritty' yeasts. This hypothesis can be further tested by the alteration of Mg:Ca ratios since magnesium and calcium are known to be antagonists and compete for binding sites on the cell surface. The increase of available magnesium ions and/or reduction of calcium ions will hypothetically reduce calcium binding and therefore reduce the activation of proteins on the cell surface which are involved in the mechanism of agglomeration, thereby reducing grit.

4.1.3 FLOCCULATION

Yeast flocculation, defined as "...the phenomenon wherein yeast cells adhere in clumps and either sediment rapidly from the medium in which they are suspended or rise to the

medium's surface" (Stewart & Russell 1981), is a reversible process and although similar to agglomeration, is not the same phenomenon (Guinard & Lewis 1993). Its similarities to agglomeration, however, requires one to have some understanding of this problem in order to address the problem of agglomeration.

Flocculation commonly occurs in the yeast *Sacch. cerevisiae* and it is a phenomenon particularly well known in the brewing industry. Due to the interest of brewing scientists in this phenomenon, research has been carried out over the last century and more intensively over the passed 20 years, in an attempt to fully understand the mechanisms of flocculation. Despite this, confusion and controversy about the biochemical, molecular and genetic basis of flocculation still remains and the causal mechanism of initiation of flocculation ability is not known. Gaining an understanding of the genetics of flocculation is obscured by the genetic complexity of the yeasts involved. However, some workers have elucidated that the genetic basis of flocculation is held in the FLO genes (Stewart & Russell 1977; Russell *et al.* 1980; Johnston & Reader 1983), in particular FLO1 and FLO5. Flocculation is not always genetically stable and mutations can occur effecting the loss of flocculant ability. Much remains to be discovered regarding the genes that govern flocculation and the existence of multiple gene copies as well as the possibility of more than one operating flocculation mechanism, highlights the difficulty in studying the genetics of the mechanisms of flocculation.

Other factors are involved in this cell-surface interaction; cell properties, fermentation environment etc. and it is the interaction of these which results in the phenotypic expression of the genetic trait. The onset of flocculation occurs around the end of the fermentation as the yeast cells reach the late stationary phase of growth (Helm *et al.* 1953). In general, it is changes in the environment which affects cell flocculation, decline in pH and the presence of ethanol are factors which increase flocculation (Amory & Rouxhet 1988; Speers 1991), while sugars e.g. mannose and its derivatives, cause a rapid dispersion of cells (Mill 1964), presumably by a direct action of the sugar on the cell wall. Smit *et al.* (1992) showed that nutrient limitation appeared to trigger an increase in cell surface hydrophobicity and

concomitantly, flocculation ability. Nitrogen appeared to be the initial growth-limiting factor but other nutrient limitations produced a similar response. Differences in flocculation character of brewing yeast arise from differences in the cell wall of yeast and Miki *et al.* (1980) describes cell-cell interactions as requiring the participation of at least two distinct cell wall components, one of which is proteinaceous or dependent on proteins with disulphide bonds for activity. The amount of dissolved oxygen can also have a significant effect on yeast cell wall properties and cell-cell physical interactions. Miki *et al.* (1982b) found that the degree of flocculation in logarithmically growing cells of a flocculent strain varied with aeration of growth medium. Other environmental factors are also involved in this phenomenon, including inorganic ions and it is known that flocculation in *Sacch. cerevisiae* is mediated by a Ca^{2+} -dependent cell surface recognition mechanism.

Several theories or hypotheses have been suggested (Mill 1964; Miki *et al.* 1982a; Miki *et al.* 1982b; Nisihara & Toraya 1987; Kihn *et al.* 1988; Kuriyama *et al.* 1991) to explain the mechanisms of flocculation, but since it would appear that a number of mechanisms are involved, the nature of these interactions are still controversial. Three main mechanisms have been invoked to explain the flocculation of susceptible strains of brewing yeast. A classic colloid theory of surface charge neutralisation was initially suggested, but this has been generally discounted because it does not account for the specific requirement for calcium in flocculation. Based on the apparent importance of calcium in flocculation, Mill (1964) suggested that calcium-bridges linked flocculent cells, presumably by joining carboxy or phosphate groups at the cell surface of adjacent cells and these bridges were believed to be stabilised by hydrogen bonds. It was also found that other metal ions promoted flocculation by ionic binding between anionic groups of adjacent cells (Miki *et al.* 1982a). After some work, however, it was thought this effect was due to an indirect effect of calcium, due to an efflux or displacement of calcium ions by other cations in suspension and these calcium ions binding to Ca-specific proteins, rather than the direct effect of these cations (Miki *et al.* 1982a; Kuriyama *et al.* 1991). This calcium-bridge model, while accounting for a divalent cation requirement, principally calcium, fails to explain the inhibition of flocculation effected by sugars. The model best describing the mechanisms of

flocculation suggested at present is the lectin-like adhesion model (LLA-model). This model accounts for the involvement of calcium, protein structures and hydrogen bonding, as well as the inhibitory effect of mannose. Elucidation of the LLA-model was presented by Miki *et al.* (1982a; 1982b), who showed evidence of specific protein-polysaccharide binding and lectin-like proteins requiring calcium for activation (although they did not isolate, or locate where these proteins were on the yeast cell). Kihn *et al.* (1988) provided further support for this model, demonstrating that flocculation could be prevented or reduced by the addition of various mannose derivatives and the results of Smit *et al.* (1992) imply both Ca^{2+}-dependent sugar binding and physiochemical interactions, in particular cell surface hydrophobicity, are involved in the flocculation process of yeast cells. They suggested, if the role of calcium consisted of only charge neutralisation then all other divalent cations would promote flocculation. This is not the case since some ions inhibit flocculation, therefore the role of calcium is thus; various cations may fit into the calcium binding site on the cell surface (the closer the ions ionic radius is to calcium the stronger the inhibition: stereochemical constraints), however calcium is the only cation which induces a protein conformation able to bind phosphomannan strongly enough to make cell adhesion possible. The positive effect of other divalent cations on promoting flocculation, as suggested for the Ca-bridge model would be to indirectly displace Ca^{2+} thus making it accessible to calcium-specific binding sites increasing the number of activated sites and correspondingly, flocculation.

4.1.4 AGGLOMERATION

Agglomeration or 'grit' formation, although a type of cell adhesion, is however distinct from flocculation (Guinard & Lewis 1993). Cell adhesion may be specific or non-specific (Marshall 1984) and may involve ionic, bipolar, hydrogen bonds and hydrophobic interactions. As observed with flocculation, yeast agglomeration seems to be the result of several factors or a combination thereof. The phenomenon is strain dependent, however DNA profiles of both 'gritty' and non-'gritty' strains have been shown to be identical

(Dumont 1995). In a comparison of the structure, composition and cell surface properties of 'gritty' and non-'gritty' cells, it was shown that calcium ions, phosphates, proteins, carbohydrates and lipids may be involved in agglomeration (Guinard & Lewis 1993).

4.1.4.1 Mechanisms of agglomeration

The mechanism of agglomeration can be divided into two main components: non-specific and specific. Non-specific components would include hydrophobicity, cell charge, cell wall compositions etc. Guinard and Lewis (1993) found no significant difference between 'gritty' and non-'gritty' yeast in cell hydrophobicity or flocculence. Phosphorous surface concentration has been observed to be higher in non-'gritty' cells than in 'gritty' cells, since phosphorous surface concentration is an indicator of surface charge (Amory *et al.* 1988a) and to physically interact yeast cells need to overcome the negative cell surface charge, the reduction noted in 'gritty' cells could be significant in effecting agglomeration, by reducing the total charge present. A reduced amount of lipids on the surface of 'gritty' yeast also occurs and although agglomeration is not simply due to a reduction in cell wall lipids, it would appear that a reduced lipid level is required for the phenotype to be expressed.

A specific adhesion model similar to cell-cell interactions in other living organisms e.g. yeasts (Miki *et al.* 1982a) and slime moulds (Garrod 1984), has been suggested (Guinard & Lewis 1993). The yeast cell wall is a complex structure consisting of proteins, lipids and polysaccharides. The protein matrixed mannan layer of the yeast cell wall is associated with several functional glycoproteins, such as enzymes (Phaff 1971), sexual agglutinins (Burke *et al.* 1980) and lectins involved in flocculation (Miki *et al.* 1980). It is therefore possible that these branched α-mannan structures may also interact as recognition and/or binding sites in the cell-cell interactions causing agglomeration (Guinard & Lewis 1993). This would imply that a separate component unique to 'gritty' cells exists and actively binds to the mannan carbohydrate, a further component unique to 'gritty' cells is thought to be proteinaceous in nature.

Actively growing cells uptake and incorporate calcium into cell structural materials, especially cell membranes. This membrane bound calcium appears to be central in regulating lipid-protein interactions with the activation of plasma membrane ATPase and maintenance of the membrane permeability barrier under adverse conditions. Calcium is known to act as an activator of proteins (this phenomenon has been shown in flocculation) and for several years Ca^{2+} has been understood as being biologically active in enzymes. Whole cell calcium positively correlated to sedimentation value indicates that calcium plays an important role in agglomeration, possibly analogous to the one calcium plays with concanvalin A (ConA); where an 'unlocked' to 'locked' conformational change occurs after binding of calcium and manganese and only the 'locked' form has any saccharide binding activity (McEuen 1982). Assuming that 'gritty' yeast possess higher levels of proteinaceous compounds at the cell surface and assuming these could be Ca-specific binding proteins, calcium ions may act as cofactors in activating the binding capacity between cells, hence promoting agglomeration.

From the extensive research on flocculation and other types of cell adhesion in eukaryotic organisms, it would appear that a number of mechanisms involved in flocculation are similar to the possible mechanisms of agglomeration. Described here, the similarities between the hypotheses suggested for mechanisms of agglomeration and flocculation, may lead one to argue they are one in the same phenomenon, however major differences between flocculation and agglomeration exist, for example:

- grit does not form in the fermentation medium, formation only occurs after pressing and re-hydrating the yeast cake (>30% solids)
- settling profile of flocculation has two components; flocs which settle rapidly passed the light beam of a spectrophotometer and free cells which sediment slowly (Miki *et al.* 1982a), in agglomeration only the slow sedimentation is observed (Guinard & Lewis 1993).
- a non-flocculent strain of *Sacch. cerevisiae* has been shown to be prone to agglomeration (Guinard & Lewis 1993).

These differences are sufficient to state that agglomeration is not an extension of flocculation of a suspension after it has been processed into a compressed yeast, it is a separate phenomenon exhibited by yeasts.

4.1.5 RESEARCH OBJECTIVE

The aim of this study therefore, was to attempt to provide some basic insight into the phenomenon of yeast agglomeration (grittiness) with a view to eventually recommending approaches to resolve the problem within the plant production process. A research approach aimed at examining the effect of inorganic ions on the problem of grit formation in baker's strains of *Saccharomyces cerevisiae* was followed and a brief look at the problem at a molecular level was included.

4.2 GENERAL METHODS

4.2.1 Yeast strain

Experiments were carried out with a baker's strain of the yeast *Saccharomyces cerevisiae*; Industrial strain C (Lallemand Inc.). The yeast was supplied as either fresh liquid cream yeast (semi-seed) or pressed yeast and maintained at 4°C, until required. Previously identified 'gritty' (7258) and non-'gritty' (1619) yeasts were used for the comparison.

4.2.2 Preparation of yeast cake

Cream yeast (min. 200ml) was vacuum filtered through a No.4 filter disc (Whatman). The cake was then wrapped in cloth and pressed to >30% solids, using a pneumatic press and stored at 4°C until further analysis.

4.2.3 Dry weight

Dry weights of cream yeast, yeast blocks or yeast cakes prepared during an experiment, were carried out using the following protocols:

Liquid Yeast: 2g cream yeast was pipetted onto an aluminium tray of a moisture analyser (Thermo Control, Sartorius GmBH), the drying cycle was initiated and the percentage dry weight recorded once drying was complete.

Pressed Yeast: 2g yeast cake was weighed out onto an aluminium tray of the moisture analyser and the exact weight recorded. The yeast cake was dissolved in a minimal amount of water and the drying cycle initiated. On completion of drying, the weight of the sample was recorded and the percentage solids calculated as follows:

$$\frac{\text{Dry Weight (final)}}{\text{Initial Weight (yeast cake)}} \times 100 = \%\ \text{Solids}$$

4.2.4 Dissolution Test

Pressed yeast cake (>30% solids) was bagged and incubated at 30°C, overnight. 25g of crumbled yeast was added to 75ml warm water in a 250ml beaker and mixed well, left to

settle for 3 minutes and mixed again. The solution/suspension was poured into a 100ml measuring cylinder and left for a further two minutes. The volume of yeast deposit formed was measured in millilitres.

4.2.5 Fermentations

Inoculum

Each fermentation was inoculated with 261.08g (dry weight equivalent) of fresh semi-seed baker's yeast (18% DW) or pressed yeast (30% DW). Exact volume or weight added depended on the percentage solids of the source yeast being used, in respect to the recipe (Table 4.2).

Fermentation Media

The initial medium consisted of 266mg $ZnSO_4$, 67mg $CaPO_4$, 106mg Thiamine, 51.4g NaCl, 4.16g $MgSO_4.7H_2O$ and 12ml H_3PO_4 (85%) in approximately 5 litres of water (exact volume dependent on recipe and water content of the yeast added, Table 4.2). The feeds consisted of: 100% cane molasses, diluted to 40-45°Brix (Reichert ABBE Mark II Digital Refractometer, Buffalo, NY. USA) autoclaved at 121°C then racked, and 5% v/v aqua ammonia (NH_4OH). Antifoam and acid (H_2SO_4, 1M) feeds controlled by computer, were utilised for the control of foam and pH, if and when required.

Alterations to the metal ion content of the final medium were carried out depending on the experiment being performed (Table 4.1). Sulphuric acid treatment involved the reduction of the wort (molasses + water) pH to 4.5 using conc. H_2SO_4, the wort was heat treated at 90-95°C/15min. and left to settle overnight. The racked molasses was then decanted into a sterile feed flask for the fermentation. Magnesium additions were made by adding a sterile solution of the appropriate final concentration of magnesium, dissolved in 1litre dH_2O, to the sterile, racked molasses made up to 4L. Following aseptic addition the final volume was approximately 5L, as per the control and this final feed was decanted aseptically into a sterile feed flask for the fermentation. Calcium additions were made in a similar manner.

Table 4.1 Alteration of metal ion content of 100% cane molasses for fermentations.

Condition	Control	- Ca	+ Ca	1	2	3	4
Code	172/183	182	177	178	179	180	181
Mg:Ca		Sulphuric					
Theory	0.14:1	acid	0.047:1	0.7:1	1.4:1	2.1:1	2.8:1
Actual	0.84:1	0.95:1	0.47:1	1.13:1	1.36:1	1.42:1	1.62:1
Increase Mg	-	nt	-	5x	10x	15x	20x
Increase Ca	-	nt	3x	-	-	-	-
g/l $MgSO_4$	0	nt	15g $CaCl_2$	3.5	7	10.5	14
Total g Mg or Ca added	0	nt	75	17.5	35	52.5	70

(nt: not tested)

Fed-batch fermentation

Fermentations were carried out in a 19 litre fermenter (Bioengineering AG, Switzerland; model D391) equipped with pH, antifoam and temperature probes (Plate 4.1). All fermentations were computer controlled using the Process Control Partner (PCP) programme (AlterSys Inc., Boucherville, Québec) to control the additions of molasses and ammonium feeds and all fermentation parameters. The recipe and feeding programme followed the schedule shown in tables 4.2 & 4.3. Feeding of the molasses, ammonium and addition of antifoam and acid was by means of peristaltic pumps, activation of which was controlled by PCP (Fig. 4.1). All fermentations were carried out at 32°C over a period of 16h with a 30min. maturation phase. Agitation was maintained at 700rpm and aeration at 20L/min., pH was monitored by an autoclavable glass electrode connected to the pH controller unit. Sterilisation of the fermenter was carried out 'in-situ' at 100°C/1h and between fermentations a caustic 'clean-in-place' (CIP) was performed.

Samples were taken at time zero and at the end of the fermentation. The final total fermentation volume was measured and yeast cells were harvested by centrifugation at 7000rpm for 5min. (Beckman Induction Drive Centrifuge, J2-21M: rotor JA10). The pellets were then washed three times with water, resuspended and pooled, then vacuum filtered on No.4 filter discs (Whatman) and pressed to >30% solids, using a pneumatic press. Yeast cakes were stored at 4°C for further analysis.

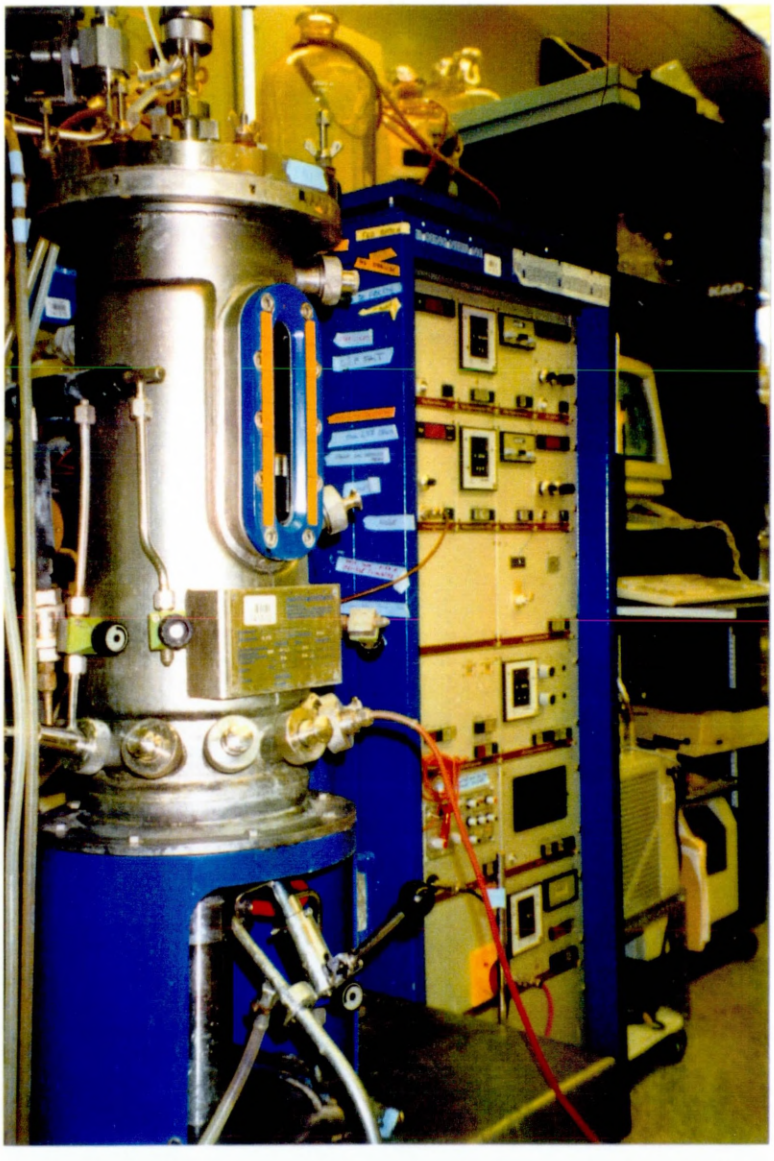


Plate 4.1 Lab scale computer-controlled fermenter (20L: Bioengineering AG.)

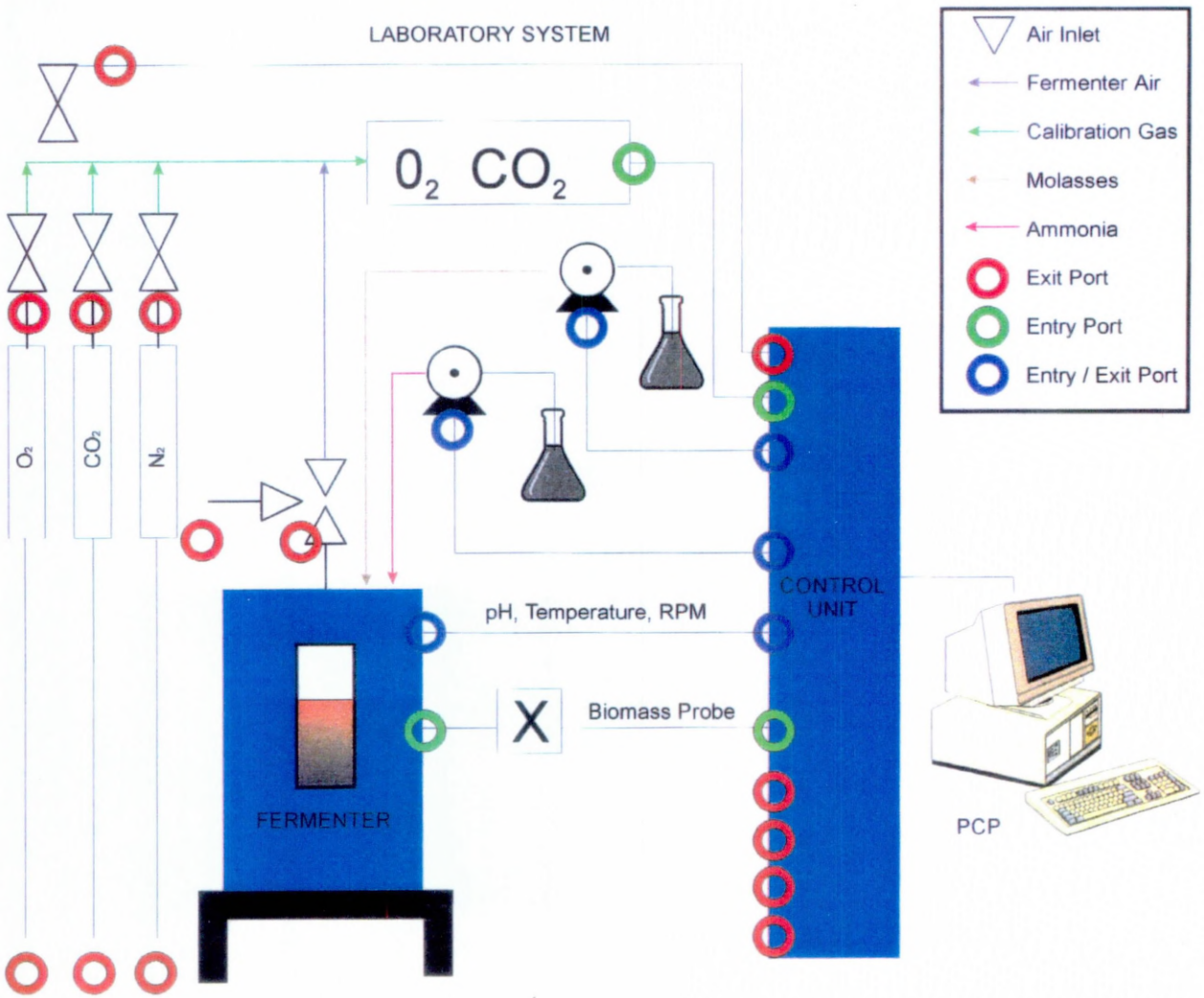


Figure 4.1 Fermentation control by PCP.

Table 4.2 Conditions of fermentation (Recipe TLB 1089).

Parameters		TLB 1089			Fermentation Sheet
Recipe		LB1089	**Volumes**	Inoculum (g)	261.076
Total Time	(min)	960.0		Total Sugar (g)	923.647
Reduction Time	(min)	.0		Molasses (g)	3638.093
Maturation Time	(min)	30.0		Molasses (ml)	3063.318
Ammonia Leakage Time	(min)	900.0		Ammonia (ml)	434.535
Phosphorus Leakage Time	(min)	60.0		Phosphorus (ml)	13.600
QT Yeast	(g @ 30)	1697		Process Water (ml)	5310.974
Sugar (Start)	(pc)	.0		Air Max. (L/min)	19.2
Ammonia (Start)	(pc)	.0		Total Liquid (L)	9.21
No. Doublages		6.50			
Protein	(pc)	53.0	**Time**	Exponential Phase (min)	298.7
Ammonia Assimilated	(pc)	84.5		Plateau Phase (min)	631.2
Phosphorus	(pc)	2.800		Reduction Phase (min)	.0
Phosphorus Assimilated	(pc)	100.0		Maturation Phase (min)	30.0
Concentration	(pc)	5.30		Total Time (min)	960.0
Estimated Yield	(pc)	74.00			
Rate of Growth		.170	**Constants**	Sugar 42.5 (pc)	26.0
Variation of Growth Rate	(pc)	.0		Brix Molasses (pc)	41.5
Air Required	(va/vl/m)	2.00		Beet/Cane (pc)	60.0
Temperature	(^{o}C)	35.0		Ammonia (pc)	10.0
				Balling (pc)	8.0

4.2.6 Analysis of fermentation samples

Cell density

Dry weight of the fermentation broth was analysed, as were cell counts of both initial and final samples. Percentages of budding and dead cells were calculated to give an indication of the health of the culture. A 15ml sample of the fermentation broth was removed for centrifuging (t=0, t=f) and the volume of yeast pellet was recorded, as an estimation of growth.

Quality control

Routine analysis for total bacterial count, coliforms, *Leuconostoc* spp. and for the incidence of wild yeast was carried out, as an indicator of levels of contamination of the fermentation. Samples were also removed for the analysis of protein (total nitrogen) and phosphates.

Formol test

15ml formaldehyde (37%), pH8.6 (pH adjusted with 0.2N NaOH), was added to 100ml fermentation broth, also adjusted to pH8.6 with 0.2N NaOH. Once stabilised, the pH of

the mixture was brought back up to pH 8.6 with NaOH and the volume of 0.2N NaOH added by burette was noted. Calculation of the level of formol was carried out using the equation:

(Volume of NaOH added) x 0.2 = Formol level

(An acceptable formol level was considered to be between 0.3 and 2.0 units).

Table 4.3 Feeding programme: PCP TLB 1089.

Fermentation Sheet				TLB1089					
Time	Air	Amm.	Phos.	Molasses	Cumm.Mol	Cumm.Amm	Centrif	Yeast	Yield
hh:mm	(ml)	(Kl/min)	(Hl/min)	(ml/min)	(ml)	(ml)	.(cc)	(g @30)	(g/g/h)
00:00	11468	2.321	2.079	1.57	.00	.00	7.9	261.07	.0000
00:30	11596	2.527	2.264	1.71	49.31	7.27	8.5	284.23	.0000
01:00	11735	2.752	2.464	1.87	103.00	15.18	9.1	309.45	1.1853
01:30	11872	2.996	.000	2.03	161.45	23.80	9.8	336.90	1.1853
02:00	12020	3.262	.000	2.21	225.09	33.18	10.6	366.79	1.1853
02:30	12181	3.551	.000	2.41	294.37	43.39	11.4	399.34	1.1853
03:00	12357	3.866	.000	2.62	369.80	54.52	12.2	434.76	1.1853
03:30	12548	4.209	.000	2.86	451.93	66.62	13.1	473.34	1.1853
04:00	12757	4.583	.000	3.11	541.33	79.80	14.0	515.33	1.1853
04:30	12983	4.989	.000	3.39	638.67	94.16	14.9	561.05	1.1853
05:00	13230	5.412	.000	3.67	744.64	109.78	16.0	610.82	1.1853
05:30	13487	5.412	.000	3.67	854.78	126.02	17.0	662.55	1.1809
06:00	13744	5.412	.000	3.67	964.92	142.25	17.9	714.28	1.1693
06:30	14000	5.412	.000	3.67	1075.06	158.49	18.9	766.01	1.1561
07:00	14257	5.412	.000	3.67	1185.19	174.73	19.8	817.74	1.1448
07:30	14513	5.412	.000	3.67	1295.33	190.97	20.6	869.47	1.1350
08:00	14770	5.412	.000	3.67	1405.47	207.20	21.5	921.20	1.1265
08:30	15027	5.412	.000	3.67	1515.61	223.44	22.3	972.93	1.1189
09:00	15283	5.412	.000	3.67	1625.75	239.68	23.0	1024.66	1.1123
09:30	15540	5.412	.000	3.67	1735.89	255.92	23.8	1076.39	1.1063
10:00	15797	5.412	.000	3.67	1846.02	272.15	24.5	1128.12	1.1009
10:30	16053	5.412	.000	3.67	1956.16	288.39	25.2	1179.85	1.0961
11:00	16310	5.412	.000	3.67	2066.30	304.63	25.9	1231.58	1.0917
11:30	16566	5.412	.000	3.67	2176.44	320.87	26.5	1283.31	1.0876
12:00	16823	5.412	.000	3.67	2286.58	337.10	27.2	1335.04	1.0840
12:30	17080	5.412	.000	3.67	2396.72	353.34	27.8	1386.77	1.0806
13:00	17336	5.412	.000	3.67	2506.85	369.58	28.4	1438.50	1.0774
13:30	17593	5.412	.000	3.67	2616.99	385.82	28.9	1490.23	1.0746
14:00	17850	5.412	.000	3.67	2727.13	402.06	29.5	1541.96	1.0719
14:30	18106	5.412	.000	3.67	2837.27	418.29	30.0	1593.69	1.0694
15:00	18363	5.412	.000	3.67	2947.41	434.53	30.6	1645.42	1.0670
15:30	18587	.000	.000	3.67	3057.54	434.53	31.1	1697.15	1.0649
16:00	-000	.000	.000	3.67	-.	434.53	-.	-.	-.

Abbreviations: Amm. = Ammonia; Phos. = Phosphorus; Cumm.Mol = Cummulative Molasses; Cumm.Amm. = Cummulative Ammonia; Centrif. = Centrifuge Test.

Fermentation calculations

Total and net dry weights of yeast produced during fermentation were calculated, as were the 30% solids total and net and these, along with the weight of molasses utilised throughout the fermentation were used to calculated the percentage yield. These calculations give a reflection of the success of the fermentation in terms of the production of yeast and therefore the suitability of the conditions imposed.

Analysis of compressed yeast samples

Prepared yeast cakes were analysed for the level of 'gritty' yeast using the protocol for the dissolution test, described earlier. Further analysis included metal ion determination by AAS and protein analysis by SDS-PAGE.

4.2.7 Yeast cell disruption

Harvested cells were washed twice with 1M TRIS buffer, pH 7.0, then resuspended into one volume of Glass Bead Disruption Buffer (Protease Inhibitor Cocktail Kit, ICN Biomedicals) and mixed with a volume of acid-washed glass beads (0.45μm diameter, Sigma). Disruption was carried out by initially vortexing to ensure no air bubbles were trapped, then homogenising for 5 min. at 4°C, using a Mini-BeadBeater (Biospec Products). Glass beads were allowed to settle out and the supernatant decanted into a fresh eppendorf tube. Two volumes of disruption buffer were added to the glass beads and mixed by inversion to wash beads, the washings were pooled with the initial supernatant. The broken cell preparation (supernatant) was centrifuged at 13000rpm for 5min. at 4°C (Eppendorf Centrifuge 5413) and sections separated:

Cloudy supernatant: cytoplasmic proteins

Pellet: cell walls and membranous debris

(Cell breakage was assessed microscopically)

4.2.8 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein analysis by SDS-PAGE was performed according to Laemmli (1970), using a 10% single concentration resolving gel with a 4% stacking gel. SDS-PAGE was conducted on

cytoplasmic extractions of whole cells and on cell wall extracts. All fractions were vortexed with an equal volume of 2X sample buffer (5ml stacking gel buffer (0.5M TRIS, pH6.8), 8ml 10% SDS, 4ml glycerol, 2ml β-mercaptoethanol, 500ml bromophenol blue, ddH_2O to a final volume of 20ml) at 20°C, before analysis. Electrophoresis was performed on minigels (Mini Protean II, Biorad Laboratories, CA. USA) at 100mV for 1-2h at room temperature (EPS 500/400; Pharmacia). A marker protein (SDS-PAGE Standard: Broad range, Biorad. Table 4.4) was included on all gels as a molecular weight standard. Gels were colourised by silver staining (Silver Stain Plus, Biorad). All stages of colourisation were carried out at room temperature on a rotary shaker (Gyrotory Shaker model G2; New Brunswick Scientific, NJ. USA). Gels were air dried between sheets of pre-wetted cellophane, held in a gel clamp (Biodesign NY. USA) overnight. (Water used in all stages of protein analysis was ddH_2O).

Table 4.4. SDS-PAGE Molecular weight standard: Broad range (Biorad)

Protein	Molecular Weight (d)
Myosin	205000
β-galactosidase	116500
Bovine Serum Albumin	80000
Ovalbumin	49500
Carbonic Anhydrase	32500
Soyabean Trypsin Inhibitor	27500
Lysozyme	18500
Aprotin	6500

4.3 RESULTS AND DISCUSSION

4.3.1 IONIC AND MOLECULAR COMPARISON OF 'GRITTY' AND NON-'GRITTY' YEASTS.

Grittiness has been a problem in North American yeast production plants for quite some years now (Mann 1985; Veilleux 1995). It is an idiosyncratic problem and can be detrimental to baker's yeast quality, therefore having important economic consequences. Work has been done by several individuals over a number of years (Mann 1985; Veilleux 1995; Dumont 1995), to look at an array of factors and possible solutions to the problem without, however, a great deal of success in gaining a definite understanding of this conflicting problem, in terms of cause or cure.

The aim of these experiments was to assess the ionic and molecular differences, if any, between 'gritty' and non-'gritty' yeasts.

4.3.1.1 EXPERIMENTAL PROCEDURE

Yeast

Experiments were carried out using fresh liquid cream yeast and pressed yeast of both 'gritty' and non-'gritty' nature.

Metal ion analysis

Whole cell samples of "gritty" and non-'gritty' cream yeasts, and the dissolved and grit portions observed following a dissolution test, were analysed by atomic absorption spectroscopy (AAS) for the metals; magnesium and calcium. Cell wall fractions isolated following yeast cell disruption were also analysed. Prior to analysis by AAS, cell pellets

(whole cell or cell wall fragments) were digested by an acid hydrolysis process using nitric and sulphuric acids (Chapter 2).

Protein analysis

SDS-PAGE was conducted on cytoplasmic extractions of whole cells and on cell wall extracts of 'gritty' and non-'gritty'. cream yeast.

4.3.1.2 RESULTS AND DISCUSSION

The ability of yeast to agglomerate is known to be strain related. The fact that this phenomenon is strain related does not however, explain the reasons behind why a yeast agglomerates or the mechanisms which cause it. Similar to the phenomenon of flocculation, metal ions are thought to be involved in both a non-specific and specific component of the mechanisms of yeast agglomeration (Guinard & Lewis 1993). This involvement is based either on the effects of surface charge of individual yeast or a specific protein activation step, therefore cellular and cell wall levels of these ions play an important role in the occurrence of grit.

The results in Table 4.5 show that calcium levels in whole cell samples of the 'gritty' (7258) and non-'gritty' (1619) cream yeast were very similar, however the total cell levels of magnesium are significantly higher in the non-'gritty' cream yeast. Cell wall levels of magnesium and calcium in the cream yeast are interesting; in the 'gritty' strain, magnesium levels are much lower than the non-'gritty' strain. These results tend to suggest that higher cellular levels of magnesium in the non-'gritty' cream yeast exhibit a protection against the calcium activation of proteins suggested to be involved in agglomeration. High levels of magnesium observed in the cell wall samples suggest that these ions are occupying surface metal ion binding sites, hence saturating the calcium-specific binding sites of the cells, preventing calcium binding and blocking activation of proteins implicated in agglomeration (Guinard & Lewis 1993) therefore reducing grit formation. In 'gritty' yeast the total cell

magnesium levels are lower and calcium levels higher in the dissolved section, with cell wall levels being much lower, but on the same level.

Table 4.5 Comparison of magnesium and calcium levels of total cell and cell wall samples of cream yeast and dissolved and 'gritty' sections of 'gritty' and non-'gritty' strains of *Saccharomyces cerevisiae*.

	Magnesium (ppm)		Calcium (ppm)	
Condition	'gritty'	Non-'gritty'	'gritty'	Non-'gritty'
Cream yeast				
Total cell	355.0	488.4	49.2	47.7
Cell wall	22.3	65.2	11.4	15.8
Dissolved section				
Total cell	164.7	417.8	58.6	38.5
Cell wall	33.9	84.0	5.0	5.9
Grit section				
Total cell	227.1	-	37.8	-
Cell wall	30.8	-	4.4	-

Overall it appears that calcium levels are slightly higher in 'gritty' than non-'gritty' yeast, giving some support to the claim that (as for flocculation) calcium ions are involved in the mechanism of agglomeration. Magnesium levels on the other hand, in both cream yeast and dissolved sections are higher in non-'gritty' strains than 'gritty', suggesting a protective effect of magnesium in non-'gritty' yeast. The presence of this ion acts in an antagonistic manner towards the calcium ions present in the surrounding media, binding to sites on the cell surface, surrounding the cells and forming a barrier to calcium ions therefore inhibiting calcium activation of saccharide binding proteins involved in agglomeration.

The close link between metal ions and proteins seems to be the best explanation for the mechanisms of agglomeration. It can be seen that protein levels in 'gritty' yeast are higher, 53.98% compared to 44.97% (Table 4.6), with higher protein levels and also higher cell calcium levels in 'gritty' yeast, this presents some evidence that both calcium ions and cell proteins are involved, in conjunction, in this problem.

Table 4.6 Comparison of protein and phosphate levels between a 'gritty' and a non-'gritty' strain of *Saccharomyces cerevisiae*.

Condition	'gritty'	Non-'gritty'
Dissolution (ml)	51	2
Protein (%)	53.98	44.97
Phosphate (%)	3.12	2.96

In an attempt to assess the molecular aspects of agglomeration *i.e.* identifying the proteins which could possibly be involved, SDS-PAGE protein gels were performed on both cellular and cell wall extracts of the 'gritty' and non-'gritty' strains. Protein profiles of cellular extracts (Figure 4.2) shows that the profiles of cream yeast and the grit and dissolved sections of pressed yeast are similar for each strain type. One can conclude, therefore, that the formation of grit is not due to the cells which actually agglomerate being different, in respect to their cellular make-up. In comparison of 'gritty' and non-'gritty' strain patterns, it can be seen that these profiles differ in terms of three bands which are absent in the non-'gritty' strain. This confirms the results of Guinard & Lewis (1993) who also observed three extra bands: α, β and γ, present in a 'gritty' strain. These bands, as the ones observed here were of the following molecular weights: α 45000, β 35150 and γ 33400 daltons. The 'gritty' cream yeast definitely shows the β and γ bands, whereas the profiles of dissolved and grit sections of this strain exhibit all three bands, although these are fainter in the 'gritty' section. In general, the colourisation of the gel is faint and therefore harder to read and ideally more work would be required to be done on the molecular aspects, to fully elucidate the involvement of these proteins. Since the gel of cell wall extracts is unreadable (due to a high level of cell wall material in the loaded extract, which obscured the protein patterns present), it is unknown whether these proteins are specifically cell wall proteins, however this idea can not be excluded since polypeptides covalently bound at the cell surface could be released during the extraction process. A close correlation between agglomeration and the existence of these three proteins was observed in this experiment, further implementing proteins in agglomeration and the results of the ionic study do not rule out a metal ion involvement, also.

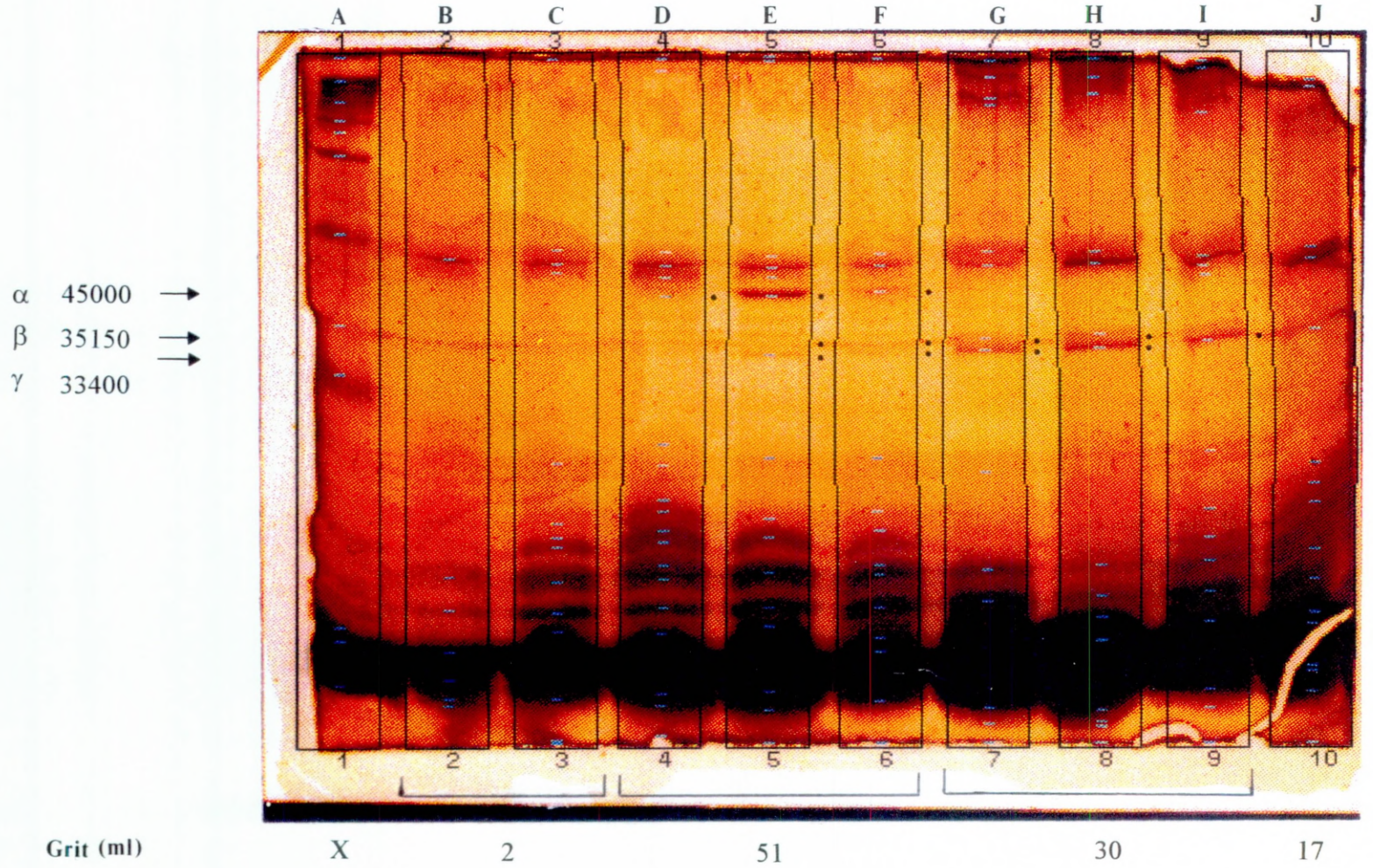


Figure 4.2 Molecular comparison of 'gritty' (7258/ 29953016M) and non-'gritty' (1619) strains of the yeast *Saccharomyces cerevisiae*. Lanes: A, Marker (Biorad); B, Cream (1619); C, Cake (1619): Dissolved section; D, Cream (7258); E, Cake (7258): Dissolved section; F, Cake (7258): Grit section; G, Cream (29953016M); H, Cake (29953016M): Dissolved section; I, Cake (29953016M): Grit section; J, 0.01-0.02U Papain (29953016M/ Cream).

4.3.2 INVESTIGATION INTO THE ROLE OF DIVALENT CATIONS IN THE OCCURRENCE OF YEAST AGGLOMERATION.

The problem of grit is affected by environmental factors during growth and appears also to be affected by fermenter design (Veilleux 1995), since varying amounts of grit can be detected in fermentations using the same strain; same source of molasses and; same or similar running conditions, in different fermenters. Environmental factors in the media are subject to some degree of control by alteration of the nutritional content of the feed, be it organically or inorganically. Since divalent cations play a large part in other types of cell adhesion e.g. flocculation (Speers *et al.* 1992), it is thought that certain ions could also play a role in the formation of grit. The aim of these experiments, therefore, was to produce baker's yeast in a lab scale fermenter (10L) under conditions of altered magnesium and calcium ion ratios, observing the effect of inorganic nutrition on the problem of agglomeration.

4.3.2.1 EXPERIMENTAL PROCEDURE

Yeast

Strains of *Sacch. cerevisiae* used and sources were as indicated earlier.

Fermentations

Alterations to the metal ion content of the final medium were carried out depending on the experiment being carried out (Table 4.1). Samples were taken at time zero and at the end of the fermentation. The final total fermentation volume was measured and yeast cakes were prepared (>30% solids) and stored at 4°C for further analysis.

Analysis of fermentation samples

Samples were analysed for cell density, contamination, formol, yield, protein and phosphates. Prepared yeast cakes were analysed for the level of 'gritty' yeast using the protocol described earlier. Further analysis included metal ion determination by AAS and protein analysis by SDS-PAGE (methodologies described earlier).

Metal ion analysis

Magnesium and calcium levels were analysed for in samples of fermentation media, extra- and intra-cellular samples from fermentations (time zero and final) and dissolved and grit portions (following a dissolution test) of yeast cakes prepared from the fermentations. Media and extra-cellular samples were supplied in liquid form and following the appropriated dilution were analysed directly. All other samples were supplied in pellet form and required digestion by acid hydrolysis prior to dilution and analysis by AAS (Chapter 2).

Protein Analysis

Protein patterns of cytoplasmic extractions of whole cells and on cell wall extracts of final fermentation samples, were observed.

4.3.2.2 RESULTS AND DISCUSSION

The ratios implied on the fermentations by the addition of magnesium or calcium were found to be different than expected, due to the fact that the theoretical levels were taken from the literature (Walker *et al.* 1994) and all sources of molasses vary greatly in their concentrations of metal ions since they are largely composed of complex plant materials. The resulting ratios can be seen in Table 4.7.

Table 4.7 Actual magnesium and calcium ratios imposed on fermentations by additions to molasses feed.

Code	Fermentation	Molasses					
		Added Magnesium (g/l)	Added Calcium (g/l)	Expected ratio Mg:Ca	Actual Magnesium (ppm)	Actual Calcium (ppm)	Actual ratio Mg:Ca
172*	Control	-	-	0.14:1	1375.8	1631.9	0.84:1
183§	Control	-	-	0.14:1	1375.8	1631.9	0.84:1
182	-Ca	-	Acidify	-	1625.4	1706.0	0.95:1
177	+Ca	-	15	0.047:1	1401.5	2985.1	0.47:1
178	Mg x5	3.5	-	0.7:1	1645.4	1452.5	1.13:1
179	Mg x10	7	-	1.4:1	2046.7	1503.7	1.36:1
180	Mg x15	10.5	-	2.1:1	2269.3	1592.4	1.42:1
181	Mg x20	14	-	2.8:1	2460.7	1513.4	1.62:1

* Control (non-'gritty')
§ Control ('gritty')

Ionic influences on the 'gritty' phenomenon had been analysed in cream yeast, but since the aim of this work was to assess what was occurring in the production of these yeast, with a view to suggesting some solution to be implemented into full scale industrial production, the phenomenon was observed in fermentations. Expression of the 'gritty' phenotype on a lab scale was assessed by altering Mg:Ca ratios. Table 4.8 shows the general fermentation characteristics resulting under the conditions of altered ion levels. In terms of basic fermentation parameters, growth was promoted in fermentations containing either higher magnesium levels; ratios 0.84-1.62:1 Mg:Ca, or sulphuric acid treated molasses (which resulted in a Mg:Ca ratio of 0.95:1). Although growth was not inhibited by increased calcium levels, the fermentation with the ratio of 0.47:1 resulted in lower yeast production suggesting these conditions were not optimum, percent yield values agree with this finding. Protein levels did not vary over much between fermentations although in general the levels were higher in fermentations with a 'gritty' strain, than that of the non-'gritty' control. Phosphate levels did not alter greatly and were essentially on a par with all the fermentations carried out.

Grit levels varied depending on the conditions imposed by the medium (Table 4.8). Increasing calcium levels in fermentation medium appears in this experiment to increase grit levels by 16.67% , this being the worse scenario. This result supports the suggestions that

calcium is involved in yeast agglomeration. The only other fermentation which gave an increase in grit formation was that of 1.62:1 Mg:Ca, an increase in grit formation of 6.67% can be explained by the non-specific involvement of cations in agglomeration. Stratford and Brundish (1990) state that in flocculation salts at low concentrations are essential for flocculation to occur, at moderate concentrations salts enhance flocculation and at high concentrations they cause reversible inhibition of flocculation. There is no reason to doubt this explanation could also be true of agglomeration and magnesium levels of 2461ppm in molasses, would therefore be acting at a concentration sufficient enough to promote agglomeration, possibly by overcoming cell surface charge and therefore allowing cells to come close enough to adhere.

Table 4.8 Fermentation characteristics under conditions of altered ion levels.

Fermentation	Control	Control ('gritty')	Sulphuric acid	Ca x3	Mg x5	Mg x10	Mg x15	Mg x20
Code	172	183	182	177	178	179	180	181
Centrifuge (ml)								
initial	8	7	7.5	6	7	7.5	6	8
final	23	20	25	21	23	24	23	25
Viability (%)	100	98.4	100	99.5	99.03	100	100	100
Formol (units)	0.5	0.7	0.46	0.42	0.22	0.42	0.5	0.44
Protein (%)	21.09	23.28	28.48	17.57	22.12	22.37	nt	28.95
Phosphate (%)	1.72	1.73	1.71	1.43	1.56	1.50	nt	1.76
Yield (%)	33.10	54.78	61.47	44.48	56.22	80.02	52.02	76.06
Grit (ml)	0	30	25	35	19	12	22	32

(nt = not tested)

Previous work in increasing magnesium levels in fermentations at 0.5x, 2x and 3x normal levels (Veilluex 1995) had no significant effect on grit. In this study levels of magnesium were increased to greater levels, the effects of 5x, 10x and 15x was to reduce the resultant levels of grit by 37%, 60% and 27% respectively, this would suggest that for 15x normal levels the level of magnesium salts was of reduced benefit. 5x and 10x increases of magnesium with a resultant Mg:Ca ratio of 1.13:1 and 1.36:1, compared to 0.84:1 of the control are more in keeping with the expected positive effect of magnesium in reducing grit, 37% and 60% reduction respectively. Comparing this to the results of Veilluex (1995), it suggests that useful levels for the reduction of grit are levels between 1.13:1 and 1.36:1,

Mg:Ca. An explanation for this inhibition of agglomeration by magnesium may be as that suggested earlier when discussing cream yeast, magnesium ions binding to the available sites therefore reducing the available sites for calcium-specific binding and reducing activation of proteins suggested to be responsible for agglomeration. Table 4.9 shows intra- and extra-cellular magnesium levels of the fermentations, final inter-cellular levels only appear to be significantly higher than initial levels in fermentations with increased magnesium levels; this can be simply explained by the fact that yeast cells will increase their uptake of magnesium ions if they are grown in an excess of magnesium. Extra-cellular levels are obviously very different since at final time all the molasses feed has been added to the medium. Total cell dissolved and grit levels were observed to be essentially similar with no significant differences between the cellular magnesium ion levels in grit or dissolved sections.

Table 4.9 Intra- and Extra-cellular magnesium levels in fermentations of altered magnesium: calcium ratios.

Fermentation	Magnesium (ppm)							
	Control	Control ('gritty')	H_2SO_4	Ca x3	Mg x5	Mg x10	Mg x15	Mg x20
Molasses	1375.8	1375.8	1625.4	1401.5	1645.4	2046.7	2269.3	2460.7
Intracellular t=0	312.1	317.5	313.4	323.0	262.9	312.4	250.6	274.1
Extracellular t=0	51.8	50.6	69.5	49.0	61.9	70.8	48.7	65.1
Intracellular t=f	356.0	335.5	381.0	332.6	359.4	402.5	425.0	388.9
Extracellular t=f	599.9	669.9	706.0	583.0	756.4	937.3	914.0	1086.4
Dissolved: Total cell	239.2	325.9	339.3	304.0	331.1	375.4	383.6	282.5
Grit: Total cell	268.4	298.8	340.3	313.4	327.0	365.4	389.0	312.9

(t=0: initial time; t=f: final time)

Calcium levels are shown in Table 4.10, as with the magnesium levels, initial levels are essentially similar. Final intra-cellular levels of controls and magnesium-supplemented fermentations are on a similar level, however 15x and 20x magnesium fermentations have much lower levels of calcium. This can be explained by the excessive availability of

magnesium ions acting antagonistically against calcium ions. Intracellular levels of yeast grown in sulphuric acid treated molasses are also reduced, despite the media levels being high, suggesting the availability is reduced and most of the calcium ions are complexed as $CaSO_4$ salts, therefore being unavailable to cells. Dissolved and grit sections of pressed yeast do not show any significant difference between levels in yeast which make up the dissolved section or those which make up the grit section, a point previously observed.

Table 4.10 Intra- and Extra-cellular calcium levels in fermentations of altered magnesium: calcium ratios.

Fermentation	Calcium (ppm)							
	Control	Control ('gritty')	H_2SO_4	Ca x3	Mg x5	Mg x10	Mg x15	Mg x20
Molasses	1631.9	1631.9	1706.0	2985.1	1452.5	1503.7	1592.4	1513.4
Intracellular t=0	10.2	10.0	11.4	10.0	6.5	12.1	5.0	6.0
Extracellular t=0	10.6	10.5	10.7	16.0	12.2	12.2	8.4	10.7
Intracellular t=f	57.3	58.9	43.0	219.2	60.5	73.0	32.8	32.9
Extracellular t=f	646.5	743.4	744.0	1200	593.2	584.1	509.8	562.3
Dissolved: Total cell	37.6	45.1	35.1	42.1	35.2	33.2	30.0	27.5
Grit: Total cell	39.4	43.6	41.6	44.0	36.3	35.9	31.2	27.9

(t=0: initial time; t=f: final time)

Levels of calcium are higher in control ('gritty') and 3x Ca fermentations than the non-'gritty' control, backing up results of previous experiments with cream yeast. Altering the magnesium levels of fermentation media has a resulting reducing effect on levels of total cell calcium, with an increase of molasses magnesium correlating to the reduction of calcium levels in compressed yeast. Overall, the increase of calcium levels in molasses causes a corresponding increase in grit formation.

A brief analysis of protein profiles of the resulting cream yeast produced from the fermentations, was carried out as a small addition to this study (Figure 4.3). Despite the faint banding patterns it can be seen that in lanes 2 and 4-9 *i.e.* all fermentations with a

'gritty' yeast strain, exhibited an extra band which corresponds to the γ band, named by (Guinard & Lewis 1993) and observed in previous work carried out in this project. It is though that the other bands: α and β, are not visual partly due to the faintness of the gel, but unfortunately, only one attempt at protein analysis was possible, in the time available. These preliminary results indicate that 'gritty' strains of the yeast *Sacch. cerevisiae* posses extra protein bands not observed in a non-'gritty' strain and this supports the more conclusive results of the comparison of 'gritty' (7258) and non-'gritty' (1619) cream yeast, earlier in this study.

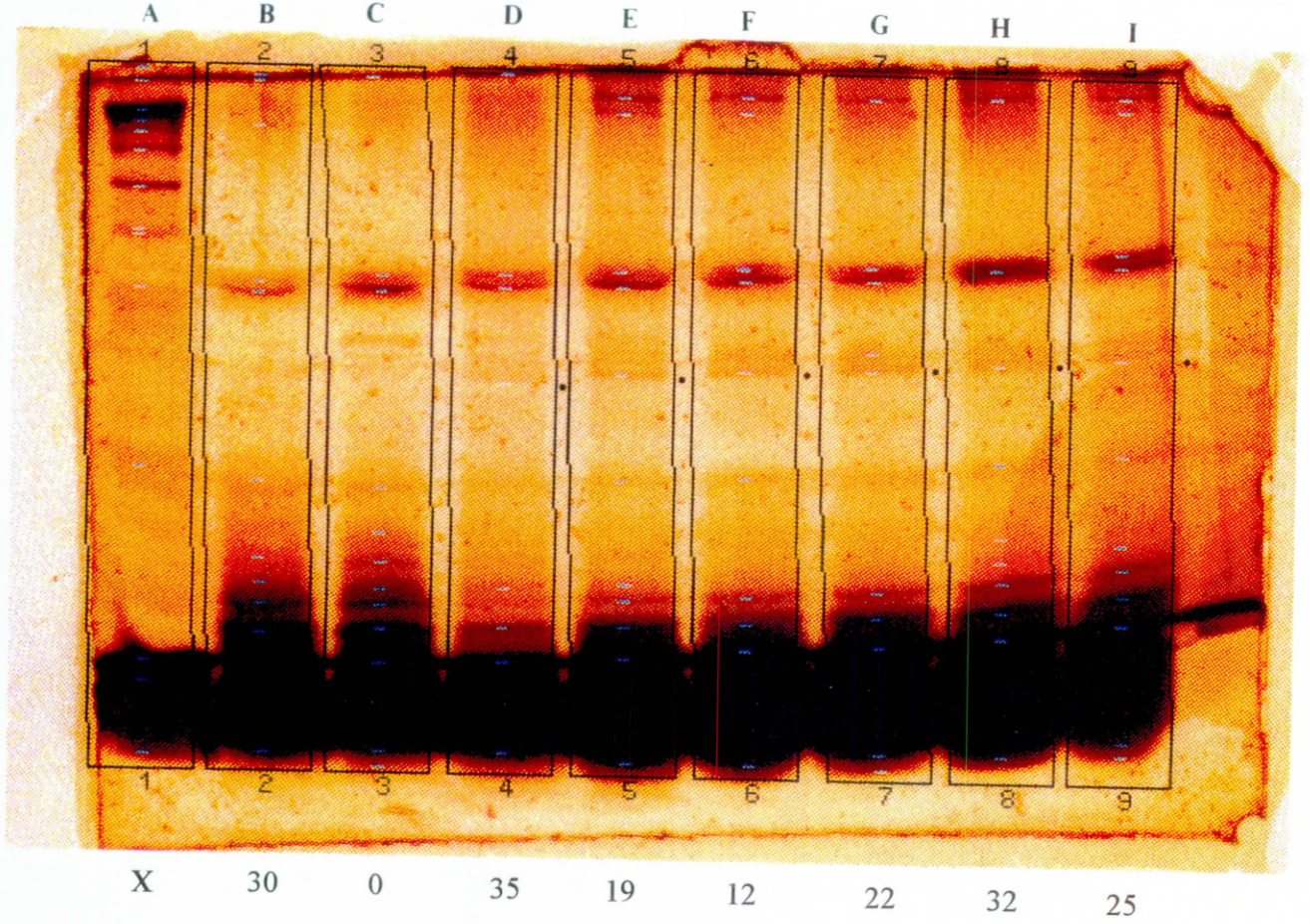


Figure 4.3 SDS-PAGE of whole cell extracts of fermentations 172-183. Lanes: A, Marker (Biorad); B, Control ('Gritty'); C, Control (Non-'gritty'); D-I, used 'gritty' yeast; D, Ca x3; E, Mg x5; F, Mg x10; G, Mg x15; H, Mg x20; I, Sulphuric acid treatment.

4.3.3 EFFECT OF THE REMOVAL OF CELL SURFACE METAL IONS ON YEAST AGGLOMERATION.

Agglomeration, similar to flocculation, is thought to involve cell surface interactions with divalent cations. Yeast cell walls are rich in mannan and glycoprotein matrices which have a myriad of binding sites involved in various factors of yeast growth. The activation of some of these sites occurs only following the binding of a cation, forming a metalloprotein complex, which alters the conformation of the protein. In the flocculation of yeast cells a lectin-like adhesion is suggested, involving Ca^{2+} activation of α-mannan which may then bind saccharide groups of adjacent cells. It is therefore hypothesised that the possibility exists of a protein-metal ion effect with regards to yeast agglomeration and in fact a significantly positive correlation between whole cell calcium and sedimentation value has previously been shown (Guinard & Lewis 1993).

The aim of this experiment was to assess the effect of the removal of cell surface metal ions on yeast grittiness using a variety of chelating agents.

4.3.3.1 EXPERIMENTAL PROCEDURE

Yeast strain

Liquid semi-seed (cream) yeast: *Saccharomyces cerevisiae*; Baker's strain C, (as described earlier) was used in this study.

Removal of cell surface metal ions in cream yeast

200ml volumes of a 'gritty' cream yeast (18% solids approx.) were centrifuged at 7000rpm for 5min. and the supernatant discarded. The yeast pellet was then resuspended in a 200ml volume of the appropriate concentration of chelator (Table 4.11), mixed well to ensure complete resuspension and left mixing for 20min., with a Teflon-coated magnetic stirrer.

The solution was then vacuum filtered through a No.4 filter disc (Whatman) and pressed to >30% solids. Resulting yeast cakes were bagged and incubated overnight at 30°C, prior to the analysis of the level of agglomeration by the method described earlier.

Table 4.11 Concentrations of chelators used to remove surface metal ions from yeast cells.

Concentration (M)	Chelator			
	EDTA	Sodium Citrate	Sodium Pyrophosphate	8-Hydroxyquinoline
0.01	-	x	x	-
0.025	x	x	x	x
0.05	x	x	x	x
0.10	x	x	x	x
0.25	x	-	-	x
0.50	x	-	-	x

x = Addition made; - = No addition made

Metal ion analysis

Whole cell samples of the dissolved and grit portions of pressed yeast, prepared from cream yeasts treated with the chelators; EDTA and sodium citrate, were analysed by AAS for magnesium and calcium. Samples were supplied in the form of pellets and therefore digestion was required prior to analysis. All AAS work was carried out by the methods described in Chapter 2.

4.3.3.2 RESULTS AND DISCUSSION

Since metal ions are implicated in the mechanisms of agglomeration (Guinard & Lewis 1993) and are heavily involved in flocculation (Kuriyama *et al.* 1991; Stratford & Brundish 1990), the concept of removing cell surface ions from yeast does not seem strange. The use of chelators on the reduction of grit formation, essentially by removing cell surface cations, had been previously attempted (Veilluex 1995; Dumont 1995). Therefore, in this study a wider range of chelators and concentrations was employed. Of the chelators studied sodium citrate and sodium pyrophosphate seemed to have a slightly positive effect on

reducing agglomeration, with a corresponding reduction in grit formation with increasing chelator concentration. Some concentrations of 8-Hydroxyquinoline reduced the levels of grit by the largest extent but the pattern of reduction with increasing concentration was observed to be too irrational and also this chemical did not have food safety clearance, so it was not further investigated (Table 4.12).

Table 4.12 Effect of chelators on grit formation in a commercial strain of *Saccharomyces cerevisiae*.

Chelator	Concentration (M)	Compressed Yeast		
		% Solids	Grit (ml)	Reduction in grit (%)
Control	-	33.1	33	-
EDTA*	0.025	32.0	19	42.4
	0.05	35.2	31	6.06
	0.10	36.9	33	0.00
	0.25	39.3	24	27.3
	0.50	44.0	27	18.0
8-Hydroxy-quinoline	0.025	32.3	29	12.1
	0.05	31.8	34	+3.03
	0.10	32.8	18	45.4
	0.25	35.6	13	60.6
	0.50	36.0	20	39.4
Sodium citrate*	0.01	32.3	32	3.03
	0.025	33.7	36	+9.09
	0.05	34.0	25	24.2
	0.10	36.1	25	24.2
Sodium pyrophosphate	0.01	30.6	30	9.09
	0.025	34.2	27	18.2
	0.05	35.5	25	24.2
	0.10	35.6	26	21.2

(* Metal ion analysis carried out)

The effects of EDTA and sodium citrate were further examined in terms of the corresponding magnesium and calcium levels in the dissolved and grit portions of pressed yeast, following treatment with the chelators. Ideally, if the chelator is doing its job, magnesium and/or calcium in yeast cells should be reduced, since both EDTA and sodium citrate have a specificity for divalent cations, particularly calcium and magnesium. The effects of EDTA on total cell levels of magnesium and calcium can be seen in Table 4.13. Calcium levels in both dissolved and grit portions, at varying concentrations are similar and

the increased concentration of EDTA reduces the levels of cellular calcium detected. Magnesium levels on the other hand, do not do not reduce correspondingly with increased EDTA concentrations, and levels are variable over the range of concentrations. This variability suggests that EDTA is not a good choice of chelator for the problem of yeast agglomeration.

Table 4.13 Magnesium and calcium levels in dissolved and grit sections of pressed yeast of a 'gritty' strain of *Sacch. cerevisiae,* following treatment with EDTA.

		Magnesium (ppm)		Calcium (ppm)	
Concentration (M)	Grit (ml)	Dissolved	Grit	Dissolved	Grit
0	33	247.0	289.8	35.0	33.0
0.025	19	270.8	282.2	13.8	13.2
0.05	31	283.1	239.3	10.0	9.5
0.10	33	290.3	290.9	10.5	10.0
0.25	24	310.8	301.7	7.8	9.1
0.50	27	229.2	275.0	8.2	7.4

Sodium citrate treated yeast was analysed for metal ion levels and it was observed (Table 4.14) that it possessed a higher specificity for calcium over magnesium (as does EDTA) with magnesium levels not being significantly different from the control, despite the increased chelator concentration. Calcium levels, however, were reduced slightly and this reduction in total cell calcium inversely corresponded to chelator concentration. The chelating capacity of sodium citrate is less than that of EDTA, but despite this calcium levels dropped and grit volumes were reduced correspondingly, with increased chelator concentration, which suggests that sodium citrate would be a better choice of chelator and supports again the claim that calcium is involved in this phenomenon.

Table 4.14 Magnesium and calcium levels in dissolved and grit sections of pressed yeast of a 'gritty' strain of *Sacch. cerevisiae,* following treatment with Sodium citrate.

		Magnesium (ppm)		Calcium (ppm)	
Concentration (M)	Grit (ml)	Dissolved	Grit	Dissolved	Grit
0	33	247.0	289.8	35.0	33.0
0.01	32	261.5	284.5	29.5	30.9
0.025	36	240.7	281.5	22.9	21.3
0.05	25	275.1	288.2	16.5	16.8
0.10	25	272.4	285.3	15.2	14.9

4.3.4 ROLE OF CELLULAR PROTEINS IN YEAST AGGLOMERATION.

Proteins have been proven to be involved in cell adhesion (Miki *et al.* 1982a; Trivedi *et al.* 1986; Nishihara & Toraya 1987). Flocculation can be irreversibly inhibited or reduced by treatment of yeast cells with mercaptoethanol or cysteine, chemicals which disrupt disulphide bonds. This loss of flocculent ability following treatment undeniably proves an involvement of cell-surface proteins in flocculation. Trevidi *et al.* (1986) states that flocculation can also be inhibited by the addition of proteinase enzymes. If this is the case for one type of cell adhesion with a protein involvement and a factor of protein involvement is suggested in agglomeration (Guinard & Lewis 1993), then it is possible that proteinase treatment could inhibit or reduce grit formation in a sensitive yeast strain.

These experiments were designed to assess the affect of the addition of a proteinase enzyme: papain on the resultant volume of grit formation, after compression of the yeast sample.

4.3.4.1 EXPERIMENTAL PROCEDURE

Yeast

Pressed yeast; *Sacch. cerevisiae*, Baker's strain C ('gritty'), from the sources described earlier, was dissolved to produce a cream yeast of approximately 18% solids, for use in this study.

Proteinase treatment of cream yeast

Range of concentrations of proteinase

A range of concentrations; 0.01-0.02U, 0.1-0.2U, 1.0-2.0U, 10-20U and 50-100U of papain were added to 200ml volumes of fresh 'gritty' cream yeast and the solutions mixed for 15min, using Teflon-coated magnetic stirrers. The solutions were then vacuum filtered

through No.4 filter paper (Whatman) and pressed to >30% solids, using a pneumatic press. Dissolution was assessed by the method previously described.

Timescale effect of proteinase

0.01-0.02U papain was added to 200ml volumes of fresh 'gritty' cream yeast and the solutions mixed for 5, 10, 15, 20 and 30 minutes respectively, using Teflon-coated magnetic stirrers. Following the appropriate time, the solutions were vacuum filtered through No.4 filter paper (Whatman) and pressed to >30% solids, using a pneumatic press. Dissolution was assessed by the method previously described. Prior to filtering, a sample was removed and viability analysis carried out using haemocytometer counts following methylene blue staining; percentage viability was calculated and recorded.

Papain Unit definition: One unit will hydrolyse 1.0μmole of BAEE per minute at pH6.2, 25°C.
(BAEE: Sodium-benzyoly-L-arginine Ethyl Ester).

4.3.4.2 RESULTS AND DISCUSSION

It has been reported that flocculation is irreversibly inhibited by the addition of proteinases. With this concept in mind and with the observation of the α, β, and γ proteins shown in protein profiles of only 'gritty' yeast, indicating a protein involvement in agglomeration, the aim of this study was to observe the effect of a range of concentrations of papain on the resulting grit formation.

Initially a range of papain concentrations were observed, Figure 4.4 represents graphically the reduction in volume of grit formation from compressed yeast samples of a 'gritty' strain of *Sacch. cerevisiae*. It is observed that a range of 0.1-10g/l papain included in the cream yeast for a short time before pressing did not give a corresponding range in the reduction of grit and a significant reduction in grit was observed even with the lowest concentration utilised. An excessive concentration of papain (50-100U) resulted in a 100% reduction in

grit, but it was suspected that this concentration may have detrimental effects on the yeast cells. From these results it was decided to observe if this effect of a proteinase enzyme, at the lowest concentration (0.01-0.02U) was specifically time related and if the action of the proteinase enzyme affected cell viability (Figure 4.5). The increasing time of exposure, correspondingly, resulted in an increased percentage reduction in levels of grit over a time period of 30 minutes and although the levels of reduction were slightly lower than the initial experiment (comparing results for the 15 min. exposure) this could be explained by the possibility that the activity of the stock solution could have been reduced by prolonged storage as a liquid stock. The fact that the study was carried out on a different source of cream yeast (with a correspondingly different level of grit) must also be considered in comparing these results.

Concern that the proteinase treatment would affect not only protein bonds between agglomerating cells, but may reduce cell viability due to the proteinase digesting important cellular proteins, prompted an assessment of the yeast cell viability. It was found (Figure 4.5) that over a period of 30 minutes, exposure to 0.01-0.02U papain in cream yeast did not adversely effect yeast viability, with viability levels being above 97.4%.

This effect of a proteinase enzyme further supports claims of a protein involvement in yeast agglomeration and if claims of the effect of viability could be assessed over a longer timescale, then this may be a simple solution to the problem.

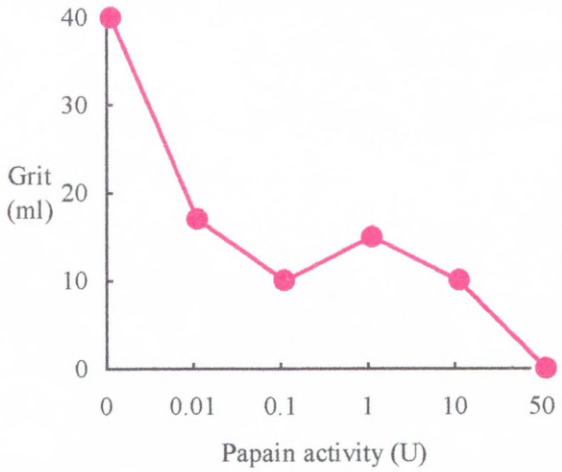


Figure 4.4 Effect of a range of concentrations of papain on grit formation in a strain of *Saccharomyces cerevisiae*. -●- grit.

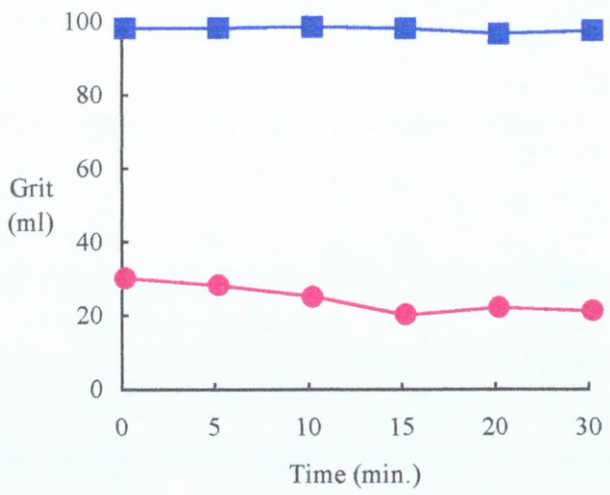


Figure 4.5 Effect of time of exposure of 0.01-0.02U papain on grit formation and percentage viability in a strain of *Saccharomyces cerevisiae*.
-●- grit (ml), -■- viability (%)

4.4 CONCLUSIONS

Yeast agglomeration is a contentious problem, its occurrence is dependent on a number of variables including strain, fermentation conditions, environment, medium and ion content thereof. No significant difference in magnesium or calcium levels was observed in the 'gritty' and dissolved sections of pressed yeast following a dissolution test, suggesting that the problem is one of the whole yeast population and not that there are two types of yeast cells, which would accordingly separate into a particular section. Overall the levels of magnesium were found to be higher in non-'gritty' cream yeast and the levels of calcium higher in 'gritty' strains, suggesting an involvement of calcium in agglomeration. If, genetically or otherwise, 'gritty' strains are able to bind higher amounts of calcium, then the activation of calcium-specific proteins implicated in agglomeration would be of a higher level and therefore a greater number of cells would agglomerate.

It was observed that increasing the magnesium content of the molasses feed by 5x and 10x (1.13:1- 1.36:1 Mg:Ca ratio) reduced the levels of grit by 36-60% respectively, suggesting that there is an antagonistic action of magnesium against calcium; the magnesium saturating the cell surface binding sites, hence blocking calcium binding. Increasing magnesium levels in the media too much has a detrimental effect on the population, in that it promotes grit formation due of the non-specific action of a large number of cations reducing the net negative charge and permitting cells to come closer together and bind more easily. The increase of calcium levels of molasses increased grit formation substantially and the comparison of all these results, appears to substantiate the claim that calcium is an important factor in promoting agglomeration.

In an attempt to solve the problem from this angle, the effect of chelators was assessed and although the effect was not outstanding, with EDTA giving variable results, sodium citrate seemed to be a possible solution to at least reducing the levels of grit, since it was observed with this chelator that an increase in concentration resulted in a corresponding decrease in

grit. The results also indicated that calcium was the major ionic cofactor in agglomeration, since sodium citrate has a higher specificity for calcium ions and reduced cellular calcium levels corresponded with reduced grit.

In general, the results of this study indicated an ionic influence on agglomeration. However, this is not the single cause of the phenomenon and a study of the protein profiles of 'gritty' and non-'gritty' yeast gave support to the suggested mechanisms of agglomeration (Guinard & Lewis 1993). SDS-PAGE gels indicated that 'gritty' and non-'gritty' strains of the yeast *Sacch. cerevisiae* differed in their protein profile by three bands; α, β and γ of 45000, 35150 and 33400 MW, only found in the 'gritty' strains. The results were most apparent in the study of a 'gritty' (7258) and non-'gritty' (1619) strain, but profiles of cream yeast produced from fermentations inoculated with a 'gritty' strain exhibited at least the γ band (gel was too faint to identify α and β bands). It is not proven if these proteins were located on the cell surface, since the gels of cell wall extracts were unreadable, but this cannot be discounted since the extraction method used may release polypeptides covalently bound on the cell surface, including these in the whole cell extract. The presence of these extra bands in "gritty' yeast and the inhibiting effect of proteinase treatment of yeast cells, indicates that there is a protein involvement in agglomeration.

It would appear therefore the best explanation for this phenomenon would be something similar to those suggested for flocculation (Miki *et al.* 1982a, 1982b; Kihn *et al.* 1988), where the effect is due to a combination of an ionic and a protein involvement, initiating binding of adjacent cells. It would appear that metal ions in moderate concentrations promote agglomeration by overcoming the cell surface negative charge between cells. A more important mechanism suggested, is the specific interaction of calcium ions in the activation of proteins involved in binding components of adjacent cells.

Further work would be required to be done on the molecular aspect of this phenomenon, to fully understand the proteins which appear to be involved. Nevertheless, from this work, two possible solutions to at least reducing the problem of grit would be; the increase of

levels of magnesium in molasses to around 1.13-1.36:1 Mg:Ca ratio (5x -10x normal) for the fermentations, an action which theoretically (based on these results) would reduce grit by levels of 35-60%. The experiments were carried out on a lab scale and scale up to production levels would be required to be tested, before it would be known if this action would be effective. The second suggestion would be to target the protein aspect of the problem, with the addition of proteinase enzymes e.g. papain, to the cream yeast. The results in this study were promising, with 30-57% reduction with a very low concentration of papain (0.01-0.02U/ room temp.) over a period of 30 minutes. Further work would have to be carried out to test whether viability of the yeast was affected with prolonged enzyme addition and as to whether the enzyme would be active at the normal storage temperature of cream yeast (4°C). In conclusion, some understanding of the problem of agglomeration has been achieved by this research and suggestions for implementation in full scale production made. It is hoped that these suggestions may at least provide, if not a solution to the problem, a method of reducing the extent of agglomeration in commercial yeast production.

CHAPTER 5

INVOLVEMENT OF SELECTED METAL IONS IN YEAST STRESS RESPONSES

5.1 INTRODUCTION

5.1.1 GENERAL CELLULAR RESPONSES TO STRESS

All living cells display a rapid molecular response to adverse environmental conditions. This phenomenon is commonly designated as the heat shock response, despite the fact that a variety of other stressors have been shown to induce synthesis of the same proteins (Craig 1985). The response must therefore be considered as a general cellular response to metabolic disturbances. The concept of a universal cellular stress response was initially considered in 1962 by Ritossa on the observation, following a brief heat shock, of puffs in the polytene chromosomes of *Drosophila busckii* (Watson 1990). However it wasn't until 1974 and the discovery of heat shock proteins in *Drosophila sp.* (Tissières *et al.* 1974) that its significance was recognised. Similar findings with prokaryotes and other eukaryotes soon suggested that the response represented an evolutionarily conserved genetic system which might be beneficial for the living cell.

The stress or heat-shock response is therefore a general property of all living organisms. Every organism examined so far has been shown to respond to a mild temperature shock (with respect to normal growth temperature) by increased synthesis of specific proteins collectively known as heat shock proteins (hsps) and this stress response is also known to occur in response to environmental stresses other than heat (Watson 1990). In unicellular organisms, stress conditions may be defined rather broadly as those environmental factors that cause a reduction of growth rate, including: nutrient starvation, oxygen, high/low temperature, heavy metal ions, high & low osmolarity, low/high pH, DNA-damaging agents, high ethanol concentrations and desiccation (Craig 1985; Lindquist 1986). In the majority of prokaryotes and of eukaryotes, stress exposure dramatically changes the pattern of gene expression. Generally, the response to stress involves the activation of a set of heat shock protein (*HSP*) genes resulting in the accumulation of hsp mRNA and the synthesis of heat shock proteins (Watson 1990). Fungi have a transient heat shock response that is

relatively short-lived, even while they are maintained at high temperature (Plesofsky-Vig & Brambl 1985). Animal systems on the other hand, are significantly different being unable to synthesise normal proteins during exposure to heat shock, as are plants which require several hours to recover normal protein synthesis at high temperature. Synthesis of a specific set of proteins, in some cases homologous proteins from quite different organisms, stimulated in response to a wide variety of environmental stresses, suggests that the stress- or heat-shock response is ubiquitous. In fact, the ability of a cell to shift rapidly to heat shock protein synthesis suggests that it is pivotal for survival, *i.e.* an "emergency response" (Lindquist & Craig 1988). Although the generally agreed function of these stress proteins is protection of cells from further, and potentially lethal, stress challenge, the complexity of these proteins makes their actual function remain in doubt despite much research. It is most likely that several types of mechanisms contribute to the phenomenon of stress tolerance of yeast and of other eukaryotes.

Industrial yeasts, during fermentation and growth, may be subject to a variety of physical and chemical insults collectively referred to as 'stress'. These stresses include: temperature stress, oxidative stress, ethanol stress, pH and osmo-stress. Yeast cells respond to these adverse environmental conditions as a survival mechanism, with a rapid molecular response; the stress response. Stress responses in yeast are characterised by the occurrence of heat shock (or stress) proteins (Miller *et al.* 1982; Lindquist & Craig 1988; Sanchez *et al.* 1992; Parsell *et al.* 1993), increases in levels of trehalose and glycerol (Gadd *et al.* 1987; Hottiger *et al.* 1987b; Attfield *et al.* 1992), alterations in membrane lipid composition (Mishra & Prasad 1989) and plasma membrane ATPase (Panaretou & Piper 1990; Rosa & Sá-Correria 1991), modulation of ion exchange processes (Petrov & Okorokov 1990; Ribeiro *et al.* 1994) and in the case of oxidative stress, the production of free radicals and superoxide dismutase (SOD) enzymes (Jozwiak & Leyko 1992; Davidson *et al.* 1996). Combinations of these factors are involved in conferring tolerance to stress and their relative importance varies according to the type of stress and the physiological state of yeast cultures.

Temperature is arguably the single most important parameter governing growth and metabolism of poikilothermic organisms. Temperature stress refers to the temperature upshift, ranging from a few degrees to 20^{o}C, above the conventional growth temperature (Heikkila 1993). Heat damages a wide variety of cellular processes and cellular structures. Biophysical studies have shown that heat shock leads to alterations to the physical states of both membranes and intracellular water resulting in increased membrane permeability (Piper 1993). Heat stress is also known to lead to changes to the lipid and protein compositions of yeast membranes. The effects of a mild heat shock on ultrastructure of *Sacch. cerevisiae* involve, contraction of nucleolus, formation of electron dense particles in the mitochondria and heat shock granules, consisting of proteins synthesised during heat shock, in the cytoplasm (Webster & Watson 1993). After extreme heat stress (52^{o}C/5min) the nucleolus was extensively disrupted and nucleolar material aggregated (Webster & Watson 1993). Heat shock was also shown to cause transient arrest in G1 phase of the cell cycle (Piper 1993). These cellular alterations and accumulation of aberrant proteins in the cell lead to the stimulation of the heat shock response, the cell's survival response to the stressful environmental conditions.

5.1.2 HEAT SHOCK RESPONSE

The heat shock response is a rapid, but transient, reprogramming of cellular activities to ensure survival during stress, to protect essential cell components and to permit a rapid resumption of normal cellular activities during the recovery period. It is characterised mainly by a rapid and massive synthesis of a few specific proteins (hsps), against a background of reduced normal protein synthesis. Heat shock genes can be activated constitutively during development as well as being induced by thermal stress and heat shock-induced hsp gene expression is developmentally regulated in numerous organisms (Heikkila 1993). For example, during ascospore development in yeast hsp26 and hsp83 were strongly induced whereas the major hsp70s were neither induced nor inducible (Kurtz *et al.* 1986). Variations occur in stress protein synthesis with respect to cell division cycle

and growth phase (Watson 1990). Stationary phase cells are much more resistant to heat shock and other stresses than rapidly growing cells (Elliot & Futcher 1993). During stationary phase *Sacch. cerevisiae* cells show long-term stress resistance which is independent of and in contrast to the transient nature of the heat shock response exhibited by exponential phase cells. Fermentative yeast cells exhibit a transient response requiring genetically functional mitochondria to survive prolonged exposure to heat shock conditions despite initial hsp synthesis (Lindquist *et al.* 1982), whereas respiratory cells display a prolonged heat shock response and are able to synthesise hsps indefinitely.

In eukaryotes, a common regulatory mechanism appears to control the transcription of all heat-shock genes. Regulation occurs through the action of the heat shock transcription factor (HSF) which is activated post-translationally and interacts specifically with its cognate DNA element, the heat shock response element (HSE), in the upstream promoter region of these genes (Mager & Moradas-Ferreira 1993). Although a universality is suggested of the heat shock response, differences exist between genus in the way the response is achieved. In yeast, the response is controlled primarily at the level of transcription (Lindquist 1986). Stress factors activate at least three transcriptional control systems of the yeast *Sacch. cerevisiae*: HSEs binding HSF, stress response elements (STREs) presumably interacting with a still hypothetical STRF and YAP-1 responsive elements (YREs) binding YAP transcription factors (Ruis & Schüller 1995). Proposed STREs are key components of the system allowing the induction of resistance to severe stress. In contrast, HSEs may play a role in stress protection by mediating the induction of synthesis of stress proteins necessary for growth under moderately stressful conditions (Ruis 1995; Ruis & Schüller 1995). Although HSEs and STREs have some functional overlap, these elements seem to have distinct overall functions (Marchler *et al.* 1993). *Sacch. cerevisiae* differs from certain other fungi in the DNA-binding properties of its HSF and in the cellular location of certain heat shock proteins (Plesofsky-Vig & Brambl 1993). HSFs of budding yeasts (*Sacch. cerevisiae*) occur as trimers in non-stressed cells and are constitutively bound to HSEs (Jakobsen & Pelham 1988; Gross *et al.* 1990). In fact, yeast HSF interacts with HSEs irrespective of the transcriptional state of the *HSP*genes (Jakobsen

& Pelham 1988). Originally defined by the 14bp consensus sequence C*nn*GAA*nn*TTC*nn*G (Pelham 1985), subsequent analysis of numerous HSEs has more precisely defined the HSE as an arrangement of a variable number of inverted *n*GAA*n* motifs (Amin *et al.* 1988; Xiao & Lis 1988). A trimer of HSF is required for efficient binding in *Sacch. cerevisiae* (Sorger & Nelson 1989) and it appears that three *n*GAA*n* repeats constitute a single binding site for the HSF complex. Constitutive binding of HSF to HSE in budding yeasts apparently mediates the constitutive expression of hsps that are essential for cell survival and growth at normal temperatures (Plesofsky-Vig & Brambl 1993). Yeast HSF undergoes a significant conformational change when cells are heat shocked, increasing its apparent cross-sectional area by 25-30% (Bonner *et al.* 1994). Transcriptional activation is dependent upon a *trans*-activating mechanism that is induced by stress in the host cell interacting specifically with the *cis*-acting promoter elements upstream of the heat shock genes (Lindquist 1986). Transcription of heat shock genes, regulated by transcription factors, is controlled by heat-shock dependent phosphorylation and activation of the factor (rather than binding to HSE as with other organisms). Activation of HSFs pre-existing in cells as a result of their extensive phosphorylation at high temperatures provides rapidity to the heat shock response (Plesofsky-Vig & Brambl 1993). The requirement for ATP during heat shock, for accumulation of hsp70 mRNA, suggests that there is an ATP-dependent step or phosphorylation involved in activation of the HSF and assembly of the transcriptional complex (Price & Calderwood 1991). Negative regulation of transcription of heat shock genes occurs when hsps have accumulated to a specific level (proportional to the severity of the heat treatment) repressing further transcription (Lindquist 1986). This inactivation of HSF, most likely by hsp70-dependent conformational change, serves to tie HSF activity directly or indirectly to the cellular need for chaperone activity (Bonner *et al.* 1994).

Heat and other stresses may trigger a variety of events (Figure 5.1) resulting in positive and negative regulation of the heat shock response. Many of the treatments which cause stress have the common property of inducing cytoplasmic accumulation of damaged, denatured or otherwise improperly folded proteins. Generation of such proteins following an environmental stress has been proposed as the major trigger for induction of the heat shock

response (Hightower 1980) thus establishing a link between cellular stress responses and intracellular protein degradation of damaged or abnormal proteins. The main sensor of protein damage is probably lack of free hsp70, as all the available hsp70 binds to the accumulations of damaged proteins in heat stressed cells (Craig & Gross 1991). DNA damage (Mitchel & Morrison 1984), changes in secondary messenger cAMP levels (Piper 1993), alterations in cytoskeletal structure (Chowdhury *et al.* 1992) and changes in cellular ion levels (Landry *et al.* 1988; Coote *et al.* 1991), can also act as signals for induction of heat shock response. Heat induced perturbations of intracellular ion levels and pH can be attributed in part to an enhanced permeability of membranes. Plasma membranes leak protons as a consequence of the stress challenge, this transient dissipation of the electrochemical pH gradient across the plasma membrane leads to a decrease in the internal pH of the cell; internal acidification (Coote *et al.* 1991). Stress-induced intracellular acidification may play a (direct or indirect) role in triggering the stress response (Coote *et al.* 1991), under normal growth conditions the pH gradient is sustained by the action of an ATP-driven proton pump, the plasma membrane ATPase (PM ATPase). Changes in H^+ concentration (detected within the first 5 min of the inducing treatment) may be involved in triggering transcriptional and/or translational control of the heat shock response (Weitzel *et al.* 1985). The signal itself may be a protein whose activity and/or permeability through the nuclear membrane is changed with a more acid pH. Analysis of membranes from stressed yeast cells revealed a 30kDa hsp that may be related to PM ATPase function (Piper *et al.* 1988). In permeabilised cells, leakage of ions other than protons also occurs. Heat shock has been shown to elevate intracellular Ca^{2+} levels (Stevenson *et al.* 1986) however raising intracellular Ca^{2+} fails to activate the heat shock response (Welch *et al.* 1983; Stevenson *et al.* 1987) indicating that despite that fact that Ca^{2+} activates HSF binding to the HSE in combination with heat shock, Ca^{2+} alone is insufficient to activate expression of the heat shock genes. Increases in cytoplasmic free Ca^{2+} have the potential to activate or destabilise the signal and the Ca^{2+} ion has been suggested as a possible mediator of the heat induced cell response (Landry *et al.* 1988). The transcriptional activation of *HS*genes has provided a useful paradigm to understand how cells detect and respond to a range of environmental and physiological stresses including; heat shock, ethanol shock, oxidative stress and

transition heavy metals, etc. The elevated synthesis of the stress-induced hsps and molecular chaperones affords a protective mechanism to the cell by regulating the transport, translocation and folding of proteins.

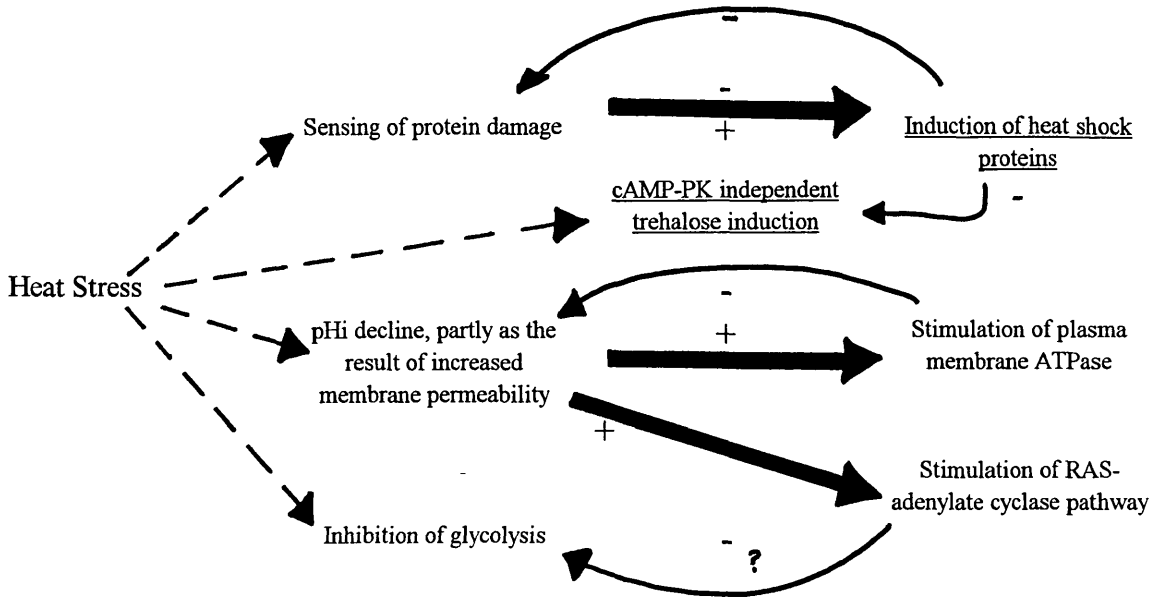


Figure 5.1 Activation of the heat shock response (Piper 1993).

5.1.3 HEAT SHOCK PROTEINS

The heat shock proteins show a remarkable conservation throughout evolution, with ubiquitin showing the highest degree of conservation in eukaryotic cells (Sharp & Li 1987). Genes encoding stress-induced proteins not only exhibit homology in their protein coding sequences but in addition, their regulatory sequences are highly conserved (Watson 1990). Heat shock proteins have been implicated in all major growth-related processes such as cell division, DNA synthesis, transcription, translation, protein folding and transport, and membrane function (Watson 1990; Mager & Moradas-Ferreira 1993). In eukaryotic cells, hsps have been implicated in the acquisition of thermotolerance and recently they have been suggested as being involved in the onset of human autoimmune diseases (Heikkila 1993). Hsps are induced by a wide variety of agents which do not have an obvious common effect (Craig 1985). Many different forms of stress proteins are produced and certain heat-shock

inducible proteins are present in unstressed cells under normal physiological conditions. These constitutive proteins play an essential role in normal growth and development of eukaryotic cells (Lindquist 1986; Watson 1990).

Many of the hsps interact primarily with other cellular proteins to facilitate their proper conformational folding, assembly into protein complexes and translocation into target organelles and because of these effects, hsps have been defined as molecular chaperones. The term 'molecular chaperone' is used for proteins which prevent incorrect interactions and assist the assembly of other proteins, without being part of the final protein structure (Ellis 1987; Georgopoulos 1992). Chaperones do not catalyse protein folding and association. However, they influence the kinetic partitioning between folding and aggregation, by keeping the concentration of folding intermediates that may aggregate low (Georgopoulos & Welch 1993). Under stress cells produce abnormal, misfolded or otherwise damaged proteins and it is proposed that increased synthesis of stress proteins is required to counter these modifications. Members of the hsp70 and hsp60 families participate in protein folding, protein translocation and higher ordered protein assembly of proteins imported into mitochondria and the endoplasmic reticulum (Watson 1990) and hsp90 family proteins play important roles in the regulation of certain transcription factors and protein kinases (Georgopoulos & Welch 1993). Although implicated in protecting the cell against the adverse effects of stress, certain hsps and their close relatives have also been shown to be essential for growth and metabolism at normal temperatures (Craig 1985; Lindquist 1986; Becker & Craig 1994). Hsps perform functions that are of common and fundamental importance to all cells, however despite this, the exact function of many of the hsps remains an enigma. One factor which introduces doubt into the role of these proteins and the stress response, is the multitude and variety of stresses which induce an elevated synthesis of stress proteins. The universality of the response and the conservation of the inducible genes throughout evolution indicates that hsps perform functions of fundamental importance (Craig 1985). Both hsp70 and hsp104 appear to participate in the regulation of primary carbon metabolism suggesting that hsps may fulfil an even more global function than was previously envisaged (Hottiger *et al.* 1992). Hsps may not be needed for stress tolerance

acquisition but for a rapid recovery from the stress-affected situation, thus serving as components of the cellular defence mechanism. It is suggested that some of the hsps may effect a regulated inhibition of metabolism and growth; growth arrest (Plesofsky-Vig & Brambl 1985). These proteins are also capable of self-regulation, repressing synthesis of hsps when required. In all systems examined, following a moderate heat shock, the synthesis of hsps is repressed and normal protein synthesis returns with a shift back to normal temperatures (Craig 1985). The repression of hsp synthesis observed after return to normal temperatures appears to be caused by the destabilisation of hsp mRNA, as well as the turn off of hsp RNA synthesis (Craig 1985). Maximum rates of heat shock protein synthesis occur 5-60min after the heat shock, after which there is a rapid decline concurrent with the onset of normal protein synthesis. However, normal protein synthesis and the normal pattern of RNA transcripts are not recovered in *Sacch. cerevisiae* until cells are allowed to synthesise the complete heat shock group (McAlister & Finkelstein 1980).

Heat shock proteins fall into three main groups: 85-110kDa, 60-80kDa and <50kDa, in addition ubiquitin, a 8.5kDa protein is a key component of the stress response in all eukaryotic cells (Table 5.1).

Table 5.1 Typical spectrum of hsps produced in yeast (Mager & Moradas-Ferreira 1993).

hsp	Cellular Site	Function (?)
hsp104	nucleus/nucleolus	stress tolerance
hsp83	cytosol/nucleus	chaperone
hsp70	cytosol/ER/mitochondria	chaperone
hsp60	mitochondria	chaperone
hsp26	cytosol/nucleus	?
hsp12	cytosol	?
Plus:		
hsp150	secretory	?
hsp30	plasma membrane	?
Ubiquitin	cytosol	protein degradation

HSP70

Stress proteins belonging to the hsp70 group are amongst the most highly conserved proteins known (Gupta & Golding 1993). High nucleotide and amino acid sequence homologies have been characterised for the proteins isolated from a wide variety of plant, animal, fungal and bacterial species and an evolutionary conservation of common functional protein domains is suggested. Highly conserved sequences (80%) are found between *E. coli, Sacch. cerevisiae, Drosophila sp.* and humans and hsps70 of eukaryotes have approximately 50% homology to dnaK, a prokaryotic hsp found in *E. coli* (Lindquist 1986). Members of the hsp70 family carry out a highly conserved molecular chaperone function in the intracellular transport of proteins and in protecting the organisms from thermal or other stress-induced damages (Lindquist & Craig 1988; Morimoto *et al.* 1990; Getting & Sambrook 1992) and they constitute essential and abundant proteins even in unstressed cells.

In eukaryotic cells, including *Sacch. cerevisiae*, *HSP70*genes exist as part of a multigene family whose genes are expressed under a variety of physiological conditions and at least eight genes related to hsp70 have been identified (Craig *et al.* 1985; Lindquist & Craig 1988; Normington *et al.* 1989; Rose *et al.* 1989). The *HSP70* multigene family in *Sacch. cerevisiae* is subdivided into four subfamilies SSA, SSB, SSC and SSD (Stress Seventy). Proteins of the SSA subfamily are indispensable for growth, SSB coded proteins are related to cold-sensitivity, the protein encoded by SSC is a mitochondrial protein (Craig *et al.* 1989) and SSD is found in the endoplasmic reticulum (Rose *et al.* 1989). In yeast, the transcriptional regulation of genes of the SSA and SSB subfamilies are different. Closely related members of the yeast hsp70 family are functionally homologous while distantly related members are functionally distinct (Lindquist 1986). Certain *HSP70*genes can substitute for others that are defective, while other *HSP70*genes cannot complement the lost function, indicating that there are separate functional groups of hsp70-related genes (Lindquist & Craig 1988). Seven of the members of the yeast *HSP70* multigene family are constitutively expressed, at different levels, in unstressed cells. These are transcribed at

elevated levels following heat shock, although not all to the same degree, and some are essential for cell viability.

Proteins of the hsp70 family are found in most cellular compartments of eukaryotes including: nuclei, mitochondria, endoplasmic reticulum (ER) and the cytosol. They appear to fulfil a variety of chaperone functions; stabilisation of unfolded precursor proteins prior to their assembly into multimolecular complexes in the cytosol for translocation into the ER and mitochondria, maintenance of newly translocated proteins in an unfolded state before folding and assembly in organelles and involvement in the rearrangement of protein oligomers and in the resolution of protein aggregates (Becker & Craig 1994). The molecular chaperone hsp70 binds to its substrate, probably via hydrogen bonds, when the polypeptide backbone of the substrate is in an extended conformation (Landry & Gierasch 1991). Hsps70 are involved in post-translational import pathways, facilitating the translocation of polypeptides across the ER and mitochondrial membranes (Chirico *et al.* 1988; Deshaies *et al.* 1988). Cytosolic/cytoplasmic hsps70 are closely associated with the synthesis, folding and secretion of proteins, mitochondrial hsp70 is necessary for the import of proteins from the cytosol into the mitochondrial matrix and a single ER-localised hsp70 protein (Kar2 in yeast), induced by the accumulation of misfolded proteins inside the ER (Normington *et al.* 1989; Rose *et al.* 1989), binds to certain polypeptide chains hence modifying or maintaining their conformation or interaction with other proteins, in all cases peptide release depends on ATP hydrolysis (Gething & Sambrook 1992). All hsps70 examined bind ATP with high affinity and posses a weak ATPase activity, which can be stimulated by binding to unfolded proteins and synthetic peptides (Rothman 1989).

There are multiple steps in the activation of transcription of hsp70 (Price & Calderwood 1991). Overexpression of hsp70 exhibited little protective effect on cell survival against heat shock, suggesting that hsp70 in yeast has some functions other than protecting cells from thermal stress (Weitzel & Li 1993). It is suggested that the function of hsp70s is to prevent aggregation of precursors and to stabilise them in an unfolded and therefore translocation-competent form (Becker & Craig 1994). Induction of hsp70 strongly

increases as a consequence of the elevated levels of 'thermally damaged' proteins allowing stabilisation of these aberrant proteins and subsequent recovery of normal cellular activity. This suggests that hsp70 is essential for the recovery of cells from the heat-stressed state and for the resumption of growth (Hottiger *et al.* 1992), rather than playing a role in preventing heat stress. Increased synthesis of hsp70 may eventually result in reassociation of the protein with HSF, which would explain the transient nature of the response (Morimoto *et al.* 1990). Also, genetic evidence has been presented that in *Sacch. cerevisiae*, 70kDa hsps repress their own synthesis and it was suggested that this is part of the mechanism that terminates the heat-shock response (Stone & Craig 1990).

HSP60

Hsp60 proteins fulfil cellular functions similar to hsps70. The hsp60 family exhibits chaperone activity and although structurally distinct from hsp70, they share general functional features as they also bind unfolded polypeptides and facilitate post-translational assembly of polypeptides (Ellis 1987). Hsp60s play a crucial role in catalysing the folding of unfolded proteins and assembling higher-order protein structures (Becker & Craig 1994). They have been identified, in *Sacch. cerevisiae*, as the protein necessary for the assembly of peptides imported into mitochondria (Cheng *et al.* 1989). Proteins imported into the mitochondria do not fold spontaneously but need hsp60 function and ATP for proper folding (Neupert *et al.* 1990). The copurification of hsp60 with mitochondrial holoenzymes, such as yeast ATPase (Gray *et al.* 1990) suggests that hsp60 may directly participate in protein assembly as well as in protein folding. Hsp60 is synthesised constitutively, as well as being induced be heat shock, and it has been shown by gene disruption to be an essential protein in *Sacch. cerevisiae* at normal temperatures (Reading *et al.* 1989). Hsp60 in yeast has been identified as showing homology (54%) to the *E. coli* groEL protein (Cheng *et al.* 1989) and characterisation of the hsp60 stress protein in *Sacch. cerevisiae* revealed the presence of an amino terminal extension of approx. 22 amino acid residues typical of many mitochondrial targeting peptides (Attardi & Schatz 1988). Hsp60

has multiple peptide binding sites and can therefore facilitate folding of already partially folded proteins while still being associated with the folding intermediate, as oppose to the single peptide binding site of hsp70 (Becker & Craig 1994), thus showing a functional distinction from hsp70.

HSP83

Yeast hsp83 belongs to the family of hsp90 proteins. The second most highly conserved group of hsps, the hsp90 family differs from hsp60s and hsp70s in that they regulate the function of folded proteins by binding to them, but similarity exists in the way that they may do so by an ATP-dependent mechanism shared by all three hsp groups (Becker & Craig 1994). The function of this family of heat shock genes is also in the encoding of chaperone-like proteins. Abundant at normal temperatures and induced by heat, two separate genes produce the constitutive and inducible forms in *Sacch. cerevisiae*, but the proteins are functionally homologous (Lindquist 1986). Hsp83 is abundantly present in the cytoplasm and a small fraction translocates to the nucleus upon the induction of heat shock (Lindquist & Craig 1988; Schlessinger 1990). Notably expression of this gene is also inducible when cells enter the stationary phase (Kurtz & Lindquist 1984) or sporulate (Kurtz *et al.* 1986). Eukaryotic hsp83 has been shown to possess 40% homology to the *E. coli* protein htpG (Lindquist & Craig 1988) again showing further evolutionary conservation of hsps between genus.

HSP104

Hsp104 is involved in the regulation of the "stress response" to a variety of agents (Boreham & Mitchel 1994). The protein is not detectable at normal growth on fermentable carbon sources, but it is expressed in respiring cells (Sanchez *et al.* 1992). It is, however, known not to be beneficial for growth or cell viability (Plesofsky-Vig & Brambl 1993) and

is strongly induced following a heat shock. A strong induction of hsp104 was observed in stationary phase cells right after diauxic shift, when glucose levels decline (Sanchez *et al.* 1992) and expression of this protein was also activated when cells enter stationary phase or are induced to sporulate (Mager & Moradas-Ferreira 1993). The protein protects cells from long exposures at temperatures just beyond their normal growth range and from short exposures at very extreme temperatures. Furthermore, it protects cells against ethanol, sodium arsenite and from long term storage at low temperatures (Sanchez *et al.* 1992). Hsp104 is most crucial under the most extreme circumstances, as the extent of stress decreases so does the importance of hsp104 in relation to other inducible tolerance factors (Sanchez *et al.* 1992). Hsp104 plays a crucial role, for induced thermotolerance, in both fermenting and respiring cells (Sanchez *et al.* 1992), in particular the *HSP104*gene is required for induced tolerance to heat in log-phase fermenting cells of *Sacch. cerevisiae* (Sanchez & Lindquist 1990). Hsp104 is therefore more closely specialised to restore/repair processes once they have been disrupted, hence it can be said to have a stress protective function and as a nucleolar protein it plays a crucial role in cell survival under heat stress.

SMALL HSPS (<50KDA)

Small hsps show only limited sequence conservation between species and a considerable variance in molecular mass. They are the most divergent group amongst the hsps in regard to molecular weight and although there appear to be multiple low molecular weight (<50kDa) stress proteins in *D. melanogaster* (Craig 1985), plant (Schoffl *et al.* 1986) and mammalian cells (Lindquist 1986), only a few small stress proteins have been characterised in *Sacch. cerevisiae*: hsp12, hsp26 and hsp30 (Petko & Lindquist 1986; Piper *et al.* 1988; Plesofsky-Vig & Brambl 1993; Piper *et al.* 1994). Generally cytoplasmic proteins the small hsps act as molecular chaperones (Jakob *et al.* 1993), they are able to prevent the irreversible aggregation of proteins under heat shock conditions. However, a cellular role of small hsps has yet to be elucidated. A universal property of the small hsps may also be their developmental regulation. Overexpression of small hsps increases not only

thermotolerance of cells (Landry *et al.* 1989) but also specifically inhibits cell proliferation (Knauf *et al.* 1992). Yeast cells contain two small cytoplasmic hsps: hsp26 (related to α-crystalin: Plesofsky-Vig & Bramble 1993) and hsp12 and they have the ability to form polymeric heat shock granules (HSGs). *Sacch. cerevisiae* has a single integral plasma membrane hsp related to PM ATPase function: hsp30 (Piper *et al.* 1988; Piper *et al.* 1994; Piper *et al.* 1996). Hsp30 is induced by several stresses including heat shock, ethanol exposure, severe osmostress, weak organic acid exposure and glucose limitation (Piper *et al.* 1996). Hsp30 induction causes a down regulation of the stress-stimulation of this PM H^+-ATPase, therefore the role of hsp30 may be one of energy conservation (PM H^+-ATPase action consumes a substantial fraction of the ATP generated by the cell), limiting excessive ATP consumption during conditions of stress (Piper *et al.* 1996). Loss of hsp30 does not affect stress tolerance, but it extends the time needed for cells to adapt to growth under stressful conditions (Piper *et al.* 1996).

UBIQUITIN

The ubiquitin system plays a key role in the response of *Sacch. cerevisiae* to various stresses. Polyubiquitin is generally considered as a heat shock protein since it displays a strongly enhanced rate of synthesis under stress conditions (Finley *et al.* 1987). All four ubiquitin genes are expressed by exponentially growing yeast cells (Ozkaynak *et al.* 1987). However, only the polyubiquitin (*UBI4*) gene is induced by heat shock. The *UBI4*gene was shown to be dispensable for normal vegetative growth in yeast, but essential for resistance to various stresses (Finley *et al.* 1987). Hottiger *et al.* (1992) further suggests that the *UBI4*gene (ubiquitin) is needed only for survival at the maximum temperatures of yeast growth, but neither for induced thermotolerance nor for recovery from the heat-stressed state. Covalent binding of ubiquitin to various acceptor proteins helps promote regulation of a number of cellular processes, many of which are related to the stress response including, selective protein degradation, DNA repair, the response of cells to nutrient starvation and amino acid analogues, and the removal of abnormal or denatured proteins

(reviewed by Watson 1990). It has been suggested that ubiquitin and the hsps are complementary methods of dealing with a common problem, *i.e.* the production of denatured protein aggregates in heat-shocked cells (Lindquist 1986).

5.1.4 TREHALOSE

Another process closely related to the stress response in yeast cells is the synthesis of trehalose. Yeast cells exponentially growing on glucose contain little trehalose (Thevelein 1984). As a result of sub-lethal heat stress, cells accumulate a large cytoplasmic pool of trehalose (α-D-glucopyranosyl α-D-glucopyranoside). This trehalose is thought to act primarily as a stress protectant, rather than a storage carbohydrate, since its levels generally show a good correlative relationship with thermotolerance (Wiemken 1990). Trehalose accumulates in vegetative *Sacch. cerevisiae* cells under conditions of reduced growth rate, nutrient limitation or exposure to physicochemical stresses and in cells undergoing physiological transition or differentiation (Attfield 1987). Trehalose accumulated very rapidly in heat shocked yeast is mobilised equally rapidly during a subsequent temperature shift down, most likely under negative regulation by hsp70 (Piper 1993). The size of the trehalose pool accumulated during heat shock must be dependent on the balance between the biosynthetic and catabolic enzymes of the trehalose cycle (Ribeiro *et al.* 1994) but none of these enzymes correspond to the primary agent responsible for this accumulation. More essential than the increase in the activity of the enzyme appeared to be the increase in the rate of flux through the pathway mediated by larger availability of substrates (Ribeiro *et al.* 1994). The trehalose cycle (Figure 5.2) in heat-shocked cells of *Sacch. cerevisiae* has been suggested to have the role of energy dissipation (Hottiger *et al.* 1987b) the cycling may not be strictly futile, indeed the accumulation of these compounds may be important in maintaining the integrity of the yeast. Enzymes responsible for trehalose synthesis and thermotolerance are regulated by cAMP-dependent phosphorylation (Thevelein 1988). However, the trehalose pathway could also be regulated by several other mechanisms, e.g. all the enzymes of this pathway require Mg^{2+} for full activity, thus the intracellular variation

in Mg^{2+} levels would be important for control of metabolic flux through the pathway (Ribeiro *et al.* 1994).

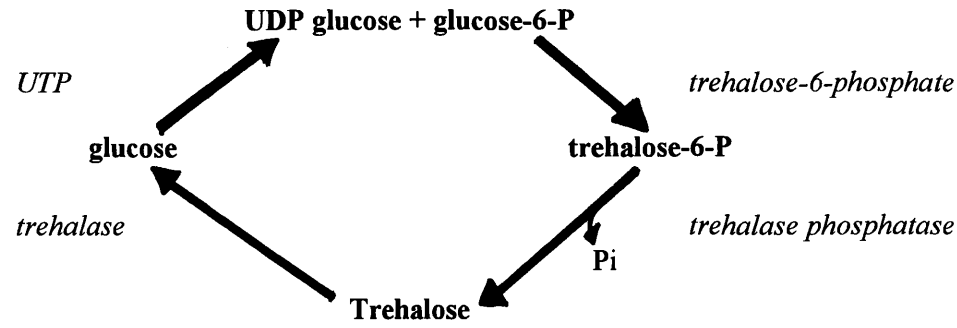


Figure 5.2 Trehalose cycle

Trehalose plays a role in the survival of yeast exposed to stress (Attfield *et al.* 1992). Observations of physiological studies on yeast and from non-cellular systems, imply that trehalose could function actively in protection or repair processes, rather than merely acting passively as a fermentable energy source for cells to access for repair processes (Attfield *et al.* 1994). A protective role of trehalose for proteins and membranes and thus in maintaining the structural integrity of the cell has therefore been suggested (Hottiger *et al.* 1989). Trehalose has been shown to play a positive role in maintaining the yeast cytoskeletal structure under various stress conditions such as heat, desiccation and frost (Hottiger *et al.* 1987a; Peixoto *et al.* 1996). It is also thought to influence membrane structure and the strength of hydrogen bonding interactions (Piper 1993). Trehalose binds to the polar head group of the lipid bilayer, where it replaces water and acts to preserve the properties of a hydrated membrane during desiccation (Crowe *et al.* 1984). Trehalose and polyols also increase the thermal stability of proteins and probably exert a similar action on membrane proteins (Piper 1993; Peixoto *et al.* 1996). Results of studies by Attfield *et al.* (1994) suggest the possibility that trehalose makes an important contribution to stress tolerance during early and mid-respiratory growth phase, but that in the latter stages, as the culture approaches stationary phase, other factors assume greater importance, e.g. hsps, etc. DeVirgilio *et al.* (1994), however, showed genetic evidence suggesting that trehalose is

important for thermotolerance of yeast cells. It is accepted that the known hsps do not seem to fully account for all thermotolerance and studies have shown that thermotolerance is partly independent of hsp synthesis (Barnes *et al.* 1990; DeVirgilio *et al.* 1991; Smith & Yaffe 1991). Hence trehalose may play an additional role in thermotolerance. There is reasonably strong genetic and biochemical evidence of a role for trehalose in both intrinsic and inducible thermotolerance (Attfield *et al.* 1994). Heat inducible trehalose accumulation appears to be influenced by heat shock proteins such as hsp70, hsp104 (Hottiger *et al.* 1992) and hsp90 (Cheng *et al.* 1993). The possibility, therefore, exists that the contribution of trehalose to intrinsic thermotolerance is influenced by the presence or absence of certain hsps, during normal growth. Elliot *et al.* (1996) suggested that trehalose and hsp104 co-operate to produce heat shock resistance and trehalose and hsp104 together, can account for most of the heat shock resistance of stationary phase cells. In budding yeasts, the heat shock dependent acquisition of thermotolerance and the concomitant synthesis of endogenous trehalose are reversible processes. In *Candida albicans*, unicellular forms display a similar behaviour during the heat shock response to that of *Sacch. cerevisiae*, except for the fact that heat shocking (30-42^{o}C) *C. albicans* did not cause any significant modification in the activity of the unique trehalase (Argüelles 1997). Argüelles (1997) showed that although trehalose decay followed a similar trend, compared to the initial increase triggered by thermal incubation, trehalase activity was practically unaffected during temperature reversion. This suggests that in *C. albicans,* trehalase is not involved in response to heat stress (Argüelles 1997). In *Sacch. cerevisiae*, however, heat stress triggers neutral trehalase, thus showing that the stress response can vary slightly from genus to genus, as well as in response to different stresses since, for example, not all chemical stressors can induce trehalose.

5.1.5 THERMOTOLERANCE

Experiments in a number of plant and animal species have indicated a correlation between the presence of hsps and the resistance of cells to a severe heat shock (Craig 1985). Heat

shock protein synthesis is a pre-requisite for the development of stress tolerance by yeast (Mager & Moradas-Ferreira 1993). Induction of hsps is often associated with increased tolerance, both to the immediate inducing agent and to other types of stress; cross-tolerance. Other conditions, e.g. ethanol stress (Plesset *et al.* 1982) which result in the synthesis of hsps, also conferred thermotolerance and the reciprocal effect, the induction of resistance to other stresses by heat treatment, has been observed in several cases (Craig 1985). Thermotolerance reflects the ability of yeast to adapt growth at heat shock temperatures, the mild response of normal yeast nucleoli to heat shock probably reflects the greater intrinsic resistance of the yeast nucleolar machinery to elevated temperatures (Webster & Watson 1993). Animal cells, in contrast, never adapt to heat shock temperatures (Welch 1990). Acquired thermotolerance is the most characteristic physiological response of microorgansims subjected to a mild temperature shock, it refers to the transient, non-inheritable acquisition of resistance to a normally lethal temperature, induced by a short prior exposure to a non-lethal heat shock.

Stimulation of stress protein synthesis does not always correlate with the acquisition of thermotolerance (Watson 1990) and in yeast, protein synthesis is not necessary for the induction of thermoresistance in cells (Hall 1983). The mechanisms of thermotolerance, rather than being single, may rely on a contribution from various factors, e.g. hsps, ATPase and trehalose (Coote *et al.* 1992). The induction of thermotolerance in yeast can occur largely independently of *de novo* protein synthesis (Piper 1993), although inactivation of heat shock genes *HSP104* and *CTT1* have been shown to reduce the thermotolerance acquired with heat shock (Sanchez & Lindquist 1990; Sanchez *et al.* 1992), yet a number of *HSP*genes when disrupted have been found not to alter basal or heat-acquired thermotolerance levels (Petko & Lindquist 1986; Piper 1993). It has been proposed that thermotolerance and heat shock are two distinct inducible states (van Bogelen *et al.* 1987). The former is believed to be a transient state associated with resistance to a high lethal temperature, while the latter is a more permanent state necessary for physiological adjustment to growth at an elevated, but non-lethal temperature. Only a small fraction of hsps are involved in conferring thermotolerance. Evidence of a possible involvement of

hsp70 (Li & Laszlo 1985), hsp27 (Landry *et al.* 1989) and calcium ions (Landry *et al.* 1988), in development of thermotolerance in mammalian cells has been shown. Heat shock proteins, like hsp70 (Li *et al.* 1992) and hsp104 (Sanchez & Lindquist 1990), which are induced via the HSF, have been shown to contribute to thermotolerance and hsp104 has previously been demonstrated to have a protective function in connection with other types of stress (Sanchez *et al.* 1992). It is however, suggested that for example, accumulation of trehalose (DeVirgilio *et al.* 1991) is more important for the induction of thermotolerance than activation of transcription mediated by HSF and that these proteins, rather than play a role in induced thermotolerance, are essential for the recovery of cells from the heat-stressed state and for the resumption of growth (Hottiger *et al.* 1992).

5.1.6 Other Types of Stress

Studies on the stress response of living cells have so far mainly been focused on the effects of heat treatment, although other stress agents can induce a similar response (review-Watson 1990). The main stress agents which affect the growth and fermentation of yeast, including wine yeasts, are; heat, ethanol, nutrient limitation, osmostress and oxidative stress.

Ethanol

The ability or inability of yeasts to tolerate the toxic effects of ethanol is of profound importance in the commercial production of alcohol. Yeast are evolutionarily adapted to ethanol, since this alcohol is a major product of their metabolic activity. However, the increasing ethanol levels during batch fermentation of *Sacch. cerevisiae* on high sugar substrates acts to cause reductions in the specific growth rate, fermentation rate and cell viability. Ethanol has been described as a non-competitive inhibitor of certain cellular functions, *i.e.* growth rates, fermentation rates, viability, etc., but the physiological and genetic basis of this inhibition is not clear (Casey & Ingledew 1986). Ethanol inhibits

glycolytic enzymes and numerous biological processes, many of which are associated with membrane lipids (Ingram & Buttke 1984). The effects of ethanol *in vivo* are not, however, primarily due to inhibition or inactivation of glycolytic enzymes. Effects on cellular membranes and membrane transport processes are now thought by many workers to be the predominant mechanism of toxicity (Pamment *et al.* 1990). The toxic effect of ethanol and other alcohols on the cell is multifarious; they inhibit biosynthesis of macromolecules, activities of glycolytic enzymes and solute transport systems and change the lipid composition of the membranes, etc. (Ingram & Buttke 1984; Dombek & Ingram 1987; Petrov & Okorokov 1990). The plasma membrane is the first cellular component to come into contact with the alcohol produced and the correlation observed between hydrophobicity of alcohols and ethanol tolerance, together suggest that the plasma membrane lipids are the prime target(s) of ethanol toxicity (Ingram & Buttke 1984; Carlsen *et al.* 1991; Mishra & Kaur 1991). Leão & van Uden (1984) described passive proton influx into yeast cells, induced by ethanol and some other alkanols, and suggested that these alcohols increased the proton permeability of the yeast PM, proposing that some phenomenon other than proton uncoupling was involved in the ethanol toxicity. Petrov & Okorokov (1990) found that neither the ATPase of the plasma membrane vesicles (PMV) nor that of the plasmalemma sheets (PMS) were inhibited by ethanol up to 0.5M. Alcohols do not essentially inhibit H^+-ATPase of yeast PM but increase its permeability for protons and anions, de-energising it and ultimately blocking secondary transport systems. The increase in PM proton permeability also leads to the imbalance of the ion composition of the cytosol (Petrov & Okorokov 1990). The composition of the cell membrane plays an important role in its tolerance to alcohols (Ingram & Buttke 1984; Mishra & Kaur 1991). There is an obvious requirement for heterogeneity of fatty acids in yeast, in order to preserve membrane structure, function and survival (Swan & Watson 1996). Ethanol-dependent modification in phospholipid fatty acyl composition has been demonstrated in *Sacch. cerevisiae* (Beavan *et al.* 1982) with a decrease in 16:0 and increase in 18:1 unsaturated fatty acids resulting in a sequential increase in fluidity of the membrane. A strong relationship exists between membrane unsaturation and cellular stress tolerance, with

sensitivity to thermal stress increasing with increased unsaturation of the membrane fatty acids (FAs).

Ethanol is also known to induce heat shock protein synthesis and thermotolerance in *Sacch. cerevisiae* (Plesset *et al.* 1982). Cross-tolerance following exposure to sub-lethal concentrations of ethanol occurs, resulting in cells which are resistant to lethal temperatures (Plesset *et al.* 1982). The reverse has also been shown, *i.e.* exposure to heat shock confers protection from other stresses, for example *Sacch. cerevisiae* cells that have been exposed to a mild heat shock, develop an increased tolerance for high concentrations of ethanol (Watson 1990). Odumeru *et al.* (1992) also showed pre-incubation at sub-lethal temperatures prior to heat shock at higher temperatures protected yeast cells from thermal death and similar results were obtained with pre-incubation at sub-lethal heat prior to incubation in 20% (v/v) ethanol. It does not appear that these examples of cross-protection solely require synthesis of hsps, thus indicating that other components of the cellular response to stress are involved in protection of cells against environmental stresses.

The addition of ethanol to *Sacch. cerevisiae* cultures has been shown to be less toxic to the yeast cells than endogenous ethanol produced by the yeast (Nagodawithana & Steinkraus 1976; Dombek & Ingram 1986a; Pamment *et al.* 1990). Intracellular accumulation of ethanol in *Sacch. cerevisiae* occurs in the early stages (3hr) of fermentation, as fermentation proceeds ethanol diffuses out through the yeast cell membrane (D'Amore *et al.* 1988). The accumulation of ethanol in yeast cultures eventually leads to decreased rates of fermentation (production of ethanol and CO_2), decreased growth rates and loss of viability (Ingram & Buttke 1984). Several alternative explanations of the apparent difference in the toxicity of produced and added ethanol have been proposed. The apparent higher toxicity of produced ethanol may be due to the high rate of change of extracellular ethanol concentration (Pamment *et al.* 1990) due partly to the inability of cells to adapt quickly enough to the rapidly changing extracellular ethanol concentration. Increases in intracellular ethanol at high osmotic pressures and the decreased growth rates and fermentative activities are associated with an increase in the ratio of intracellular to extracellular ethanol (D'Amore *et*

al. 1988). However, nutritional requirements and the accumulation of toxic by-products may play a contributing role. When compared at equal rates of change of ethanol concentration, differences in toxicity between added and produced were shown to be due mainly to nutritional deficiencies which arise when ethanol is produced, e.g. magnesium deficiencies (Pamment *et al.* 1990).

HEAT/ETHANOL

The involvement of heat concurrent with ethanol shock and its effect on the stress response of yeast cells is also pertinent. Alcohol and temperature exert a synergistic effect on the cells heat shock response, with alcohols mediating a reduction in the minimum and maximum temperatures required for the induction of the stress response in *Sacch. cerevisiae* (Curran & Khalawan 1994). While ethanol concentration is sufficiently low, the yeast population has its normal cardinal temperatures, during fermentation while ethanol concentration increases, the optimal, final $T_{max.}$ and initial $T_{max.}$ decrease. Several of the changes induced in yeast by exposure to stressful ethanol levels are identical to those caused by a heat stress (Piper *et al.* 1994). It is not surprising, therefore, that ethanol acts in a synergistic way to increase the damage caused by heat. Both heat and ethanol cause membrane disordering and protein denaturation (Casey & Ingledew 1986; Piper 1993), inhibition of glycolysis and glucose transport (Leão & van Uden 1982; 1985) and alkanols enhance thermal death by acting in a non-specific way on the membrane lipids (van Uden 1983). Results of work by Curran & Khalawan (1994) implicated a perturbation of the cell membrane in heat-induced activation of this heat shock sensitive promoter and support the view that thermal stress may be transduced into a cellular signal at the level of the cell membrane. Ethanol is believed to interact with membranes by insertion into the hydrophobic interior, increasing the polarity of this region, weakening the hydrophobic barrier to the free exchange of polar molecules and affecting the positioning and type of membrane components (Ingram & Buttke 1984). Yeast cells exposed to heat and ethanol shock had a significant increase in the concentration of mono-unsaturated fatty acids and a

corresponding proportional decrease in the concentration of saturated FAs, resulting in an increase in the ratio of unsaturated to saturated FAs in cells (Odumeru *et al.* 1993). Both stresses increase the permeability of the plasma membrane, resulting in an increased passive proton flux that acts to dissipate the electro-chemical potential gradient that the cell maintains at this membrane. This is reflected in the decline in intracellular pH that is observed both with ethanol addition (Leão & van Uden 1984; Petrov & Okorokov 1990) and with heat shock (Coote *et al.* 1991). These stresses therefore adversely affect those vital functions for which a plasma membrane electrochemical gradient is essential, e.g. nutrient uptake, regulation of intracellular pH, etc. (Piper *et al.* 1994). Protective responses induced by heat shock and ethanol show a high degree of similarity. Heat and ethanol both induce hsps in yeast (Plesset *et al.* 1982) and the form of hsp90 and hsp70 induced by ethanol are identical to those induced by heat (Piper *et al.* 1994), and these two stresses are also shown to have similar effects on the levels of two major integral plasma membrane proteins; ATPase and hsp30 (Piper *et al.* 1994).

NUTRIENT LIMITATION

Stress tolerance of *Sacch. cerevisiae* varies greatly with the physiological state of cells and culture conditions. Cells growing rapidly are more sensitive to stresses, e.g. temperature, osmotic and chemical stresses, than cells that are growing slowly or resting (Craig 1985; Lindquist 1986; Watson 1990). *Sacch. cerevisiae* when cultured on glucose in batch culture exhibits several distinct phases of growth. There are two separate periods of exponential growth; the first and most rapid of these, respiro-fermentative growth, involves mainly fermentative metabolism of glucose to form alcohol, whereas the secondary, exponential growth phase, respiratory growth, produces a slower growth rate and involves oxidative metabolism of the accumulated ethanol. Nutrient limitation is the major factor responsible for the decline in fermentative activity during the early stages of fermentation in yeast (Dombek & Ingram 1986a). The limiting nutrient will more than likely be an ionic species essential for enzyme activity, e.g. magnesium, or some other essential cellular

process, or it will be the exhaustion of nitrogenous compounds or the C-source itself. Whatever the cause, nutrient stress is a serious environmental stress and as such triggers an action from the stress response. Walker & McWilliams (1989) found that transient proteins induced by nitrogen starvation represented a stress response of cells in the fission yeast *S. pombe*. The resultant stress proteins play a similar role to heat shock proteins in protecting cells from environmental insults. The existence in *S. pombe* of hsps and nitrogen starvation proteins which co-migrate on polyacrylamide gels at around 46 and 27kDa lead them to suspect that these are representative of proteins synthesised as a general response to physical and chemical stresses (Walker & McWilliams 1989). They also showed a functional relationship between nitrogen starvation and heat shock in *S. pombe*, with the conference of thermotolerance by prior nitrogen starvation of cells.

In a similar response, new proteins are synthesised by *E. coli* at the onset of carbon starvation and such protein synthesis is beneficial for survival during starvation (Groat & Matin 1986) due to the role of hsps in effecting a rapid recovery after heat shock and other stresses (Mager & Moradas-Ferreira 1993). In *E. coli* hsps are synthesised in response to several different stresses (Craig 1985; Lindquist 1986) and common signal molecules are known to mediate their expression. *relA*-mediated guanosine tetraphosphate (ppGpp/Magic Spot I) accumulation in amino acid starved *E. coli* has been proposed as a factor affecting heat shock protein synthesis (Grossman *et al.* 1985). Since ppGpp also accumulates during glucose limitation (J. Leitch- personal communication) this nucleotide might also affect protein synthesis during carbon/energy starvation (Groat & Matin 1986). It is thought therefore, that Magic Spot I (ppGpp) may be tentatively described as a stress metabolite.

Chelating agents cause induction of the stress response in a variety of cell types due to the alteration of intra- and extra-cellular ionic levels, most particularly that of Mg and Ca. A23187 is a divalent cation ionophore, which acts as a freely mobile carrier to equilibrate calcium and magnesium across various membranes. It promotes their passive transport across biological membranes, therefore affecting the equilibrium of divalent ions between the cell sap and the surrounding medium counteracting any active accumulation of ions

within the cell (Reed & Lardy 1972). It inhibits mitochondrial ATPase by releasing endogenous magnesium, while uncoupling oxidative phosphorylation by a calcium-dependent mechanism (Reed & Lardy 1972). A23187 and EDTA inhibit cell division in yeast by limiting Mg^{2+} supply (Walker & Duffus 1979) as do sodium pyrophosphate and citric acid. Magnesium and calcium play important roles in the cell division cycle, stress proteins and G_0 phase induction, etc., therefore alterations in the intracellular levels of these ionic species would be highly detrimental to cell viability and stress survival.

5.1.7 PROTECTIVE EFFECT OF MAGNESIUM

As has been discussed previously, various stresses can cause disruption of the cellular ionic composition thus risking reduced productivity and even cell death. Magnesium is known to be beneficial to yeast cells. As an essential inorganic ion it plays important roles in yeast cell physiology. It is involved in many physiological functions, including growth, cell division and enzyme activity (Walker 1994). Magnesium ions have been implicated, by several studies, in the amelioration of the detrimental effects of ethanol, high osmotic pressure and temperature inhibition in *Sacch. cerevisiae* (Dombek & Ingram 1986c; D'Amore *et al.* 1988; Dasari *et al.* 1990). Identified as a principle growth-limiting nutrient (Dasari *et al.* 1990). The pronounced effects of magnesium, the influence of the temperature of fermentation and the rate of change of ethanol concentration on viability, raises the question of whether these factors influence ethanol-enhanced thermal death, ethanol-induced death or both (Dasari *et al.* 1990). Magnesium ions have been shown to exert a protective effect or at least enhanced recovery from a variety of stresses. Magnesium ions decrease the proton and especially anion permeability of the plasmalemma (Petrov & Okorokov 1990) and it is believed that Mg^{2+} interacts with membrane phospholipids, resulting in stabilisation of the membrane and a decrease of lipid bilayer fluidity. These observations of a protective effect of Mg^{2+} in response to environmental stress (Petrov & Okorokov 1990) suggest possibly that magnesium plays a more important role in the protection of and recovery of cells from stress than was previously envisaged.

5.1.8 RESEARCH OBJECTIVE

The aim of this chapter was to assess the protective effects of elevated levels of magnesium against a variety of stress agents, including: temperature, ethanol and nutrient stress. This was accomplished by monitoring changes in cell viability, cell surface topology, trehalose levels and stress protein biosynthesis, in a selection of industrial strains of the yeast *Sacch. cerevisiae*.

5.2 EXPERIMENTAL APPROACH

5.2.1 CULTURES AND CULTURE PREPARATION

Sacch. cerevisiae (L-2226), and in comparative studies strain DBVPG2168, were grown up in complete modified minimal medium (CMMM) to serve as an inoculum for the experimental pre-cultures. From a 48hr liquid inoculum (CMMM), cells were grown up overnight in CMMM, at 30^{o}C in an orbital incubator. In experiments requiring a magnesium pre-conditioned inoculum, cells were grown orbitally overnight at 30^{o}C in CMMM containing the respective concentration of magnesium. Following a cell count by haemocytometer, a volume of this pre-culture was used to inoculate 10ml volumes of fresh pre-warmed minimal media to give an initial cell number of approx. 3 x 10^{6} cells/ml and these cultures were incubated orbitally at 30^{o}C for 4hrs.

Following the 4hr incubation period, flasks were placed into the experimental conditions, allowed to equilibrate for 1min and then labelled with L-[^{35}S]-Methionine in aqueous solution (Amersham International, UK) to give a final radioactivity level of 1μCi/ml at the initiation of the respective stress conditions. Stress conditions were imposed on the yeast cultures for a period of 60min, with intermittent shaking. Subsequent to the 60min stress exposure, cells were harvested by vacuum filtering on 0.45μm Millipore filters (Whatman, UK).

Samples were removed prior to harvesting cells and analysed for radioactivity level. 10μl of culture was pipetted onto filter paper squares (3MM Chromatography paper: Whatman, UK) and allowed to air dry. Filters were then washed for 5min in ice cold 5%w/v trichloroacetic acid (TCA), followed by a 10min wash in 90^{o}C TCA (5%w/v) and a final rinse in ice cold TCA (5%w/v), prior to being oven dried (100^{o}C). Radioactivity levels were measured using a scintillation counter (LKB 1216 Rackbeta Liquid Scintillation Counter: Wallac) by immersing filters in a non-organic scintillant (Ecoscint A: National

Diagnostics, USA) and counting counts per minute (cpm) based on a ^{35}Sulphur standard. Information from these counts were used in order to load a balance of radioactivity across an individual gel, for subsequent exposure to X-ray film.

N.B. Quench correction was carried out on all samples to allow for sample balancing in experiments

5.2.2 EXPERIMENTAL CONDITIONS

5.2.2.1 HEAT STRESS

Sacch. cerevisiae (L-2226) was exposed to heat shock (30^{o}C - 42^{o}C shift) under conditions of concurrent additions and pre-conditioning of cells with various levels of magnesium. Magnesium concentrations analysed were; 5/10/15/20 and 50mM Mg. Concurrent additions involved introducing the elevated magnesium level to the culture at the moment of initiation of shock conditions (42^{o}C). Pre-conditioned cells were grown in media containing that level of magnesium prior to the initiation of shock conditions. A 30^{o}C control and a 42^{o}C control (2mM Mg, normal medium level) flask was included in the heat shock experiments.

5.2.2.2 ETHANOL STRESS

Initially the effect of a range of ethanol concentrations on *Sacch. cerevisiae* (L-2226) was analysed. Concentrations of 0/5/10/15 and 20% ethanol were included in the media and cultures were incubated at 30^{o}C, with intermittent shaking.

Following this experiment, the yeast was exposed to ethanol shock (10%) under conditions of concurrent additions and pre-conditioning of cells with various levels of magnesium. Magnesium concentrations analysed were; 5/10/15/20 and 50mM Mg. Concurrent additions involved introducing the elevated magnesium level to the culture at the moment of initiation of shock conditions (10%). Pre-conditioned cells were grown in media containing

the respective concentration of magnesium prior to the initiation of stress. A control containing 10% ethanol was included along with a media control flask, in the ethanol stress experiments. All flasks were incubated at 30°C.

5.2.2.3 NUTRIENT AND METAL ION RELATED STRESS

NUTRIENT STARVATION

Starvation of several nutrients was imposed on cultures of *Sacch. cerevisiae* (L-2226), for a period of 60min, except in the case of nitrogen starvation where the shock period was extended to 75min for optimum stress protein production (Walker & McWilliams 1989). Nutrient starvation conditions imposed were; nitrogen starvation (N_2 free media), carbon starvation (carbon free media), carbon limitation (CMMM with 250μM glucose), calcium starvation (Ca free media) and magnesium starvation (Mg free media). A control (CMMM) was included in the experiment and all cultures were incubated at 30°C.

MAGNESIUM : CALCIUM RATIOS

CMMM media was adjusted to contain a range of magnesium : calcium ratios and cultures of *Sacch. cerevisiae* (L-2226) were exposed to these conditions for 1hr. The ratios utilised were;

1000:1 (100mM Mg : 0.1mM Ca)
100:1 (10mM Mg : 0.1mM Ca)
10:1 (1mM Mg : 0.1mM Ca)
1:1 (0.1mM Mg : 0.1mM Ca)
0:0 (No Mg or Ca source added)
1:10 (0.1mM Mg : 1mM Ca)
1:100 (0.1mM Mg : 10mM Ca)
1:1000 (0.1mM Mg : 100mM Ca)
Wine (2.38mM Mg : 2.65mM Ca)

A control culture in complete CMMM was included and all cultures were incubated at 30°C.

CALCIUM SHOCK

Sacch. cerevisiae (L-2226) was exposed to conditions of calcium shock over a range of calcium concentrations. Levels to result in final calcium concentrations of; 1/2.5/5/10/50 and 100mM Ca were added to cultures, to initiate shock conditions and the production of stress proteins was analysed by polyacrylamide gel electrophoresis and autoradiography. All flasks were incubated at 30°C and a control flask (CMMM) was included in the experiment.

METAL ION REMOVAL: CHELATORS

Cultures of *Sacch. cerevisiae* (L-2226) were exposed to the chelators; EDTA (30mM), EGTA (15mM), citric acid (40mM), 8-Hydroxyquinoline (0.05mM) and sodium pyrophosphate (40mM), for 60min duration. Control cultures of CMMM and CMMM containing (b): 0.1M NaOH neutralised with HCl and (c): 0.1M HCl, were also run. All cultures were incubated at 30°C.

METAL ION REMOVAL: IONOPHORES

Sacch. cerevisiae (L-2226) cultures were treated with varying concentrations of two commercial ionophores; Magnesium Ionophore (Calbiochem) and A21387 Ionophore (Sigma), for 60min. The concentration range analysed included: 0.05/0.25/0.5/0.75 and 1.0μg/ml of the respective ionophore. Controls of CMMM and CMMM containing an equal volume of acetone/ethanol were included in the experimental set up. All cultures were incubated at 30°C.

5.2.2.4 COMPARATIVE STRESS RESPONSES IN WINE YEASTS

Responses to various stress conditions were compared in the *Sacch. cerevisiae* strains L-2226 and DBVPG2168. Stress conditions were imposed for 60min at 30°C, unless otherwise stated. Comparisons were made between; control (30°C), heat shock (42°C), ethanol stress (10%), excess calcium (100-1000mM) and deionised media (chelator: citric

acid (40mM); ionophore treatment: Mg Ionophore and A21387 (1μg/ml); ion exchange treatment: Chelex 100). Samples were analysed by SDS-PAGE and subsequent autoradiography, for the identification of stress proteins. Western blotting of samples was also carried out using antibodies specific to hsp27, hsp60 and hsp70.

5.2.3 SAMPLING METHODOLOGY

5.2.3.1 SAMPLING PROCEDURE

Samples from heat stress and ethanol stress experiments were analysed for cell numbers, % viability, trehalose levels and ion leakage (Mg and Ca: enzymatic kits), following the methods described in Chapter 2.

Presence of stress proteins was analysed for by SDS-PAGE and subsequent autoradiography. Immunoblotting to identify production of the specific proteins hsp27, hsp60 or hsp70 was also carried out. DNA analysis by CHEF was carried out on both strains of *Sacch. cerevisiae* (L-2226 & DBVPG2168) with heat shock, ethanol shock and the combined effect of these two stresses being analysed. Samples of *Sacch. cerevisiae* (L-2226), heat and ethanol shocked, and shocked under these conditions in media (CMMM and grape must) containing elevated magnesium levels (20mM) and pre-conditioned with 20mM magnesium, were also analysed by scanning and transition electron microscopy. Methods for these analyses may be found in the proceeding section.

Nutrient and metal ion related stress experiments were analysed for the production of stress proteins by SDS-PAGE and subsequent autoradiography, following the procedures described subsequently.

All SDS-PAGE gels are displayed as autoradiographs in this chapter. Qualitative and quantitative analysis of autoradiographs may be carried out using the Phoretix software described later.

5.2.3.2 SPECIFIC METHODOLOGY

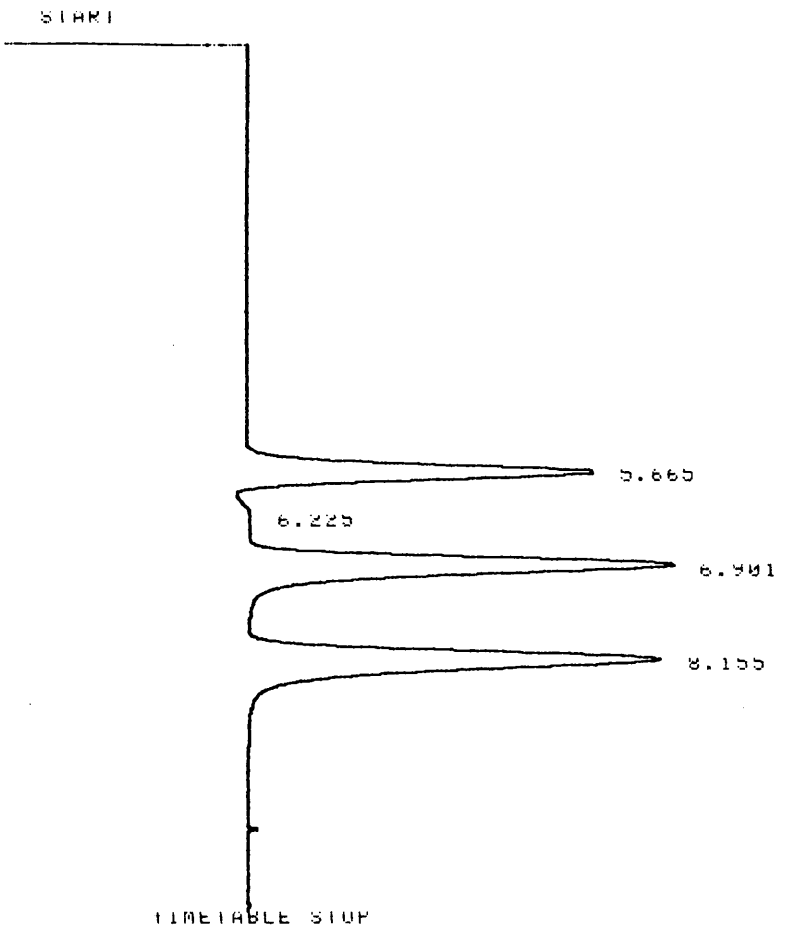
TREHALOSE DETERMINATION

EXTRACTION OF TREHALOSE FROM YEAST CELLS

10ml aliquots of experimental culture were removed and centrifuged (IEC Centra 4B Centrifuge, International Equipment Company, USA) for 10min at 3000rpm, the resultant supernatant was discarded and the cells resuspended in 0.5ml of deionised water (18Ω). Samples were boiled for 10min in a boiling water bath to disrupt the cells, after which the samples were centrifuged as before and the supernatant removed to a sterile eppendorf tube. Dry weights were measured of a 10ml culture aliquot by vacuum filtering (Vacuum Pump; N022AN18: Whatman) through GF/C filters (Whatman) and then weighing the resultant filters using a moisture analyser (Mettler LP16).

DETERMINATION BY HPLC

The levels of trehalose in the samples were determined by Isocratic HPLC using an Aminex® Ion Exclusion HPX87H column (Biorad). Samples were drawn into a syringe fitted with a 0.22μm micropore filter (Whatman) to remove any residual particulate matter. 0.5ml of the supernatant was then loaded onto the column (300mm x 7.8mm) and results were displayed in mg/ml on the integrator (Hewlett Packard HP3396A). Identification of trehalose by retention time, from an external sample mixture (using a three point calibration), was achieved (Figure 5.3). Trehalose concentrations were calculated as; mg Trehalose/g DW cells.



RUN# 12/03 FEB 24, 1997 11:39:51

TREHALOSE ANALYSIS

ESTD-HEIGHT

RT	TYPE	AREA	WIDTH	HEIGHT	CAL#	MG/ML	NAME
5.665	BP	2311290	.233	165521	1R	1.964	GLYCOGEN
6.901	PB	317024	.268	19569	2R	1.839	TREHALOSE
8.155	BB	3365133	.295	190388	3R	1.820	GLUCOSE

TOTAL HEIGHT= 558462
MUL FACTOR=1.0000E+00

Figure 5.3 HPLC Calibration Profile for Trehalose

PROTEIN ANALYSIS

YEAST CELL DISRUPTION

Harvested cells were washed twice with 5ml volumes of ice-cold 10mM TRIS buffer, pH6.8, then resuspended in 200µl Yeast Homogenisation Buffer (Protease Inhibitor Cocktail Kit, ICN Biomedicals) and mixed with 0.50g of acid-washed glass beads (0.45µm diameter, Sigma). Disruption was carried out by vigorously vortexing (maximum resonance) for 5 x 1min intervals, with intermittent cooling on ice. A hole was pierced in the base of the eppendorf tube containing glass beads and broken cell preparation, this tube was fitted through the lid of a 10ml plastic centrifuge tube and centrifuged at 2500rpm, 15min/4^{o}C (Cooled Centrifuge: CR312, Jouan). The supernatant containing cytoplasmic proteins (cloudy) was removed and cell breakage was assessed microscopically. 100µl supernatant was mixed with an equal volume of 2X Sample Buffer (0.0625M TRIS-HCl; pH6.8, 2.0% SDS, 5.0% β-mercaptoethanol, 10% glycerol and 0.001% Bromophenol Blue) and this mixture boiled for 4 min, to completely dissociate proteins. Water used in all stages of cell disruption was ultra-pure dH_2O (18Ω).

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Protein analysis by SDS-PAGE was performed according to Laemmli (1970), using a 10% single concentration resolving gel with a 3% stacking gel (Appendix 5.1). SDS-PAGE was conducted on cytoplasmic extractions of whole yeast cells. All fractions were vortexed with an equal volume of 2X Sample Buffer (0.0625M TRIS-HCl; pH6.8, 2.0% SDS, 5.0% β-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue) and boiled for 4min in a 100^{o}C water bath, to completely dissociate proteins. Electrophoresis was performed on large slab gels (SE600; Hoeffer Scientific Instruments, CA, USA) at 30mA/gel, 30min: Stacking gel, then 15mA/gel for 3-4hrs at room temperature (PS500X: Electrophoresis Power Supply; Hoeffer Scientific Instruments, CA, USA). A radiolabelled marker protein (Rainbow™ [^{14}C] methylated protein molecular weight marker: High Molecular Weight Range 14300 - 220000; Amersham International Plc., UK. Table 5.2) was included on all

gels as a molecular weight standard. Gels were colourised by silver staining. Water used in all stages of protein analysis was ultra-pure dH_2O (18Ω).

Table 5.2 SDS-PAGE Molecular weight standard:
High molecular weight range 14300 -220000 (Amersham)

Protein	Molecular Weight
Myosin	220 000
Phosphorylase b	97 400
Bovine Serum Albumin	66 000
Ovalbumin	46 000
Carbonic Anhydrase	30 000
Trypsin Inhibitor	21 500
Lysozyme	14 300

SILVER STAINING OF SDS-PAGE GELS

Gels were stained following the silver staining method of Blum *et al.* (1987). Gels were placed in 250ml fix (50% methanol, 12% acetic acid and 0.5ml 37% formaldehyde solution in 1L distilled water) for at least 60min at $4^{o}C$. The staining procedure commenced with three washes of 20min duration in Wash A (50% ethanol) followed by 1min in Pretreat*# (0.2g sodium thiosulphate.$5H_2O$/L), three 20sec rinses* in ultra-pure water and then a 20min Impregnate*# (2g silver nitrate (anhydrous), 0.75ml 37% formaldehyde solution/L). After two 20 second rinses* in ultra-pure water, the gel was developed# (60g sodium carbonate (anhydrous), 0.5ml 37% formaldehyde solution, 4mg sodium thiosulphate.$5H_2O$/L) for as long as required to colourise the protein bands. Two rinses of 2min duration in ultra-pure water were made prior to a 10min period in Stop solution (50% methanol, 12% acetic acid/L), followed by a wash of 20min duration in Wash B (50% methanol).

All stages of staining were carried out at room temperature on a rotary shaker (Orbital Shaker 501; Stuart Scientific Co., UK). Gels could be stored in 50% methanol at 4°C for 3-4 weeks, or dried immediately. Timing of stages marked * was critical. Solutions marked # had to be made fresh immediately prior to use.

DRYING OF SDS-PAGE GELS

Stained gels were placed in Predry 1 (30% methanol) on a rotary shaker (Orbital Shaker 501; Stuart Scientific Co., UK) at 4°C for 30min, followed by a 30min wash in Predry 2 (3% glycerol) at 4°C.

Radioactive gels were then laid on 3MM Chromatography paper (Whatman, UK), covered with a sheet of acetate and placed in the slab gel dryer (LKB Bromma 2003 Slab Gel Dryer; Hoeffer Scientific Instruments, CA, USA). Gels were dried for 75min under a vacuum of 15mbar.

Non-radioactive gels were laid between pre-wetted cellophane sheets (Pharmacia Biotech, CA, USA) and rapidly air dried using a gel dryer (Easy Breeze Gel Dryer; Hoeffer Scientific Instruments, CA, USA).

AUTORADIOGRAPHY

Dried gels were laid down against X-ray film (Hyperfilm™ β-max; Amersham, UK) in a gel cassette for a defined period of time, dependent on the levels of radioactivity (initially 10 days). Autoradiographs were developed and fixed using, 5min immersion in developer (D19 Developer, Kodak), 1 minute wash in running water, followed by 5min immersion in fixative (Hypam; Ilford, UK) and a final 1min wash in running water. Films were then allowed to air dry. All stages of this procedure were carried out in a photographic darkroom.

ANALYSIS OF AUTORADIOGRAPHS

Autoradiographs were scanned onto computer disc using a Hewlett Packard ScanJet 4P scanner linked to the software; Corel PhotoPaint. Images (200 x 200 resolution: 256 grey scale) were manipulated using a Quantimet Image Analyser (Quantimet 600S; Leica, UK) and the software package; Quantimet Q600S. Autoradiograph images were then analysed using the Phoretix 1D Lite software (Phoretix International Ltd, UK). Lane analysis and measurement of molecular weights of protein bands present was carried out by comparison to and calculation from the molecular weight marker (Table 5.2) lane run on each gel (Figs 5.4 & 5.5). Each sample lane of the autoradiographs analysed was treated in the same manner and molecular weight values recorded for all protein bands (Fig 5.6). Identification of shock proteins was accomplished by matching comparison to the control (30^{O}C) lane of the respective gel, any protein band present in the sample lane and not present in the control lane was deemed to be a shock protein (Fig 5.7).

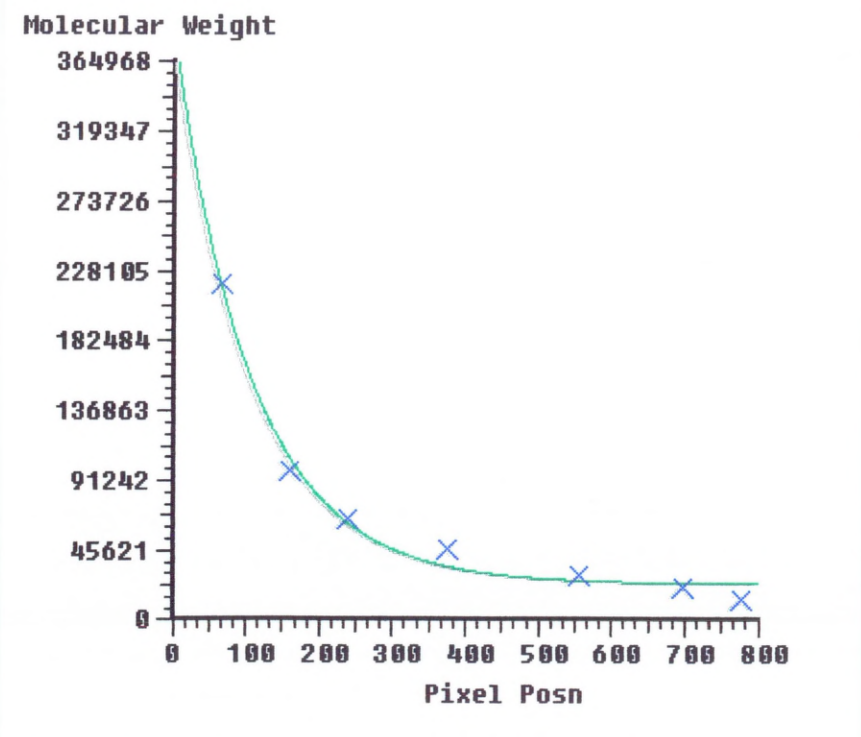


Figure 5.4 Molecular weight calibration curve of Rainbow™ molecular weight marker: 14300-220000 (Amersham).

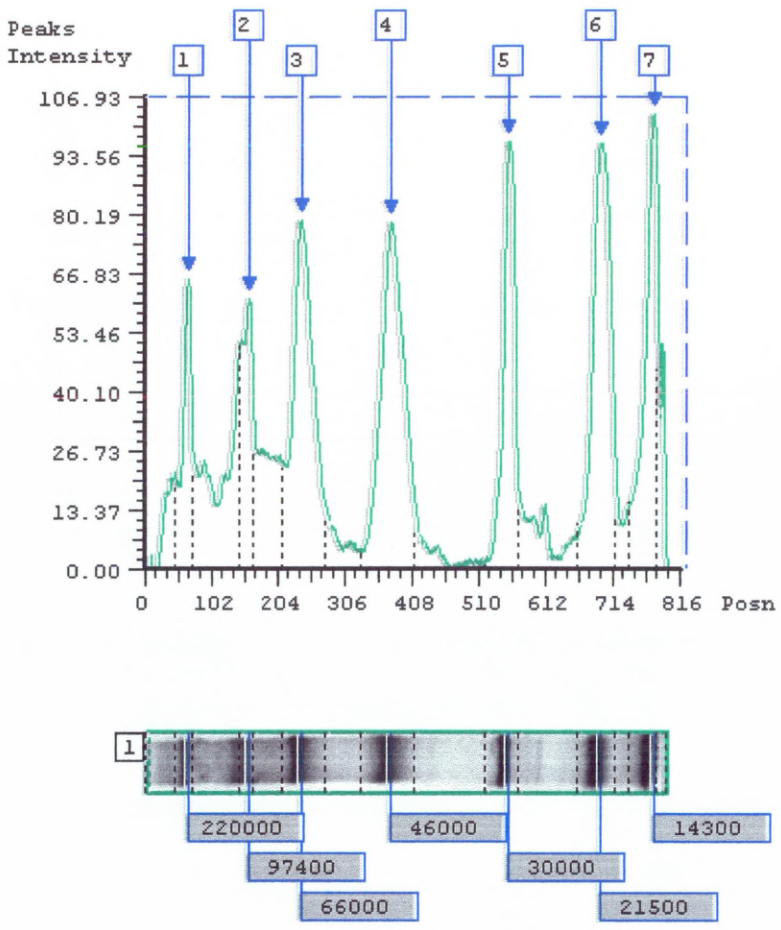


Figure 5.5 Lane analysis profile of Rainbow™ [^{14}C]-methylated protein molecular weight marker: High molecular weight range 14300-220000 (Amersham).

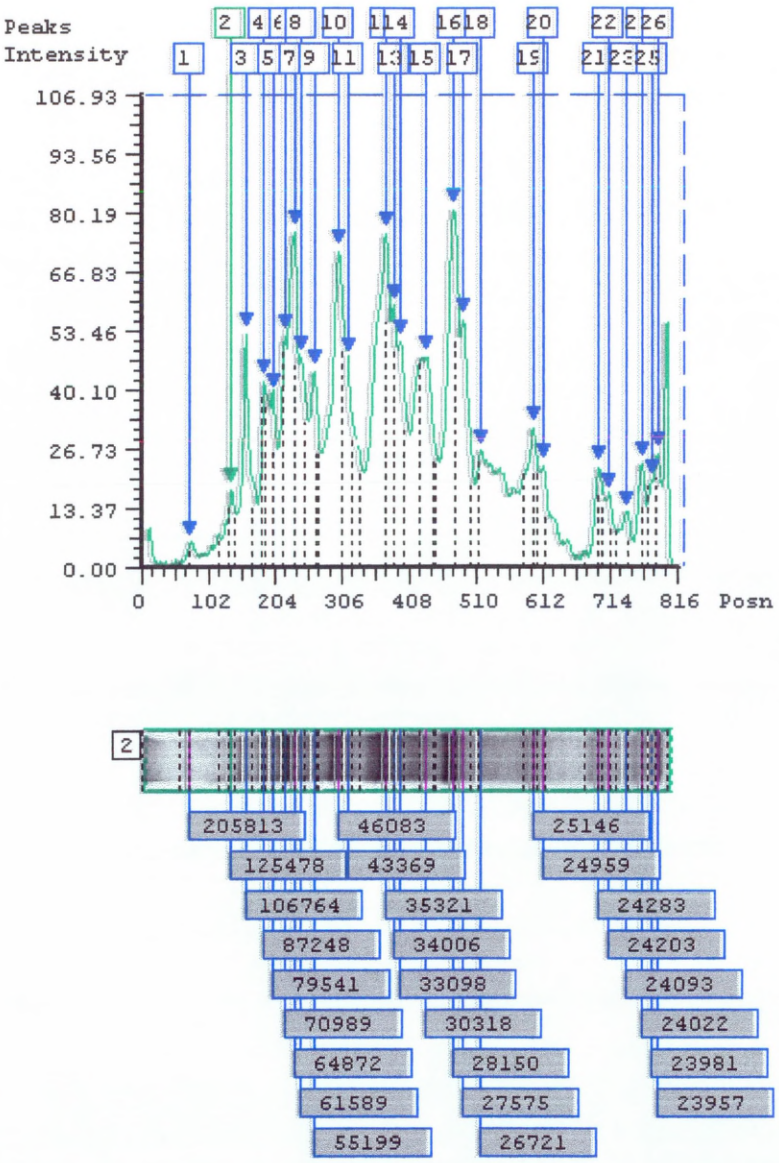


Figure 5.6 Typical lane analysis profile for heat shocked (42°C) yeast (*Sacch. cerevisiae* L-2226).

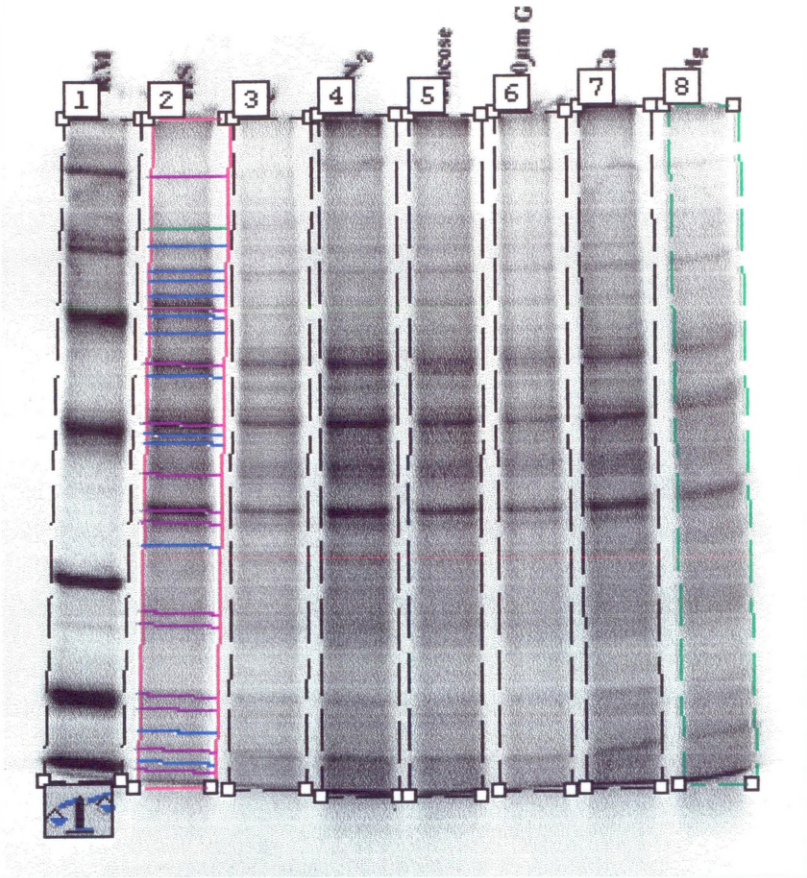


Figure 5.7 Typical example of matching protein profiles of heat shocked (42^{o}C) and control (30^{o}C) yeast (*Sacch. cerevisiae* L-2226).

IMMUNOBLOTTING - WESTERN BLOTTING OF PROTEINS

BLOTTING PROCEDURE

Electrophoresis was performed on mini-gels (Dual Mini Vertical System: FEC185, Fissons) at 100mV for 1-2hr at room temperature (Electrophoresis Power Supply E455, Consort). Electrophoretic transfer to nitrocellulose transfer membrane (0.45μm pore size; HyBond™ NC: Whatman) was carried out at 100V for 1hr (Mini Trans Blot Transfer Cell, Biorad; Appendix 5.2). Nitrocellulose sheets were rinsed briefly in TBS, then blocked with blocking solution (Appendix 5.2) for 1hr with agitation and rinsed in TBS, prior to the primary antibody incubation (rabbit anti-hsp70 & anti-hsp60, human-specific rabbit anti-hsp27 and control; normal rabbit serum; 1:1000 dilution in TBS: DAKO Corporation, UK) for 1hr at room temperature with constant gentle agitation. This incubation stage was followed by four washes of 5min duration in TBS and then the species-specific second antibody (HRP-linked anti-rabbit; 1:500 dilution in TBS: DAKO Corporation, UK) incubation for 1hr at room temperature with constant gentle agitation. Two washes in TBS-Tween20 were followed by two washes in TBS, all of 5min duration and the blot was then developed in accordance with the species-specific second antibody.

DEVELOPMENT OF IMMUNOBLOTS

Nitrocellulose sheets following immunoblotting (HPO labelled second antibody) were developed using chromogen (Appendix 5.2). Developing reagent was added to the nitrocellulose sheet in a rocking shaker (Platform Shaker STR6; Stuart Scientific, UK) and colour development was monitored (approx. 5-30min). The developing reaction was stopped by three washes in dH_2O for 60min duration each. Nitrocellulose sheets were then dried and photographed (B/W film: 400ASA).

DNA ANALYSIS

EXTRACTION OF WHOLE CHROMOSOMAL DNA

Whole chromosomal DNA was extracted from yeast cells following the protocol of Cardinali (1993; Cardinali *et al.* 1995), modified from the method of Schwartz & Cantor (1984). 2 x 10^9 cell/ml of *Sacch. cerevisiae* culture was centrifuged (J2-21 Centrifuge, Beckman) at 3000rpm/3min and 0^oC. Cell pellet was resuspended in 5ml ddH_2O, pre-cooled to 4^oC and then centrifuged as before. Cells were then resuspended in 5ml 50mM EDTA, pH7.5 (pre-cooled to 4^oC) and centrifuged. Resuspension of cells in 1ml SPGZ (137mg NaH_2PO_4, 50ml glycerol, 50ml ddH_2O + 5-6mg/sample of lysing enzyme: *Rhizoctonia solani*) was followed by incubation at 40^oC for 60min, until approximately 60% protoplast formation had occurred (determined microscopically). For each sample, 1ml 125mM EDTA, pH7.5 containing 1% Agarose was prepared and mixed with the cells in SPGZ, poured into a pre-cooled (4^oC) mould and placed in the fridge for 15-20min. Plugs were then incubated in 2-3ml LET (90ml EDTA 500mM, pH7.5; 1ml TRIS-HCl 1M, pH7.5; 9ml ddH_2O) at 37^oC for 1-4hr. LET solution was replaced with 1.5ml of NDSP (180ml EDTA 500mM, pH9.0; 2ml TRIS-HCl 1M, pH7.5; 2g SDS; 18ml ddH_2O + 1mg/ml Proteinase K: Sigma) and the plugs incubated for 4-16hr at 50^oC. Following this incubation period, plugs were washed three times with 50mM EDTA, pH7.5 at 4^oC and then stored in 500mM EDTA, pH9.0 at 4^oC, until required for analysing by electrophoresis.

CHEF ANALYSIS OF DNA

Contour Clamped Homogeneous Electric Field Pulse Field Gel Electrophoresis is an advanced pulse field system based on the CHEF design of Chu (1989).

A 1% Agarose (Type II-A EEO, Sigma) gel was prepared in 0.5X TBE (0.045M TRIS-Borate, 0.001M EDTA, pH8.0). Sample plugs were loaded into the gel and the gel orientated into the CHEF electrophoresis unit (CHEF-DRII, Biorad Laboratories, CA, USA). Electrophoresis was carried out at 200V, 120mA for 18hr/90sec pulse and

12hr/60sec pulse, 120^{o} angle, 6.0Vcm^{-1}. Gels were stained with ethidium bromide (0.1%, Sigma) for approximately 30-45min at 4^{o}C. Following a brief rinse in ddH_2O (4^{o}C) gels were visualised on a transilluminator (Variable Intensity Transilluminator TVL312A; 312nm Ultraviolet: Spectroline) and photographed using a red filter with a Polaroid camera (B/W 667 Film, ASA 3000/36^{o}).

The chromosome standard strain for *Sacch. cerevisiae* (YNN295; Biorad) was also run on every CHEF gel as a standard lane (Table 5.3).

Table 5.3 Molecular weights of chromosomes of *Sacch. cerevisiae* standard strain (YNN295, Biorad)

Chromosome No.	Molecular Weight (Kbp)
XII	2200
IV	1600
XV, VII	1125
XVI	1020
XIII	945
II	850
XIV	800
X	770
XI	700
V	630
VIII	580
IX	460
III	370
VI	290
I	245

ANALYSIS OF CHEF GELS

Images of CHEF gels were captured using a KAPPA (Kappa Messtechnik GmbH: Gleichen, Germany) B/W video camera connected to a Macintosh Quadra 950 computer by

means of a NuVista+ video card. After capture, images were inverted and converted to 256 grey scale (TIFF format) using the imaging software; Adobe Photoshop. Densitometric analysis was carried out with the NIH-Image package (NIH: Bethesda, USA) by subtracting the background with the "2D-rolling ball" algorithm and marking gel lanes. Peaks were marked (density vs. migration plot) and molecular weights automatically calculated from a known marker (Table 5.3) lane, using a polynomial 3rd degree regression equation. Molecular weight values for each single band were transferred to a Kaleida Graph file (Macintosh).

Statistical Analysis of CHEF Gels

CHEF gels were analysed by Local Significant Difference (LSD) analysis of gel banding patterns (Cardinali & Martini 1997). The LSD procedure compares differences of molecular weight between test and control samples with differences of molecular weight between two standard patterns, also used to produce the molecular weight vs. migration distance calibration curve. Corresponding bands from the two marker lanes have by definition the same molecular weight, however different values of calculated molecular weights can be obtained if migration distances are not identical. The basic assumption is that differences of band migration, and therefore molecular weight, in electrophoretic experiments can be due to some sort of error. Differences between the calculated molecular weight of corresponding bands of the marker patterns produce a series of Local Reference Values (LRVi) representing the level of error in each area of the gel. LSD is defined as follows:

$$LSDi = \delta i \geq 2LRVi$$

Therefore, by pairwise comparison, two bands belonging to two patterns can be regarded as different only when the difference between them is at least 2 times the difference found in that region of the gel between corresponding bands of two identical molecular weight standards, *i.e.* the reference value of that area.

SCANNING ELECTRON MICROSCOPY (SEM)

SAMPLE PREPARATION FOR SEM

Fixation/Dehydration

Cells were harvested by centrifugation (J2-21 Centrifuge, Beckman). Cell pellets were washed in Cacodylate buffer (0.1M, pH7: Appendix 5.3) at 4^{O}C, 3 drops of 3% gluteraldehyde were added, the suspension vortexed and then centrifuged (3000rpm/4min). Cells were resuspended in 3% gluteraldehyde in cacodylate buffer for 1hr at 4^{O}C, washed twice with cacodylate buffer (8min duration) and then resuspended in 3% gluteraldehyde overnight at 20^{O}C. Cells were resuspended in two passages of 50% ethanol for 10min each and then centrifuged. Dehydration through a graded alcohol series involving a 10min passage in 70% ethanol, followed by 10min in 80% ethanol and a final 15min passage in 100% ethanol was carried out, with centrifugation following each step of the dehydration procedure. The final ethanol concentration was discarded and the cells were placed in a filter paper pocket.

Filters were placed inside a Critical Point Dryer (Balzers Union) and drying was carried out for 5min at 40^{O}C/62' Atms. Filters were removed after drying and opened carefully. EM stubs with double-sided sticky tape discs were lightly pressed onto the surface of the filter to pick up cells.

Gold Coating and Viewing of Samples

Stubs were placed in a SEM Coating Unit (ES100: Polarton Equipment Ltd) for gold coating. Coating was carried out for 3min to a thickness of 18.3Å Gold. Samples were then ready for viewing by Scanning Electron Microscopy (Philips 501 SEM). Samples were viewed at various magnifications up to 5000x. Photographs of cells were taken on black and white film ISO 25/15^{O} (AGFA AGFAPAN APX25, 120 Professional).

TRANSITION ELECTRON MICROSCOPY (TEM)

SAMPLE PREPARATION FOR TEM

(Based on a modified protocol of the Centraalbureau voor Schimmelcultures, Netherlands).

Fixation/Dehydration

Cells were harvested by centrifugation (J2-21 Centrifuge, Beckman). Cell pellets were washed in Cacodylate buffer (0.1M, pH7: Appendix 5.3) at $4^{O}C$, 3 drops of 3% gluteraldehyde added, the suspension vortexed and then centrifuged (3000rpm/4min). Cells were resuspended in 3% gluteraldehyde in cacodylate buffer for 1hr at $4^{O}C$, washed twice with cacodylate buffer (0.1M, pH7) and each wash was left for 8min after vortexing, prior to centrifuging. Cells were then resuspended in 1% OsO_4 (Osmium tetroxide) in cacodylate buffer for 2hr at $4^{O}C$. A final washing of cells in cacodylate buffer (2x, 8min washes) was carried out prior to dehydration in ethanol. Dehydration steps were carried out in centrifuge tubes, through a graded alcohol series. A 10min passage in 50% ethanol was followed by a 10min passage in 70% ethanol, then a 10min wash in 80% ethanol, 15min in 100% ethanol and finally a second 15min passage in 100% ethanol. Centrifugation followed each step of the dehydration procedure and the supernatants were discarded.

Embedding and Viewing of Samples

The final ethanol wash (100%) was removed and propylenoxide was added to the cell pellets. After 15min, propylenoxide was refreshed and left for a further 15min at room temperature, this was then removed and replaced with a mixture of propylenoxide and resin (1:1 ratio) for 3hrs at room temperature. After this time, the mixture was then removed and replaced with pure resin (Appendix 5.3) and left overnight. A loopful of this mixture was placed towards the top of a resin capsule and the resin polymerised by incubation of the mould at $60^{O}C$/48-72hr. Capsules were then ready for ultra-thin sectioning using an ultra-microtome. Following sectioning, samples were mounted on copper grids, stained and viewed by Transition Electron Microscopy (Philips EM400T TEM), at various magnifications up to 28000x. Photographs of cells were taken using Kodak™ electron microscope film (ESTAR™ Thick Base 4489; Kodak, NY, USA).

5.3 RESULTS AND DISCUSSION

Industrial yeast, during growth and fermentation may be subject to a variety of physical and chemical insults collectively referred to as "stress" and wine yeasts are no exception. Yeast cells respond to such adverse environmental conditions initiating a survival instinct which is multifaceted. Stress responses in yeast are characterised mainly by the occurrence of heat shock proteins (hsp's) or stress proteins (Miller *et al.* 1982). This, however, is not the only response to stress executed by cells; increases in levels of trehalose and glycerol (heat and osmostress), alterations in membrane lipid composition (heat and ethanol stress), modulation of ion exchange processes (pH and ethanol stress), production of free radicals and superoxide dismutase (SOD) enzymes (oxidative stress) are also observed in response to the indicated stresses (Mishra & Prasad 1989; Petrov & Okorokov 1990; Attfield *et al.* 1992; Jozwiak & Leyko 1992; Ribeiro *et al.* 1994; Davidson *et al.* 1996). Combinations of these factors are involved in conferring tolerance to stress and their relative importance varies according to the type of stress and the physiological state of the yeast cultures.

Magnesium is known to be beneficial to yeast cells. As an essential inorganic ion it plays important roles in yeast cell physiology. It is involved in many physiological functions including growth, cell division and enzyme activity (Walker 1994). Magnesium ions have been implicated, by several studies, in the amelioration of the detrimental effects of ethanol, high osmotic pressure and temperature inhibition in *Sacch. cerevisiae* (Dombek & Ingram 1986; D'Amore *et al.* 1988; Dasari *et al.* 1990).

5.3.1 HEAT STRESS

The rate of yeast growth and alcoholic fermentation increases as the temperature increases, with maximum rates generally occurring at temperatures between 20^{o} & 25^{o}C (Amerine *et al.* 1980) however, temperatures above $35^{o}/40^{o}$C are generally regarded as a sub-lethal

heat shock and it is at these temperatures where problems occur. It must be stressed that the fermentation itself generates heat. Unless cooling is applied, the fermentation will not be isothermic and a temperature increase of 5-10^{o}C may occur from the beginning to the end of fermentation (Fleet & Heard 1993). Cooling control is particularly important if there is a possibility of the temperature increasing above 30^{o}C, where heat stress may occur and the sensitivity of *Sacch. cerevisiae* to ethanol is enhanced, possibly resulting in a premature arrest of fermentation (van Uden 1989). The financial implications of cooling on a large scale leads on to the search for other methods of yeast cell protection.

The cellular response to heat stress is varied, affecting many aspects of cellular metabolism and physiology. The most obvious effect of a sub-lethal heat stress on yeast cells is the effect on viability. Figure 5.8 shows a dramatic reduction in viability of *Sacch. cerevisiae* (L-2226) at 42^{o}C of around 55% over 5 hours, at media magnesium levels of 2mM. Elevation of Mg^{2+} in the growth medium to 20mM on initiation of the shock conditions, reduced the mortality effect of prolonged heat shock, with viability levels remaining above 55% following five hours of temperature shock. Viability levels at 30^{o}C over this period remained high (>90%). The long term protection (24h) of this yeast can also be seen in Figure 5.8, where at 2mM Mg viability dropped to 2.6% yet, a 10-fold improvement in survival of cells with 20mM Mg addition was observed (22.5% @ 24h). Control (30^{o}C) levels over this extended period remained high (2mM: >60%; 20mM Mg: >85%).

Cells react in response to physical or chemical stresses, with several types of mechanisms contributing to the phenomenon of stress tolerance in yeast. One such process thought to be closely related to the stress response in yeast cells is the synthesis of trehalose. Yeast cells exponentially growing on glucose contain little trehalose (Thevelein 1984). As a result of sub-lethal heat stress, cells accumulate a large cytoplasmic pool of trehalose, this is thought to act primarily as a stress protectant, rather than a storage carbohydrate, since levels generally show a good correlative relationship with thermotolerance (Wiemken 1990). The exact role of trehalose is unknown as yet, it may play a role in protection or repair processes or as a fermentable energy source for cells in repair (Attfield *et al.* 1994).

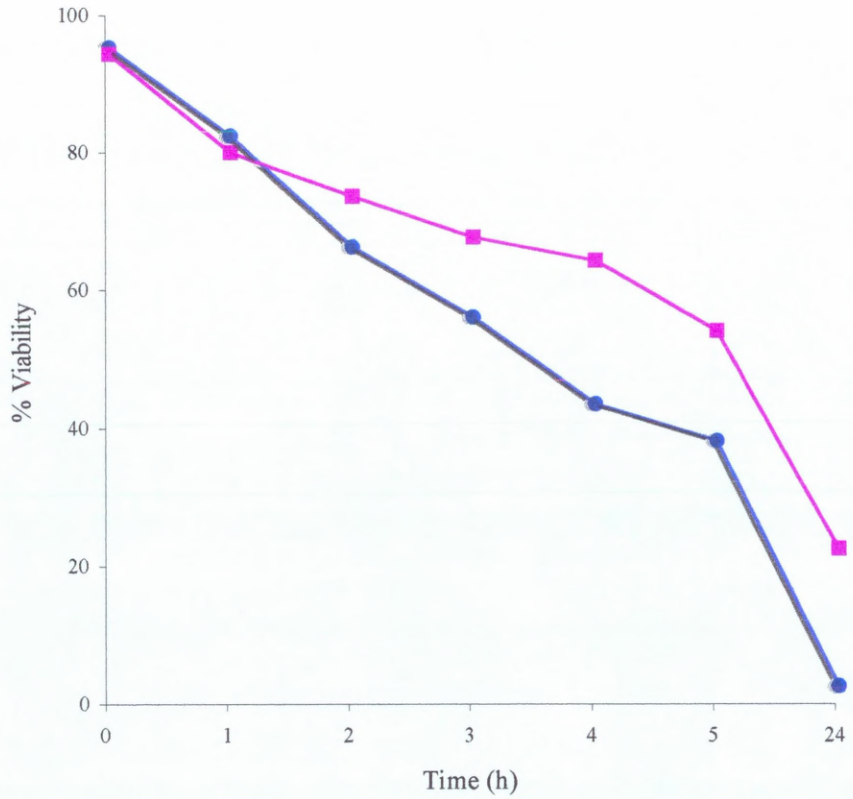


Figure 5.8 Magnesium induced thermo-protection (42^{o}C) of wine yeast (L-2226). -●- 2mM Mg, -■- 20mM Mg.

The enzymes responsible for trehalose synthesis and thermotolerance are regulated by cAMP-dependent phosphorylation (Thevelein 1988). Irrespective of the exact route of the trehalose pathway all enzymes require Mg^{2+} for full activity, thus intracellular variation in magnesium levels would be thought to be important for control of metabolic flux through the pathway (Ribeiro *et al.* 1994). Figure 5.9 shows the levels of trehalose in the wine yeast (L-2226) at high and low magnesium. It can be observed that levels of the carbohydrate did not differ greatly on elevation of temperature to a sub-lethal shock level. Trehalose levels at $30^{o}C$ remained constantly low over a five hour shock period, irrespective of magnesium concentration on initiation of shock, except for an initial drop over the first hour of shock in 2mM Mg cultures. Both cultures (2mM Mg & 20mM Mg) exhibited a rise in trehalose levels following prolonged growth (24h) at $30^{o}C$. On elevation of growth temperature to $42^{o}C$ (sub-lethal heat shock) cellular trehalose levels steadily increased following an initial drop over the first hour of shock, to a level of 2% (w/w) after 5h heat shock in cultures grown in 2mM Mg. An increase of magnesium levels in the growth medium to 20mM, kept trehalose levels fairly constant after an initial rise in levels during the first hour, with levels remaining under 1% (w/w) over prolonged shock (24h). These results suggest that trehalose plays a role in the heat shock response, however, the elevation of magnesium levels counteracts to some degree the requirement for this carbohydrate and thus levels are maintained at a low level.

In altering extracellular magnesium levels to observe the effect on the stress response it was thought pertinent to consider the ionic status of the cells during the shock period. Extracellular magnesium and calcium status was observed over a 1h shock period and Figure 5.10 shows cellular magnesium leakage during heat shock of *Sacch. cerevisiae* (L-2226) grown in various concentrations of Mg^{2+}. Control levels (no shock) rise initially in the first 15min of shock and then remain relatively constant during the shock period. A similar response is exhibited of cultures grown in 2mM Mg (media control). Elevation of magnesium concentration to 5/10/15 or 20mM, on initiation of heat shock, results in an increase in extracellular magnesium levels over the first 15min of shock which then levelled off until the last 15min of the shock period where a drop in levels occurred, except in the

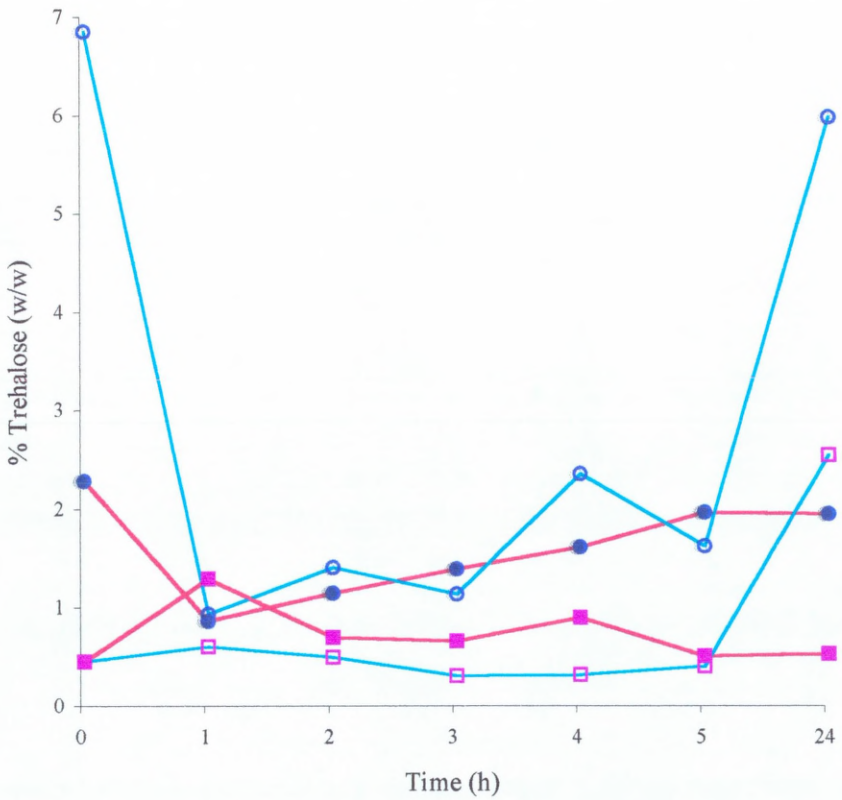


Figure 5.9 Trehalose levels during heat stress (42°C) of wine yeast (L-2226) at high and low magnesium. ●/○ 2mM Mg, ■/□ 20mM Mg, — 30°C, — 42°C.

case of 10mM Mg. Results of ion concentrations under conditions of 50mM Mg appear very erratic and these discrepancies may be due to experimental error. Patterns of extracellular calcium under similar conditions (Fig. 5.11) generally also appear to mimic that of the control (no shock) culture with, as in magnesium leakage studies, an initial rise over the early stages of shock, followed by a shorter constant period and then a dramatic drop to near zero levels. Cultures grown in 5/15 and 20mM however, exhibited a rise in levels of extracellular calcium in the latter stages of shock. From these results it would seem that the situation of calcium leakage from cells appears to be inconclusive, however, with magnesium leakage in the initial stages of shock cells grown in the lower concentrations of magnesium continue to leak essential ions out of the cell whereas other cells actively or otherwise uptake ions for cellular processes.

On a more subtle level the heat shock response is activated to protect or repair cells under stress. This is a transient molecular response cumulating in the alteration of normal patterns of protein synthesis and the production of heat shock proteins (hsp's). These hsps can be split into three broad groups; 85-110kDa, 60-80kDa and <50kDa and proteins found in these various groups have a range of functions. The effects of a heat challenge on protein synthesis were assayed by determination of TCA-precipitable incorporation in pulse-labelled extracts. Figure 5.12 shows the effect of altered magnesium concentration on production of hsps at 42^{o}C in wine yeast (L-2226). Table 5.4 lists the presence of known yeast hsps across the range of the three main groups. Following 1 hour of heat shock in *Sacch. cerevisiae* (L-2226) hsps were visible at: 150, 104, 90, 83, 70, 60, 46, 30 and 26kDa, compared to the 30^{o}C control lane (Fig. 5.12). Repression of these hsps was exhibited on concurrent addition of levels of Mg^{2+} as low as 5mM and certainly over the range 5-20mM (Fig. 5.12/Table 5.4). At high levels of magnesium (50mM) certain shock proteins remain visible, however, this may be due to a combination of stresses *i.e.* both heat stress and a shock effect occurring due to excess ion levels.

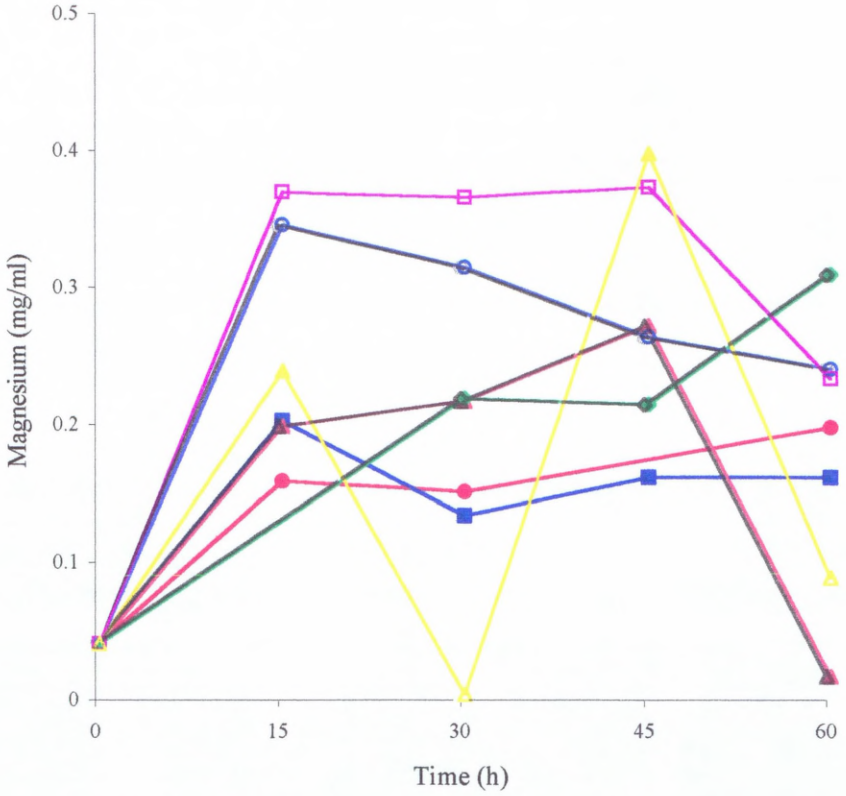


Figure 5.10 Leakage of cellular magnesium during a 1h period of heat shock (42°C) with concurrent magnesium addition. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

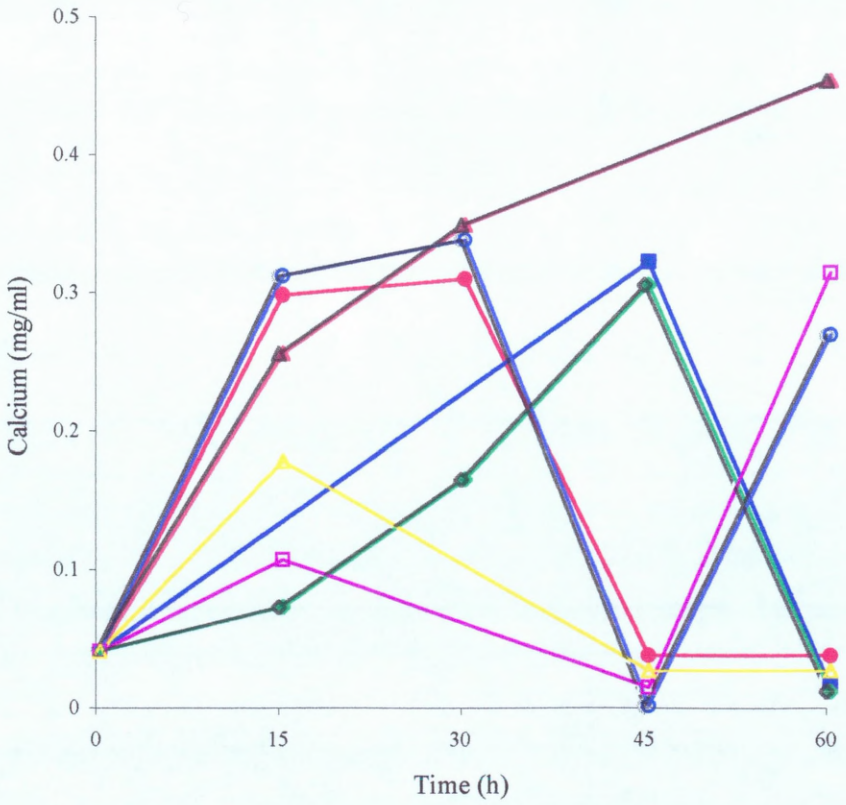


Figure 5.11 Leakage of cellular calcium during a 1h period of heat shock (42°C) with concurrent magnesium addition. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

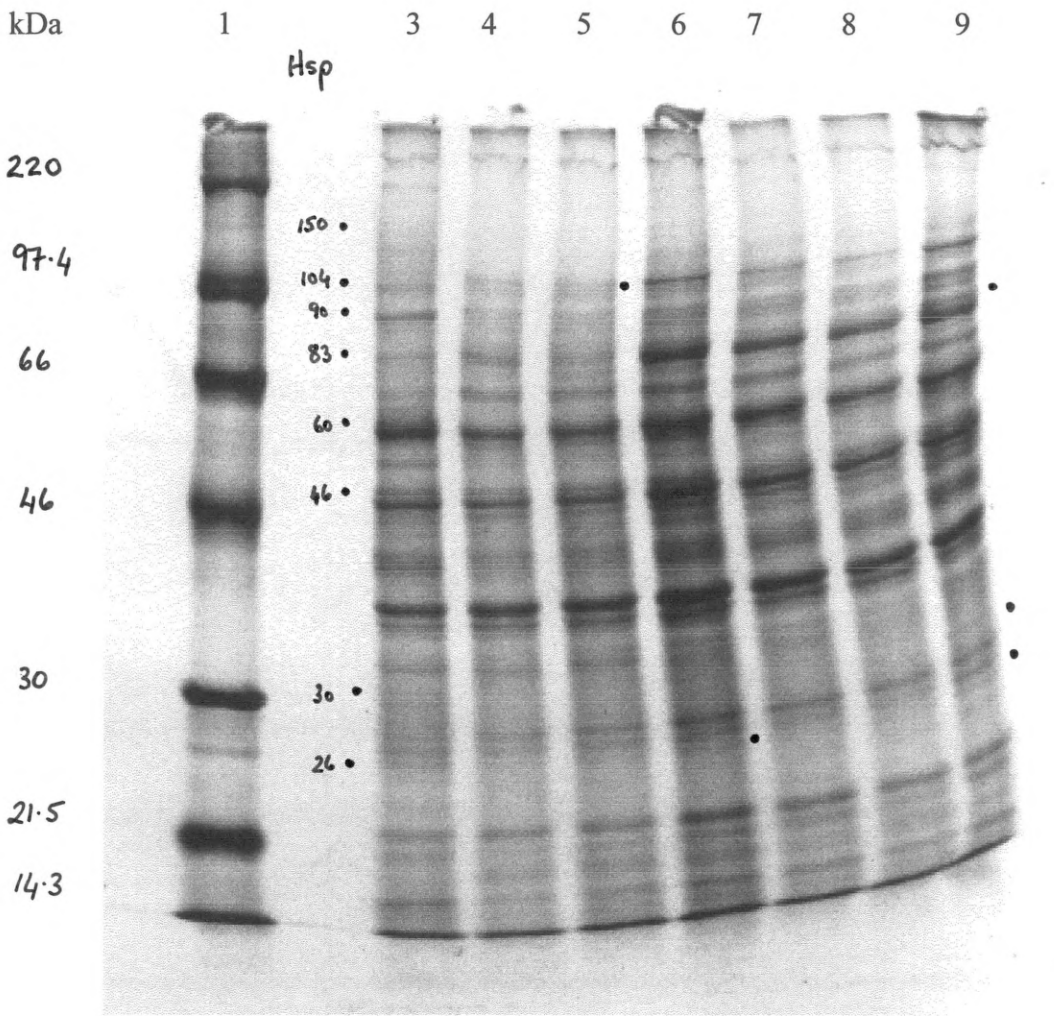


Figure 5.12 Effect of altered magnesium concentration on production of heat shock proteins at 42^{o}C in wine yeast (L-2226). Lanes: 1) Rainbow™ Marker, 3) 2mM Mg, 4) Control (30^{o}C), 5) 5mM Mg, 6) 10mM Mg, 7) 15mM Mg, 8) 20mM Mg and 9) 50mM Mg.

Table 5.4 Effect of concurrent additions of varying concentrations of magnesium on heat shock protein production at 42°C in wine yeast (L-2226).

Heat Shock Protein	Concurrent Magnesium					
(kDa)	2mM	5mM	10mM	15mM	20mM	50mM
hsp150	✓	×	×	×	×	×
hsp104	✓	✓	×	×	×	×
hsp90	✓	×	×	×	×	×
hsp83	✓	×	×	×	×	✓
hsp70	✓	×	×	×	×	×
hsp60	✓	×	×	×	×	×
hsp46	✓	×	×	×	×	×
hsp30	✓	×	×	×	×	✓
hsp26	✓	×	✓	×	×	✓
hsp12	×	×	×	×	×	×

Note: ✓ = protein present, × = protein absent

Preconditioning of cells with elevated concentrations of magnesium was also investigated to assess whether the involvement of magnesium in yeast stress responses was predominantly intra- or extra-cellular. Overnight preconditioning of yeast cells in concentrations of magnesium equivalent to the ensuing experimental conditions was carried out in an attempt to saturate the intracellular stores of this essential ion prior to exposing cells to heat stress. Elevation of Mg^{2+} in the growth medium to 20mM (Fig. 5.13) was found to reduce the mortality effect of prolonged (5h) heat shock, with the maintenance of viability levels above 60% following temperature elevation. This beneficial effect was not, however, held over long term (24h) shock and although survival levels of *Sacch. cerevisiae* (L-2226) remained higher than that of normal media (2mM Mg) levels there was still a dramatic drop in the percentage of survivors compared to control (30°C) levels (2mM: >65%; 20mM: >85%).

Trehalose levels (Fig 5.14) on magnesium-preconditioning followed a pattern very similar to that exhibited by cells undergoing stress with concurrent magnesium additions. Under

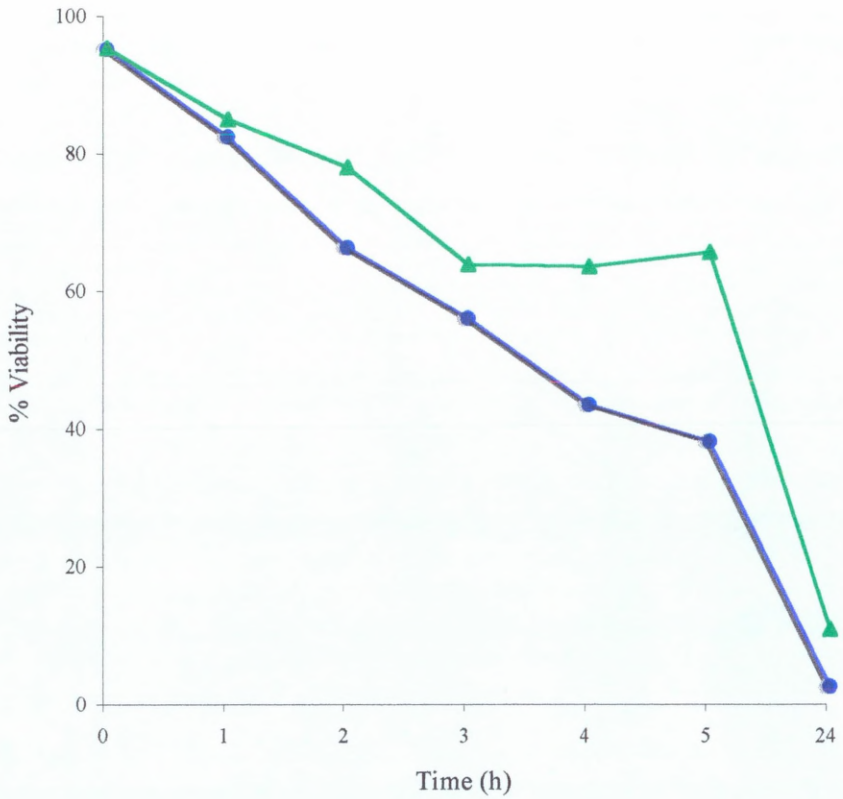


Figure 5.13 Effect of magnesium preconditioning on the induction of thermo-protection (42^{o}C) in wine yeast (L-2226). -●- 2mM Mg, -▲- 20mM Mg.

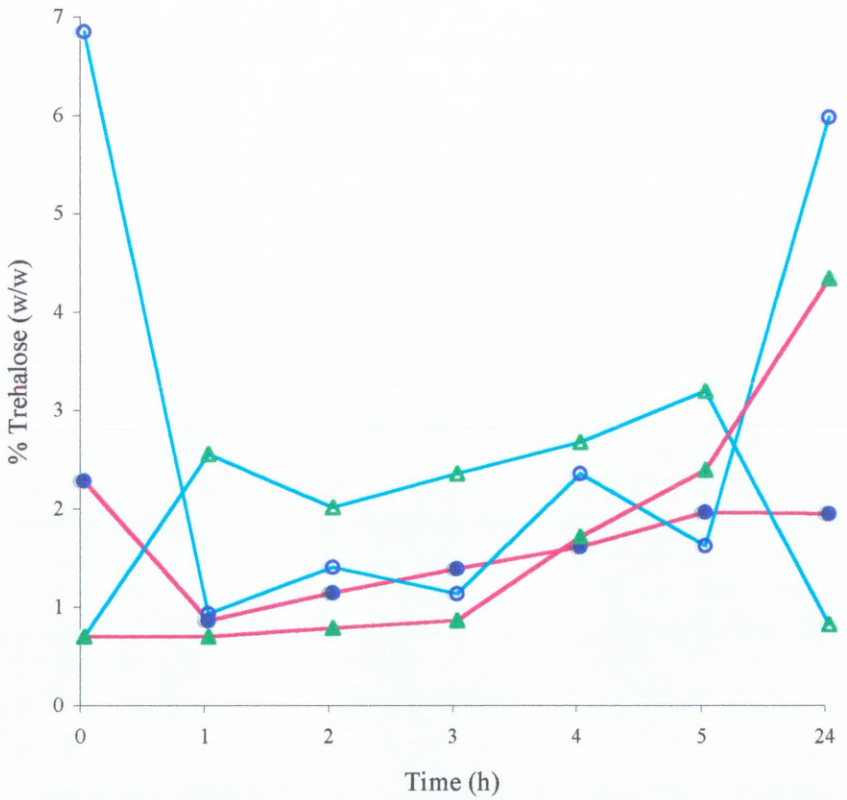


Figure 5.14 Trehalose levels during heat stress (42°C) of magnesium-preconditioned wine yeast (L-2226). ●/○ 2mM Mg, ▲/△ 20mM Mg, — 30°C, — 42°C.

conditions of heat shock, trehalose production increased steadily over the period of shock. In control conditions, levels in magnesium-preconditioned (20mM) cells remained constant over the 5h shock period dropping off to levels <1%w/w after 24h of growth, suggesting a utilisation of this storage carbohydrate.

Ion leakage studies, as with cells undergoing concurrent magnesium additions and heat stress, showed for magnesium (Fig. 5.15) fairly constant extracellular levels during the shock period. Control (no shock) cells gradually released magnesium until towards the end when a slight uptake in levels was observed. Under conditions of heat shock a pattern of magnesium release over the initial stages of shock was also observed, however, generally the occurrence of ion uptake commenced sooner, *i.e.* half way through the stress period, with cells preconditioned with higher magnesium concentrations, *i.e.* 15mM, 20mM and 50mM Mg. The pattern of calcium leakage in preconditioned cells (Fig. 5.16) is very different, with extracellular levels in the control dropping steadily over the 1h shock period. On magnesium-preconditioning an initial increase in extracellular levels was witnessed in all bar 5mM Mg-preconditioned cells, suggesting calcium is released on initiation of shock to a much greater degree than Mg^{2+}. This pattern then levelled off or dropped slightly during stress prior to a dramatic uptake of calcium in the latter stages of heat stress. The situations observed in ion leakage from cells grown in media with elevated magnesium concentrations, whether by concurrent additions (Figs. 5.10 & 5.11) or preconditioning (Figs. 5.15 & 5.16), is such that generally less vital cells absorb magnesium to cope with stress whereas cells in an unstressed state will release magnesium and then at later stages reabsorption of ions will occur. These results agree with findings of other work on yeast viability (F. Mochaba: personal communication).

Heat shock protein synthesis as an 'emergency response' occurs rapidly on initiation of shock and maximum expression can be seen following 60min shock. Figure 5.17 illustrates this and shows the effect on heat shock protein production at 42^{o}C of magnesium-preconditioning of the wine strain *Sacch. cerevisiae* (L-2226). Hsps were visible at: 150, 104, 90, 83, 70, 60, 46, 30 and 26kDa (although bands at 83 and 70kDa in this case were

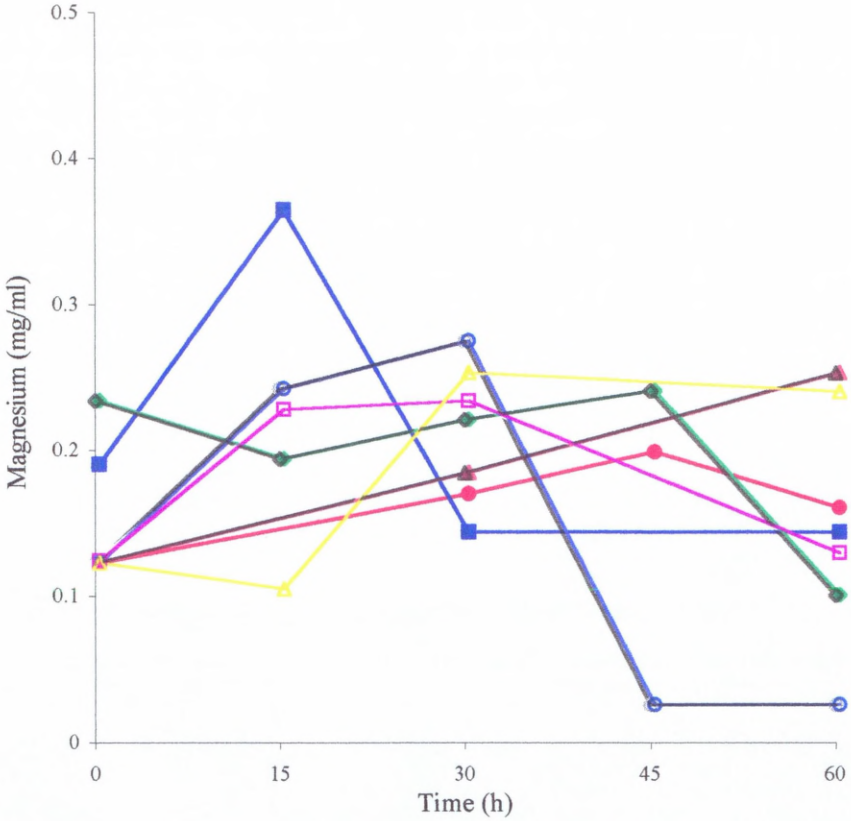


Figure 5.15 Leakage of cellular magnesium during a 1h period of heat shock (42°C) under magnesium-preconditioning. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

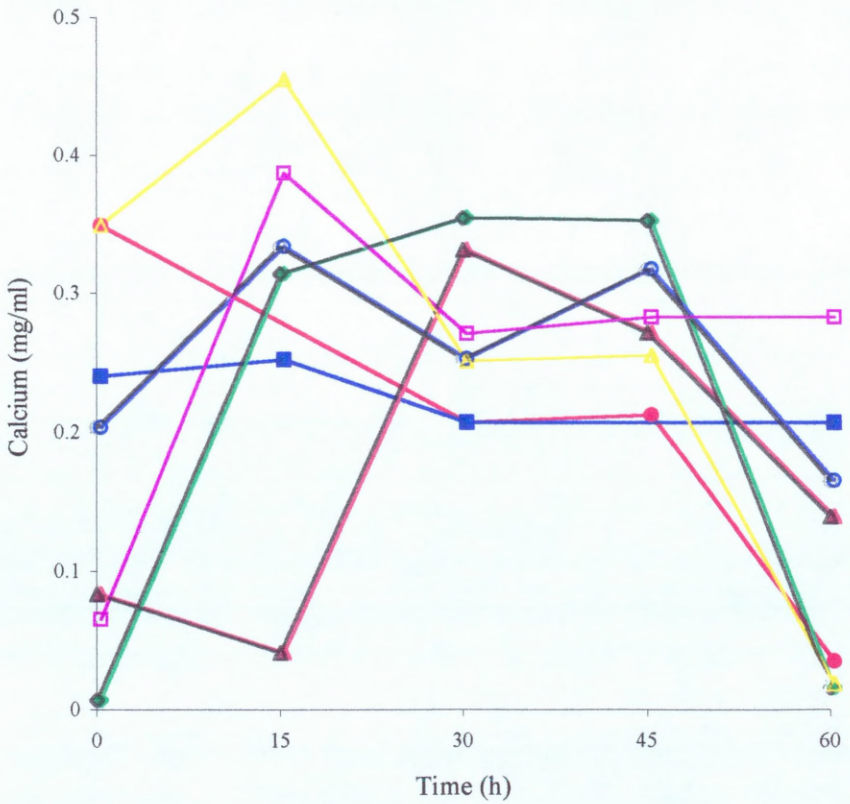


Figure 5.16 Leakage of cellular calcium during a 1h period of heat shock (42°C) under magnesium-preconditioning. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

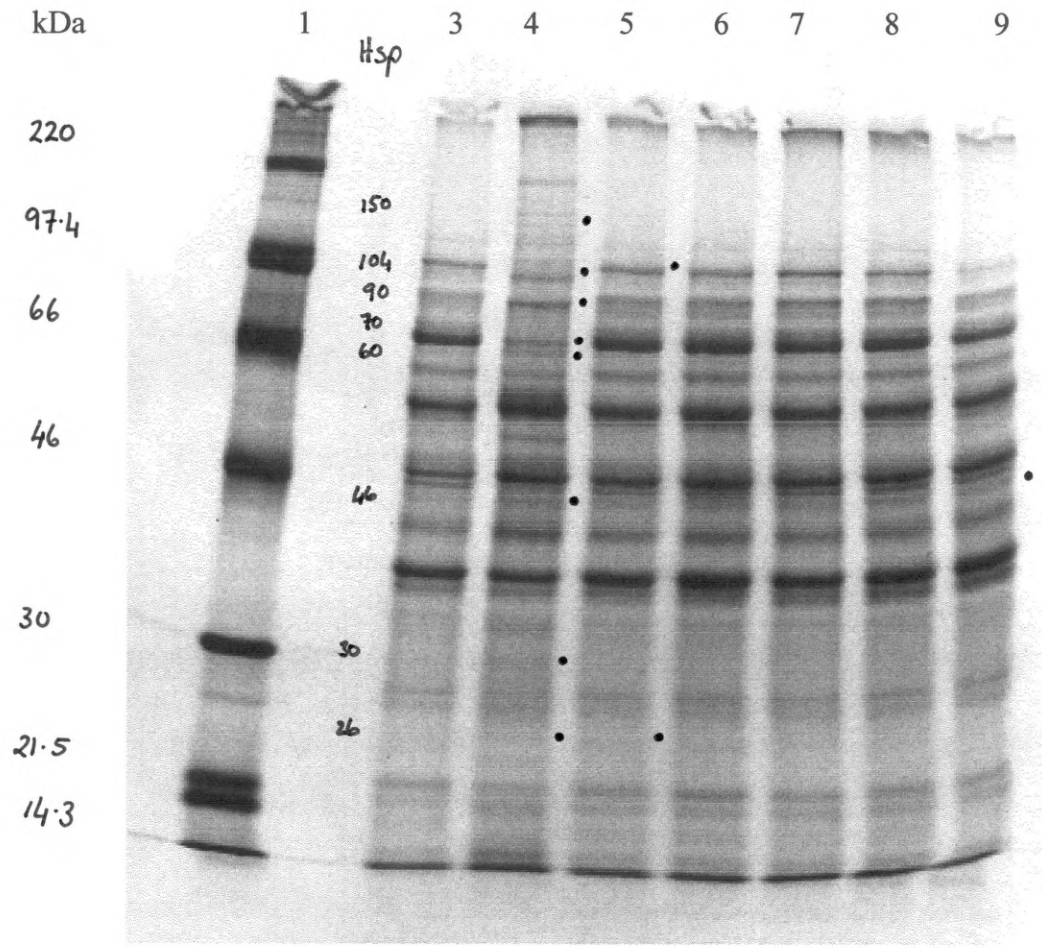


Figure 5.17 Effect on heat shock protein production at 42°C of magnesium-preconditioning of wine yeast (L-2226). Lanes: 1) Rainbow™ Marker, 3) Control (30°C), 4) 2mM Mg, 5) 5mM Mg, 6) 10mM Mg, 7) 15mM Mg, 8) 20mM Mg and 9) 50mM Mg.

quite weak), compared to the 30°C control lane. It can be seen that repression of these hsps was exhibited on preconditioning of cells with levels of magnesium across the range 5-20mM (Fig. 5.17). Table 5.5 shows the occurrence of typical stress proteins at the various magnesium concentrations. Repression of these hsps is quite dramatic with very few hsps remaining following preconditioning above 10mM Mg. Levels as low as 5mM do not appear to repress all these repair/response proteins. The results of concurrent addition of similar levels of magnesium on initiation of stress (Fig. 5.12/Table 5.4) displayed this same phenomenon, indicating that magnesium exerts a protective effect on cells undergoing stress. As observed in Figures 5.12 and 5.17 cells cultured with levels of Mg^{2+} >10mM exhibit repression of most stress proteins, suggesting that Mg^{2+} counteracts the requirement for formation of damage repairing stress proteins. Elevation of magnesium to these levels (>10mM) but not excessive levels (as this in itself could subject cells to stress) appears to negate the requirement for stress protein synthesis in response to elevated temperatures suggesting that magnesium protects cells from stress damage.

Table 5.5 Effect of magnesium-preconditioning on heat shock protein production at 42°C in wine yeast (L-2226).

Heat Shock Protein	Preconditioned Magnesium					
(kDa)	2mM	5mM	10mM	15mM	20mM	50mM
hsp150	✓	×	×	×	×	×
hsp104	✓	✓	×	×	×	×
hsp90	✓	×	×	×	×	×
hsp83	✓	×	×	×	×	×
hsp70	✓	×	×	×	×	×
hsp60	✓	×	×	×	×	×
hsp46	✓	×	×	×	×	✓
hsp30	✓	×	×	×	×	×
hsp26	✓	✓	×	×	×	×
hsp12	×	×	×	×	×	×

Note: ✓ = protein present, × = protein absent

In contemplating the degree of importance of intra- and extra-cellular magnesium on the heat shock response, comparison was made of concurrent addition to and preconditioning of cells on various stress responses in wine yeast. In comparing the effect of addition time of magnesium on the induction of thermo-protection (42^{o}C) in *Sacch. cerevisiae* (L-2226), it can be seen (Fig. 5.18) that elevation of magnesium levels to 20mM reduced the mortality of cells during prolonged (5h) shock when compared to normal growth medium levels of magnesium (2mM). Preconditioning of cells showed little benefit over cells grown normally and then exposed to elevated levels of this essential ion on initiation of shock and this leads one to conclude that both extra- and intra-cellular magnesium are of equal importance in terms of their beneficial effects on cells undergoing heat stress.

Confirmation of this and further to the results of the SDS-PAGE gels, western analysis was carried out on heat shocked samples and those heat shocked in the presence of elevated levels of magnesium (concurrent and preconditioned). Analysis using antibodies specific to hsp70, hsp60 and hsp27 was carried out. Western blots of yeast material when probed with human-specific hsp27 gave no detectable signal, suggesting the yeast hsp26 (an hsp27-related protein) may be present but does not exhibit a shared epitope. In analysis using hsp70 antibodies no signal was observed in yeast samples, indicating either the band detected at 70kDa by SDS-PAGE autoradiography may not be an authentic member of the hsp70 family or the yeast hsp70 simply does not share an epitope with the antibody used. Western analysis with hsp60 however gave interesting results. Figure 5.19 illustrates the positive response with GroEL (positive control for hsp60) in lanes 2 and 9 and also confirms the presence of hsp60 in heat shocked yeast at normal magnesium levels (2mM). On culturing cells with elevated magnesium (20mM) this protein cannot be detected, again suggesting that magnesium plays a protective role due to its repression of heat shock protein synthesis.

In an attempt to assess the mode of action of heat stress on yeast cells and the possible action of magnesium in the protective role suggested, the gross morphological status of the cells was considered. Cellular morphology in both minimal and complex media was

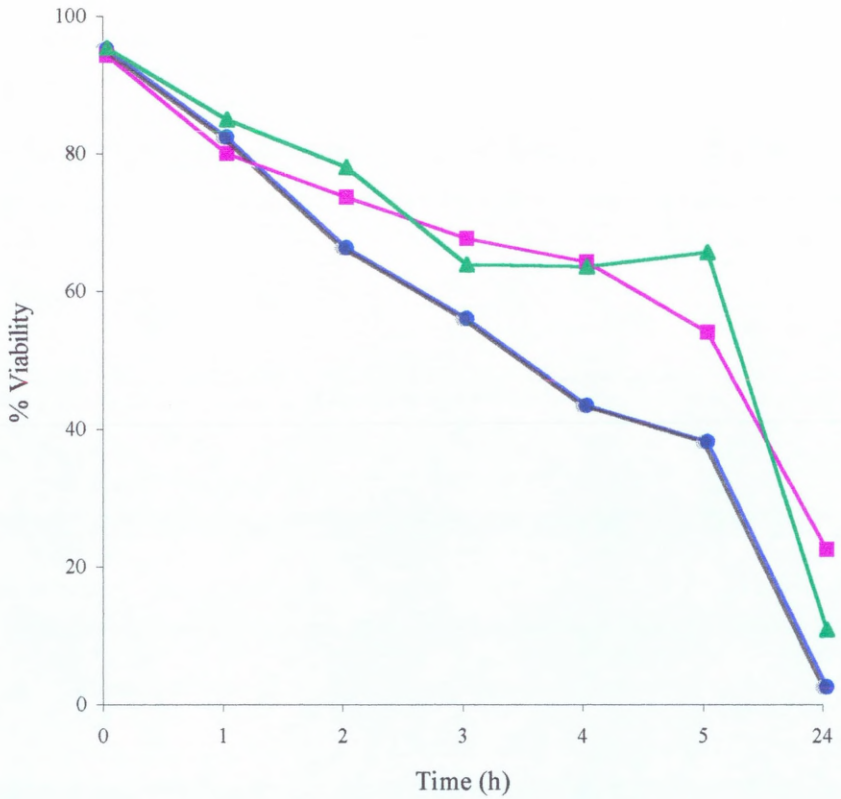


Figure 5.18 Comparison of the effect of the addition time of magnesium on the induction of thermo-protection (42°C) in wine yeast (L-2226).
-●- 2mM Mg, -■- 20mM Mg concurrent, -▲- 20mM Mg preconditioned.

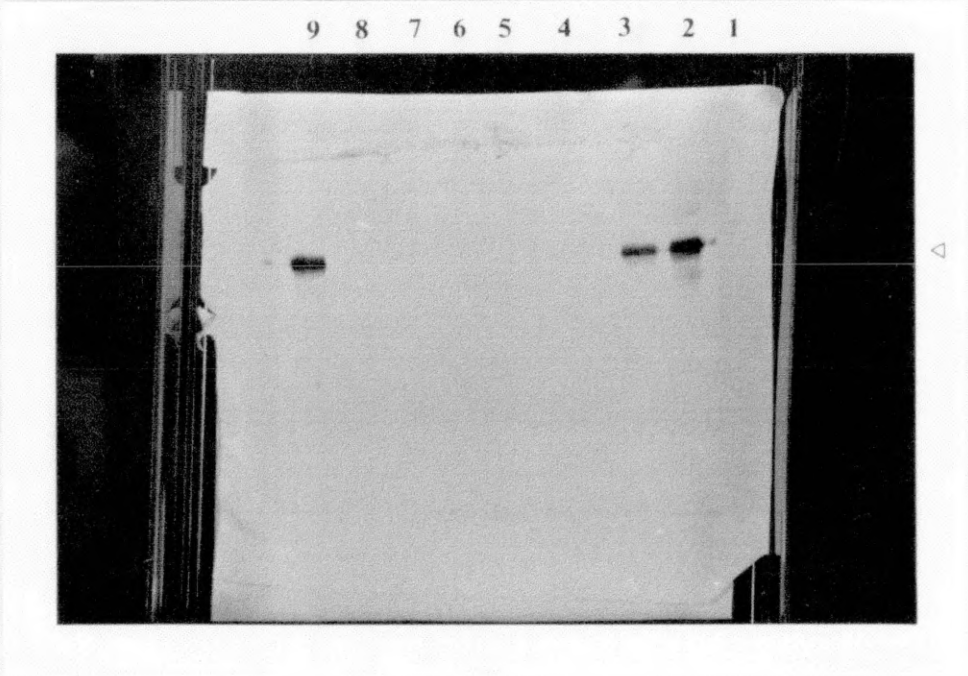


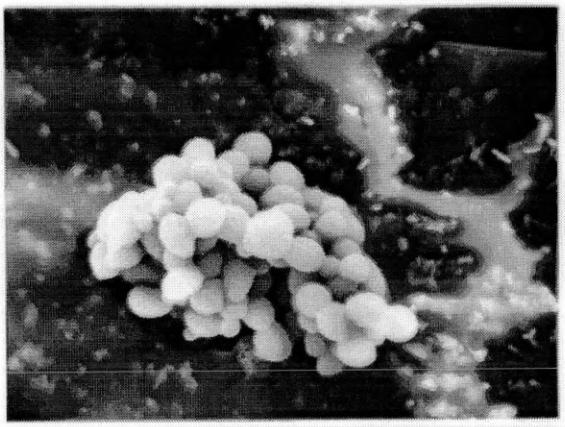
Figure 5.19 Western analysis of heat shocked (42°C) wine yeast (L-2226) specific for hsp60. Lanes: 1) Rainbow™ Marker, 2) GroEL, 3) 2mM Mg, 4) Control (30°C), 5) 5mM Mg, 6) 20mM Mg, 7) preconditioned 5mM Mg, 8) preconditioned 20mM Mg and 9) GroEL. Lanes 3), 5)-8) @ 42°C

assessed since it is in such conditions that the cells are most likely to experience stress, therefore one must consider if these laboratory results extend to a more industrial situation. Cell surface damage experienced by yeast cells on switching from 30-42^{o}C can be observed in Plates 5.1a)-d). Plate 5.1b) shows the surface damage experienced by cells following 1h heat shock at 42^{o}C. Cracking and gross cellular damage can be visualised compared to the control (Plate 5.1a). The protective effect of Mg^{2+} can be observed in Plates 5.1c) and 5.1d) where the visual damage following 1h heat shock at 42^{o}C in minimal media was reduced, the cells appear smother and less cracked. This phenomenon is also observed with *Sacch. cerevisiae* cells grown in grape must (Plate 5.2a)-d). A dramatic wrinkling and pitting of cells is seen on exposure to sub-lethal heat shock (Plate 5.2b). Elevation of magnesium concentrations in grape must reduces the damaging effects of heat on the cell surface and the cells appear more akin to the control (30^{o}C) cells, with a smoother, more complete appearance (Plate 5.2c & d). These studies suggest that magnesium may offer physiological protection to yeast cells in terms of membrane stabilisation and in preventing cell wall damage.

Heat damages a wide variety of cellular processes and structures. Intracellular disruption of *Sacch. cerevisiae* (L-2226) in grape must also occurs on exposure to sub-lethal heat stress. The effects of a mild heat shock on the ultrastructure of *Sacch. cerevisiae* involve; contraction of the nucleolus, formation of electron dense particles in mitochondria and presence of heat shock granules in the cytoplasm (Webster & Watson 1993). It can be seen from Plate 5.3b) that these such occurrences transpire, in particular the occurrence of electron dense particles in the cytoplasm of the cell. In comparison elevation of magnesium levels (Plate 5.3c & d) countermands the necessity for these responses and cells shocked under these conditions resemble those of the control conditions (Plate 5.3a).

The final avenue to consider following assessment of viability, cell topology and stress protein production, all as part of a multi-varied heat shock response, was that of a DNA response. The question posed was one of what, if anything, occurred at the DNA level on exposure of cells to shock and to compare the protein and chromosomal pattern diversity

a)



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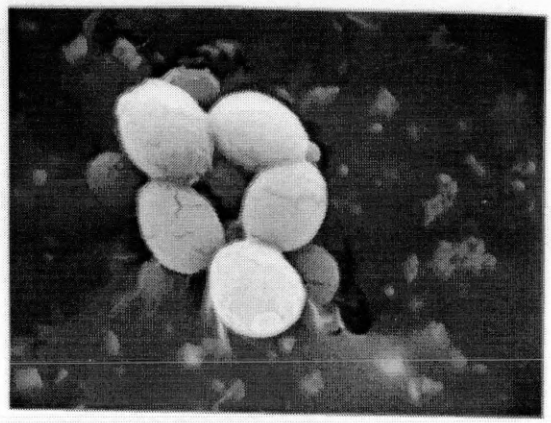

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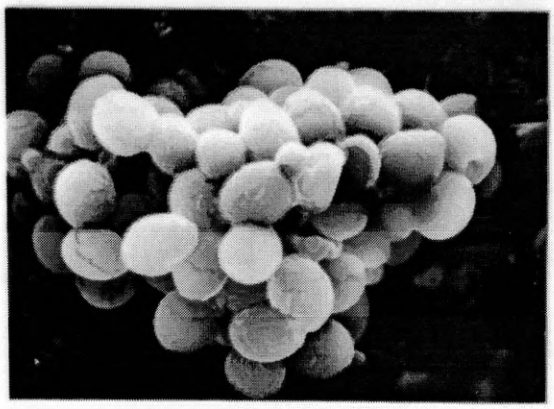

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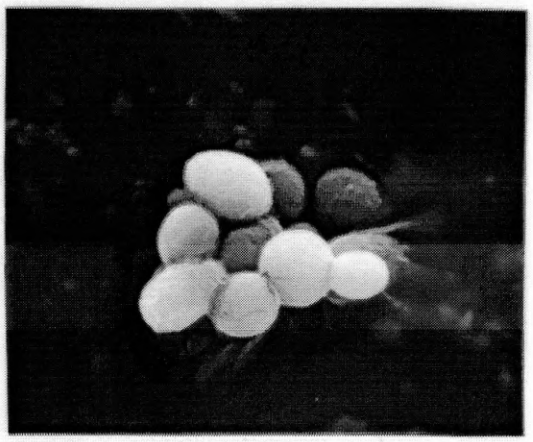
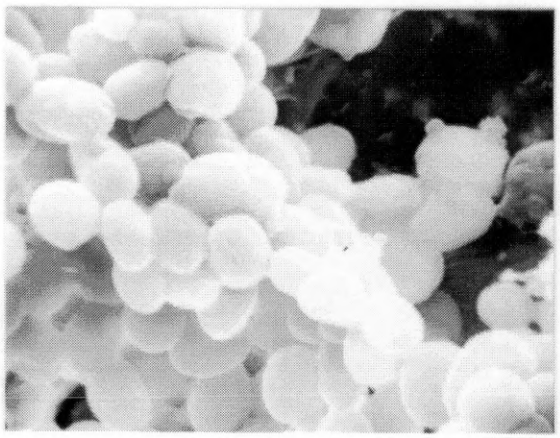


Plate 5.1 Cellular damage of *Sacch. cerevisiae* (L-2226) in minimal media, under conditions of; a) Control (30^{o}C), b) Heat shock (42^{o}C), c) Heat shock (42^{o}C) with elevated magnesium (20mM) and d) Heat shock (42^{o}C) preconditioned with elevated magnesium (20mM). (Magnification: a) x1582.5, b) x3165, c) x2373.7, d) x3165).

a)



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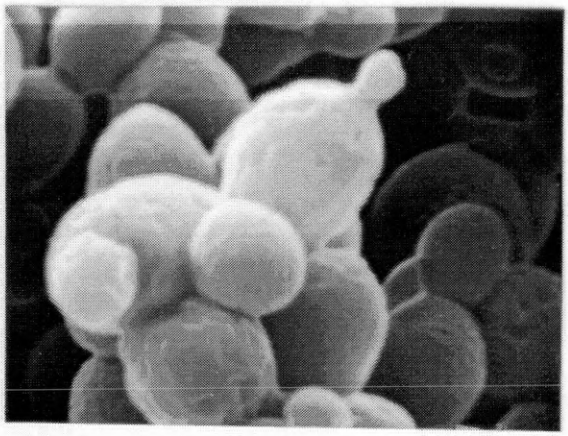

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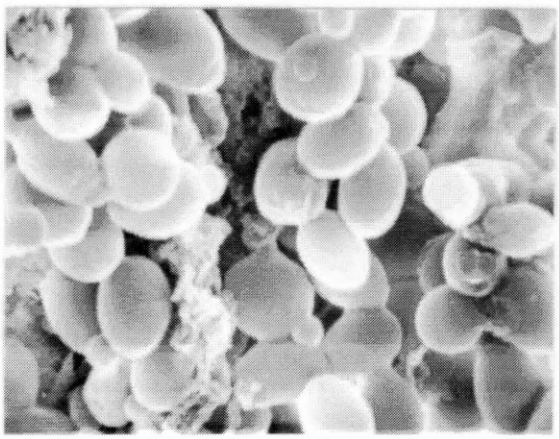


d)

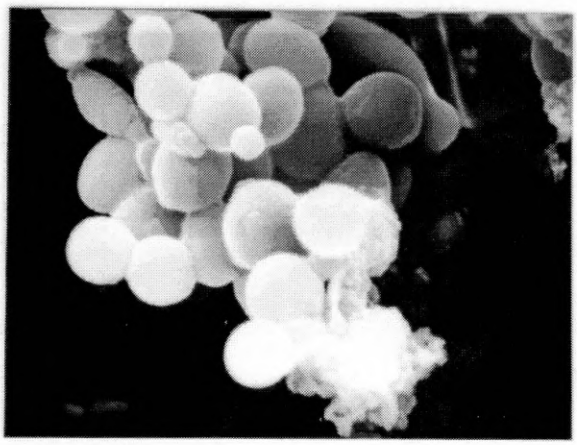


Plate 5.2 Cellular damage of *Sacch. cerevisiae* (L-2226) in grape must, under conditions of; a) Control (30°C), b) Heat shock (42°C), c) Heat shock (42°C) with elevated magnesium (20mM) and d) Heat shock (42°C) preconditioned with elevated magnesium (20mM). (Magnification: a) x3165, b) x6330, c) x3165, d) x3165).

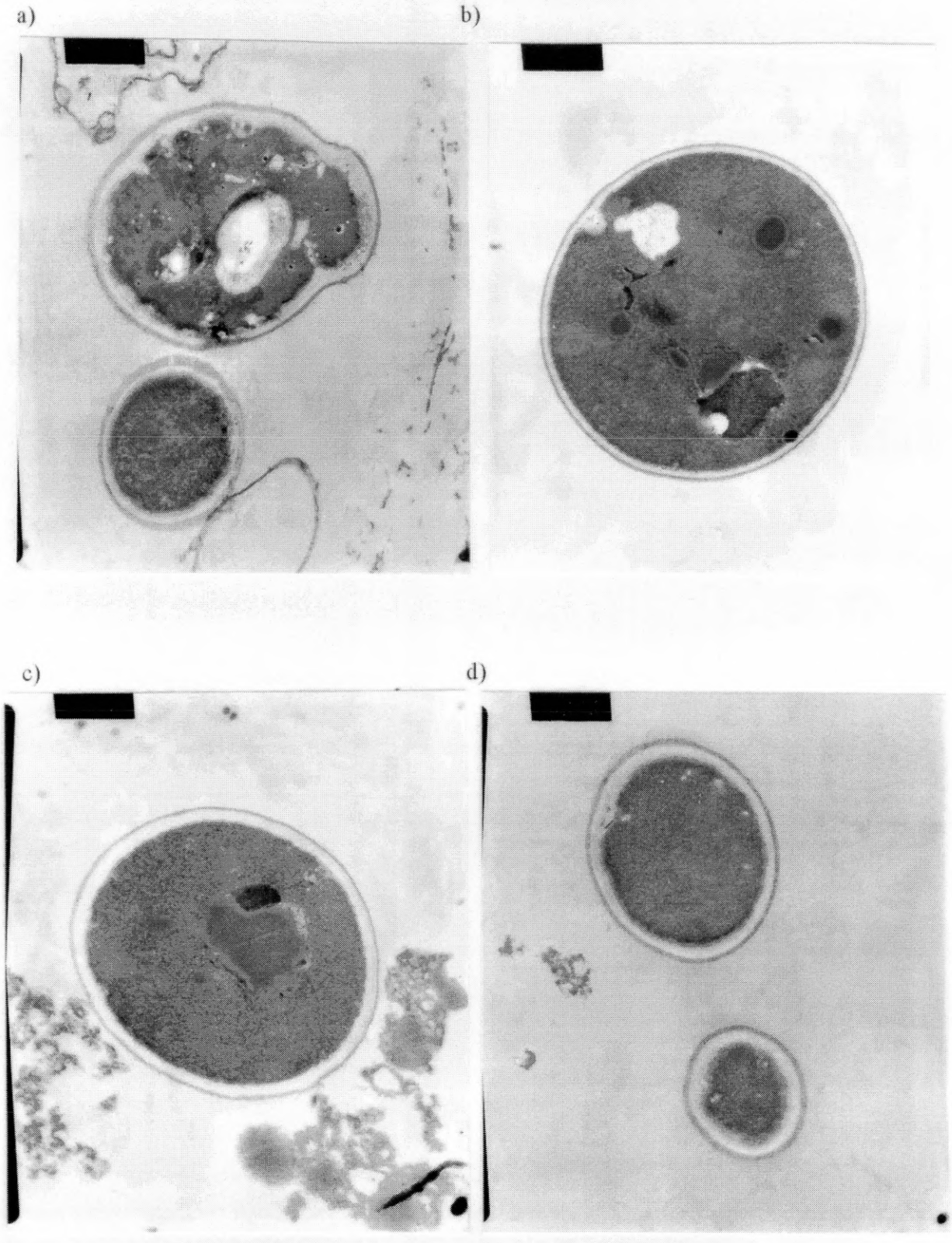


Plate 5.3 Intracellular disruption of *Sacch. cerevisiae* (L-2226) in grape must, under conditions of; a) Control (30°C), b) Heat shock (42°C), c) Heat shock (42°C) with elevated magnesium (20mM) and d) Heat shock (42°C) preconditioned with elevated magnesium (20mM). (Magnification: a),b) x17000, c) x22000, d) x12000).

after stress. CHEF analysis has been suggested to be effective to detect chromosomal variations among pure culture of industrially fermenting strains. In this case, a pool of stressed cells was utilised to search for a mass effect rather than for low frequency mutants. Inherent of the heat shock response is that it is a transient short-term mechanism involving transcription and post-translational regulation neither of which is visible at the DNA level. From LSD analysis of Figure 5.20 it can be seen that there is no significant difference between corresponding bands of patterns obtained from pools of heat stressed cells. Thus comparison between CHEF and pulse-chase experiments confirms that heat shock has an effect at the level of gene expression (transcription, translation) and/or on protein stability, but not at the level of chromosome integrity, since as can be seen no chromosomal shift occurred (Fig. 5.20). This result was as expected due to the transient nature of the heat shock response and the speed at which the mechanisms of the response are put into action.

In conclusion therefore, elevation of magnesium levels in the growth medium leads to a reduction in the detrimental effects of heat stress on yeast cells. Magnesium appears to exert a protective effect on cells undergoing heat stress and this is illustrated in improved viability levels, reduction of trehalose synthesis, reduction in cellular ion leakage, repression of hsps and reduction in cell surface and intracellular damage on exposure to heat shock experienced by cells grown in elevated Mg levels. Comparative studies showed that extra- and intra-cellular magnesium levels are equally important in terms of the beneficial effect of Mg^{2+} on stress. It is suggested that magnesium plays a role in protection from stress rather than the repair role stress proteins, etc., play. It is thought that magnesium may offer physiological protection to yeast cells in terms of membrane stabilisation and preventing stress damage of cells generally.

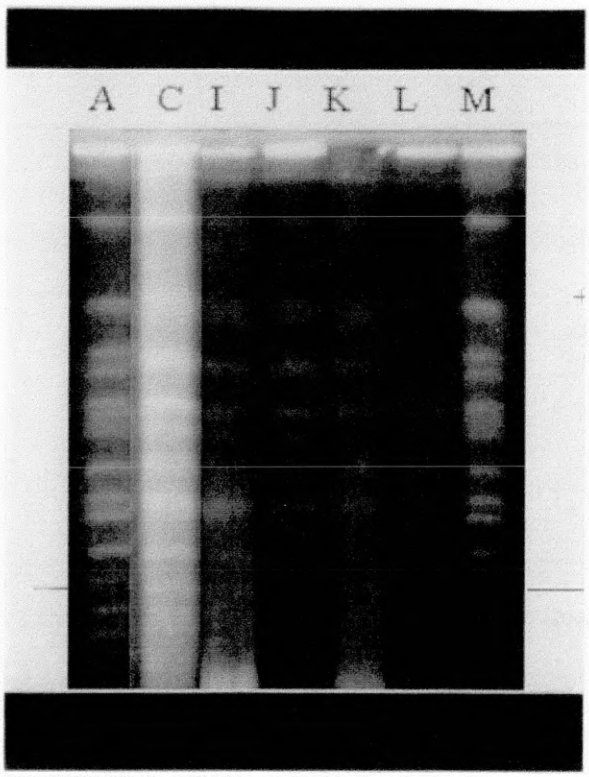


Figure 5.20 Analysis of *Sacch. cerevisiae* (L-2226) following heat (42^{o}C) and ethanol (10%) shock by contour-clamped homogeneous electric field electrophoresis (CHEF). Lanes: A) DBV6768 (CHEF DNA Standard), C) L-2226 (YEPG) I) L-2226 (CMMM)-Control (30^{o}C), J) L-2226 (CMMM)-Heat shock (42^{o}C), K) L-2226 (CMMM)-Ethanol shock (10%), L) L-2226 (CMMM)-Heat and Ethanol shock, M) DBV6768 (YEPG).

5.3.2 ETHANOL STRESS

The ability or inability of yeast to tolerate the toxic effects of ethanol is of profound importance in the commercial production of alcohol. Yeasts are evolutionarily adapted to ethanol, since this alcohol is a major product of their metabolic activity. Yet, the increasing ethanol levels during batch fermentation of *Sacch. cerevisiae* on high sugar substrates acts to cause reductions in the specific growth rate, fermentation rate and cell viability.

During fermentation, ethanol levels are constantly increasing, putting cells under increasing stress. The stress effect of a range of ethanol concentrations on wine yeast was assessed for a variety of responses. Viability was assessed following 60min ethanol stress over the concentration range 0-20% v/v ethanol. Figure 5.21 illustrates that viability of *Sacch. cerevisiae* (L-2226) declined as ethanol concentration increased. However, on elevation of Mg levels to 50mM on initiation of stress, a significant improvement in survival of cells is noted. This phenomenon is similarly observed, in some cases with greater effect, with cells pre-conditioned to a similar level of magnesium. Long term (48h) protection of wine yeast against ethanol toxicity by Mg^{2+} ions can be seen in Figure 5.22. Over a range of ethanol concentrations from 0-16% v/v, the percentage survivors declined as ethanol concentration increased, but a significant improvement in survival of cells with 1mM/3mM and 6mM Mg^{2+} additions was observed. The most profound differences being seen with higher Mg^{2+} concentrations.

Cellular magnesium leakage over an increasing range of ethanol concentrations remained relatively low and followed the pattern of control cells (0% ethanol) except for perhaps a slight uptake towards the end of the stress period (Fig. 5.23). Calcium levels over the same range follow the pattern of control cultures only up to 5% v/v ethanol, above this concentration the level of ethanol has an effect on cellular calcium leakage, with cells treated with 15% and 20% v/v ethanol exhibiting a profound uptake of calcium after about 30min of stress (Fig. 5.24). The leakage of both ions up to this point coincides with the damaging effect ethanol has on cell membranes and the subsequent ion leakage which would

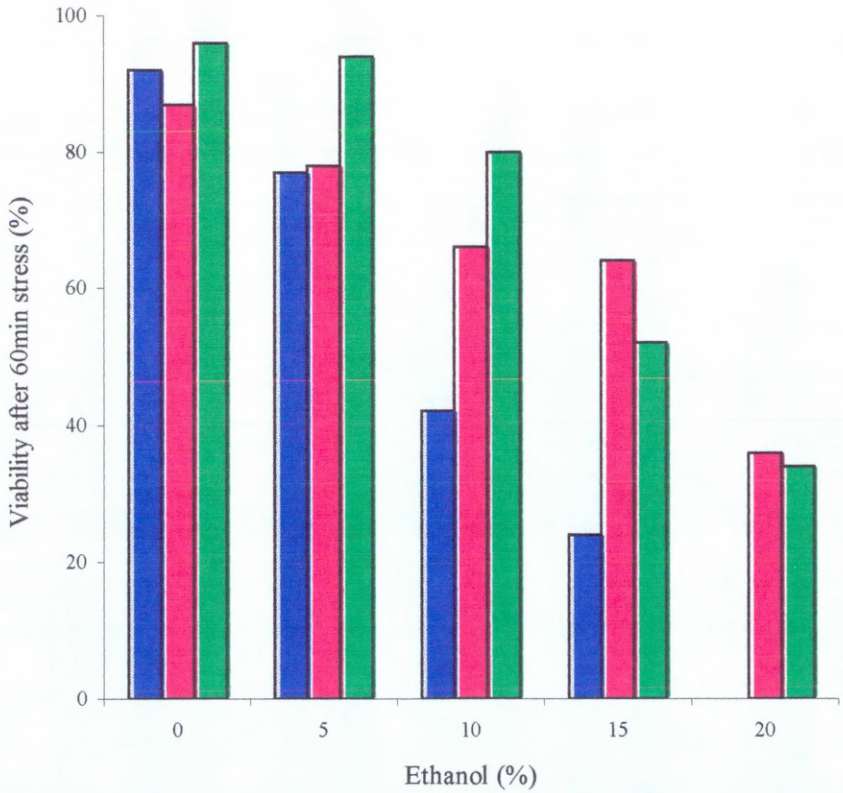


Figure 5.21 Viability of wine yeast (L-2226) after 60 min stress at various concentrations of ethanol. ■ 2mM Mg, ■ 50mM Mg concurrent, ■ 50mM Mg preconditioned.

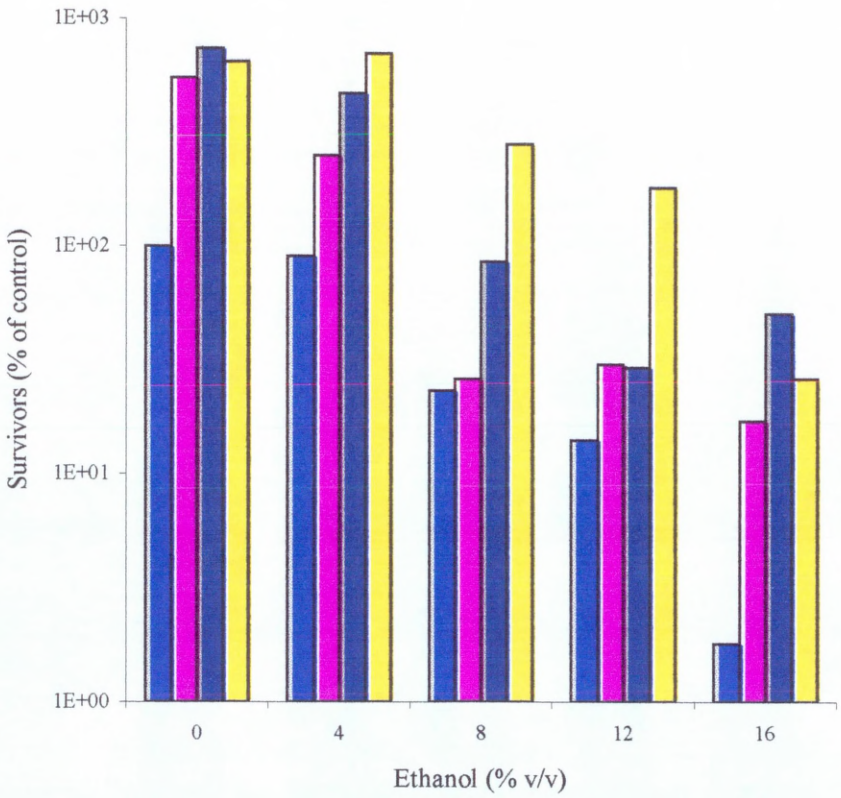


Figure 5.22 Long-term (48h) protection of wine yeast (L-2226) against ethanol toxicity by magnesium. ■ 0mM Mg, ■ 1mM Mg, ■ 3mM Mg, ■ 6mM Mg.

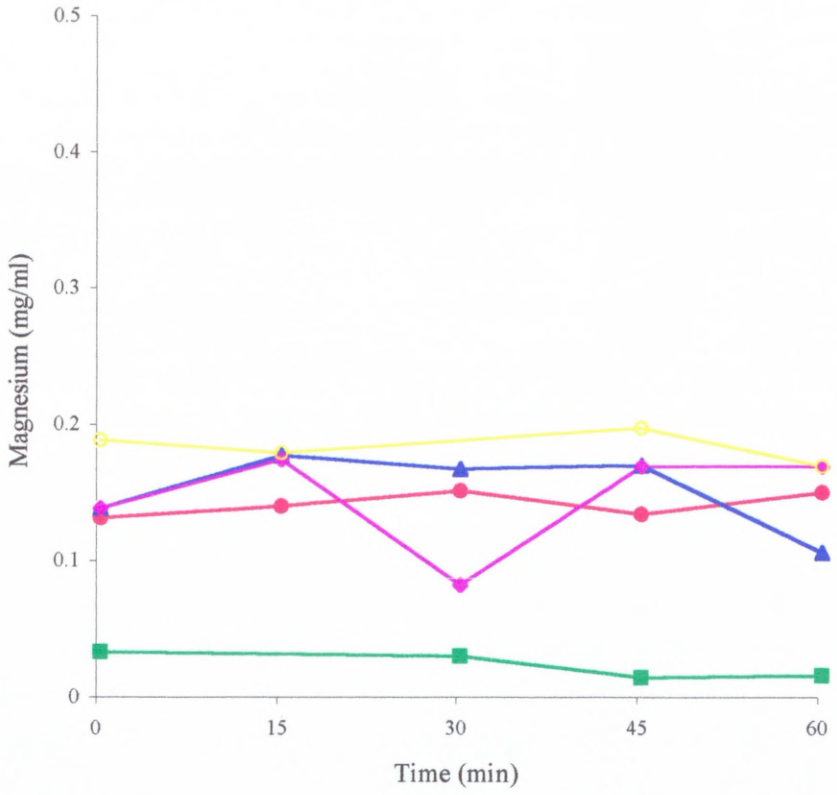


Figure 5.23 Cellular magnesium leakage due to increased ethanol concentration.
●0%, ■5%, ▲10%, ◆15%, ●20%.

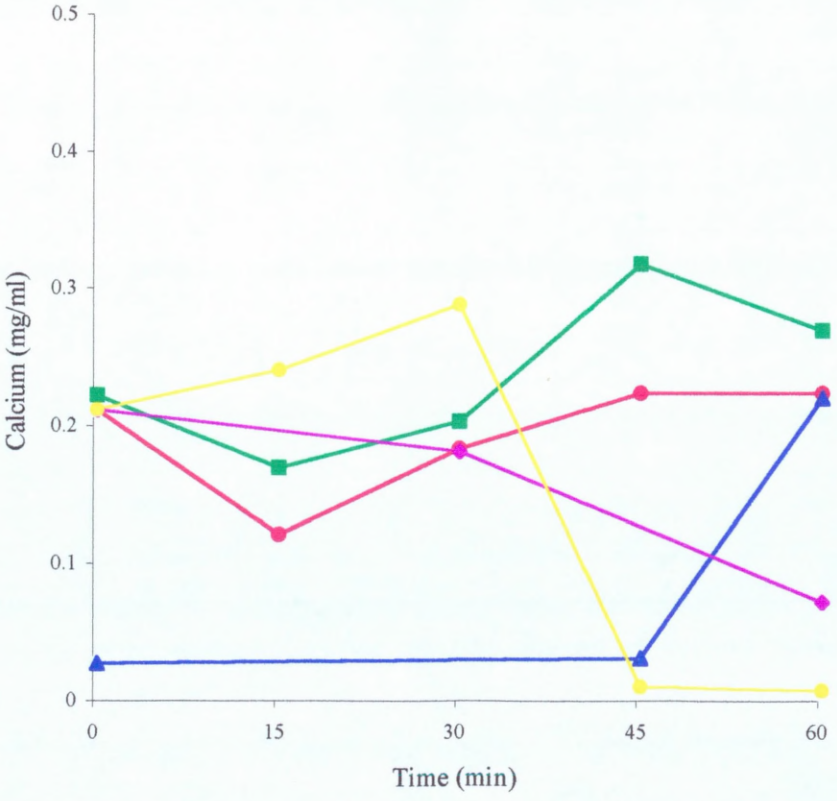


Figure 5.24 Cellular calcium leakage on result of increased ethanol concentration.
●0%, ■5%, ▲10%, ◆15%, ●20%.

therefore ensue. The uptake of ions towards the end of the shock period may be explained by the cells importing essential ions for the use in enzyme activation or as a requirement for some repair mechanism or cellular function.

The stress effect of increased ethanol concentration can probably best be illustrated on a molecular level by Figure 5.25. This shows the effect of increased ethanol concentration (0-20% v/v) on protein profiles of *Sacch. cerevisiae* (L-2226). The initial point to consider from this autoradiograph is one which confirms the viability studies previously illustrated (Fig. 5.21 & Fig. 5.22), that concentrations of 15% and 20% ethanol reduces viability of cells to such an extent that protein profiles were unable to be obtained. The results for 5% and 10% ethanol (Fig. 5.25/Table 5.6) however, clearly show the presence of hsps or stress proteins at: 104, 70, 46, 30 and 26kDa compared to the control (30^{o}C; no shock) and notably induction is progressively increased as ethanol concentration increases as shown in Figure 5.25. These results correspond to those of Piper *et al.* (1994) who identified the induction of hsp104, hsp70 and hsp26 with ethanol stress. The involvement of hsp30 in this stress is not altogether surprising also, since it is associated with cell membranes and ethanol is known to affect membrane stability, the mode of action being one of membrane permeabilisation.

Table 5.6 Effect of ethanol concentration on stress protein production in wine yeast (L-2226).

Stress Protein (kDa)	Heat Shock (42oC)	Ethanol Concentration 0%	5%	10%	15%	20%
hsp150	✓	×	×	×	No Growth	No Growth
hsp104	✓	×	✓	✓		
hsp90	✓	×	×	×		
hsp83	✓	×	×	×		
hsp70	✓	×	✓	✓		
hsp60	✓	×	×	×		
hsp46	✓	×	✓	✓		
hsp30	✓	×	✓	✓		
hsp26	✓	×	✓	✓		
hsp12	×	×	×	×		

Note: ✓ = protein present, × = protein absent

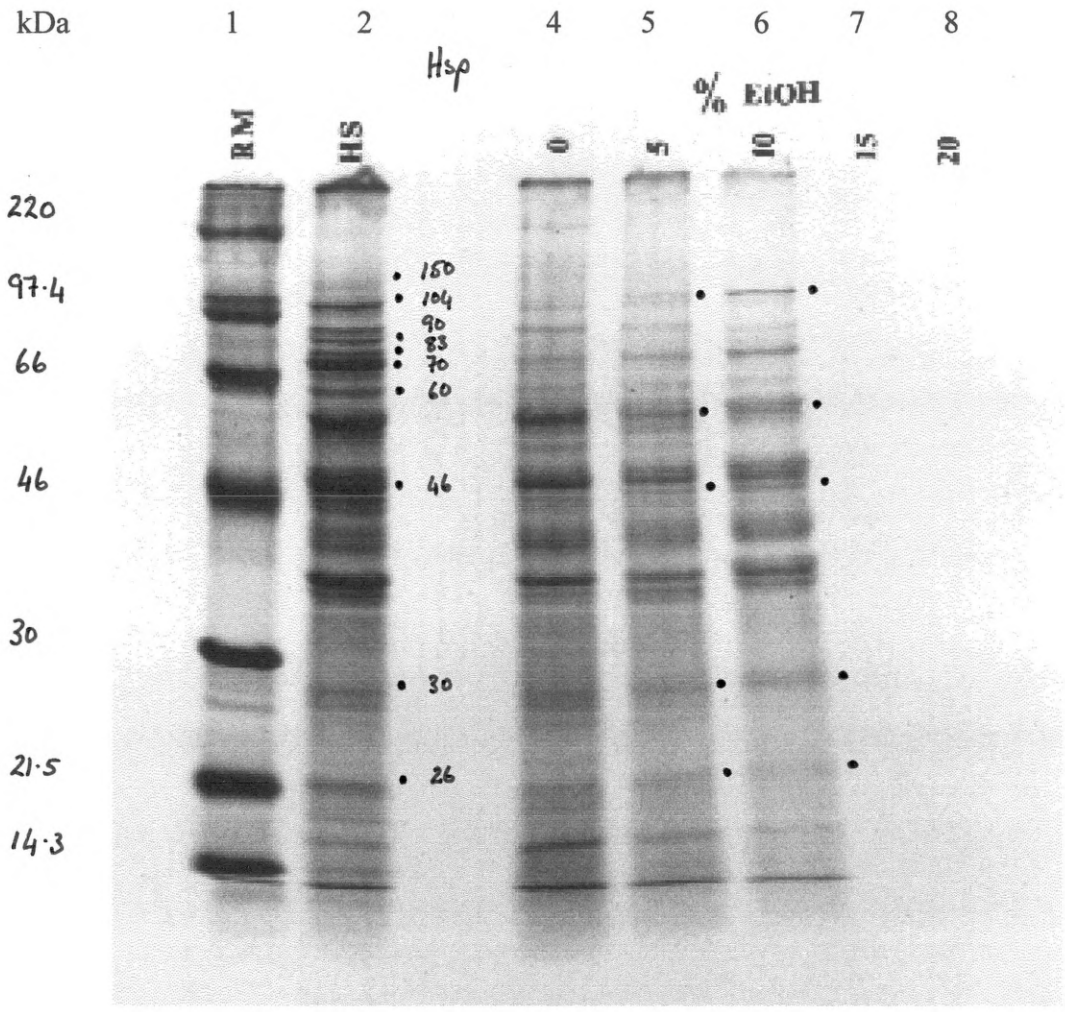


Figure 5.25 Effect of increased ethanol concentration on protein profiles of wine yeast (L-2226). Lanes: 1) Rainbow™ Marker, 2) Heat shock (42^{o}C), 4) 0% Ethanol, 5) 5% Ethanol, 6) 10% Ethanol, 7) 15% Ethanol and 8) 20% Ethanol.

As it is now established that ethanol constitutes a stress effect on yeast despite it being an important metabolite, one must consider ways to protect the cells from its detrimental effects. Ethanol is a valuable commodity industrially and if one can maximise ethanol production by maintaining viability at high levels or preventing the need for cellular activation of the stress response this alcohol causes, then this can only benefit the yeast and the industry as a whole. Magnesium induced protection of wine yeast (L-2226) to 10% ethanol can be seen in Figure 5.26. Viability levels over both intermediate (5h) and long (24h) term are maintained at >50% on the elevation of medium magnesium levels at the time of shock. This is a significant factor since long term exposure of yeast cells to this concentration of ethanol at normal media levels of Mg (2mM) resulted in absolute cell death. Viability levels in control flasks remained high over this period (>80%). These results, as with those of heat stress, suggest that magnesium exercises a protective role in the growth of yeast cells under stress, enabling culture viability to remain at high levels for prolonged periods.

Magnesium is therefore able to maintain levels of survival at an improved degree, so the consideration turns to the effect of magnesium on trehalose production during ethanol stress (10%). At 2mM Mg (normal media levels) control cultures exhibit a drop in trehalose levels over the first hour, followed by a fairly constant period and then a stark rise in levels over a 24h growth period (Fig. 5.27). On initiation of shock (10% v/v ethanol) levels again drop over the first 1h of shock and follow a similar pattern to the control at a subdued intensity. Levels at elevated magnesium concentrations of 20mM exhibit a rise during the first 1h of shock, followed by a subsequent decline over prolonged periods (up to 24h). Control levels remained constant and low over the same period (Fig. 5.27). This suggests trehalose plays a small role in the response to ethanol stress the wine yeast *Sacch. cerevisiae* (L-2226) and that alteration of magnesium levels has little effect on trehalose concentration under these conditions.

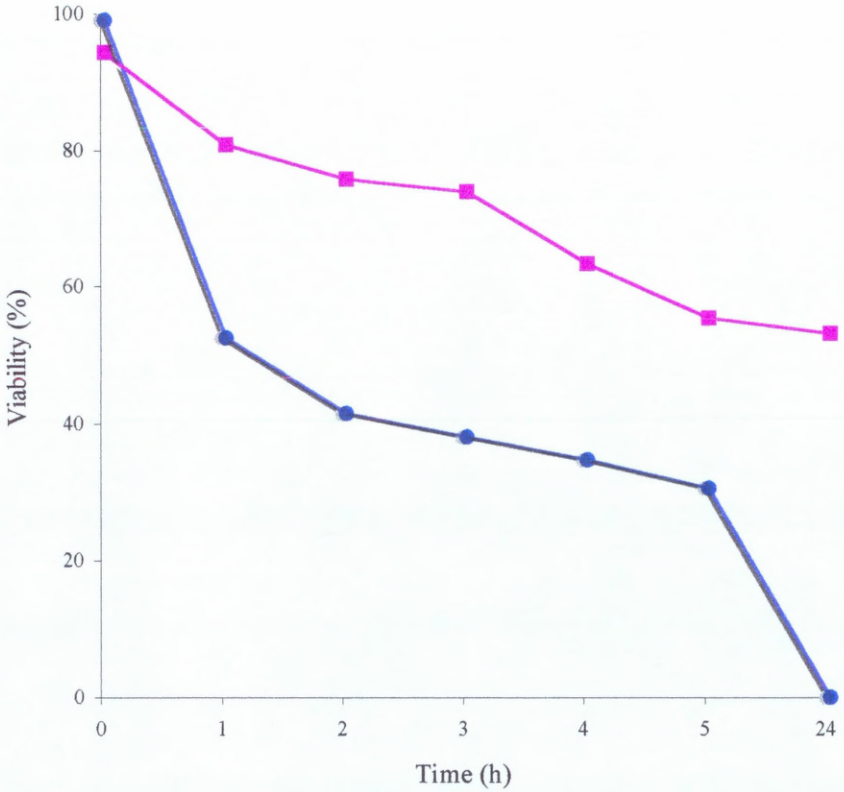


Figure 5.26 Magnesium induced protection of wine yeast (L-2226) to ethanol (10%). -●- 2mM Mg, -■- 20mM Mg.

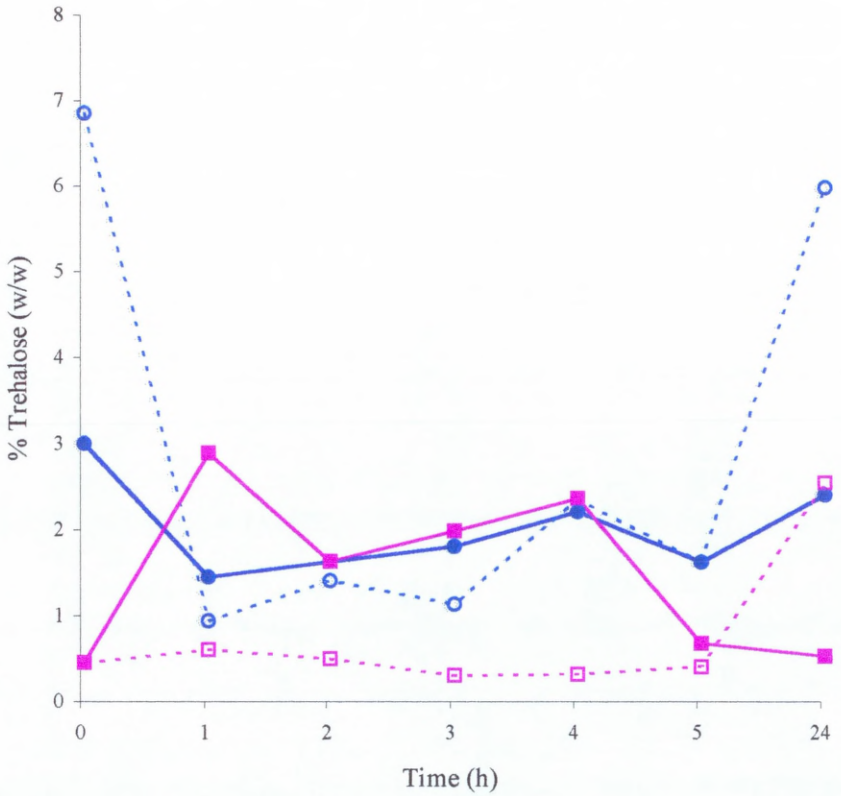


Figure 5.27 Trehalose levels during ethanol stress (10%) of wine yeast (L-2226) at high and low magnesium. ●/○ 2mM Mg, ■/□ 20mM Mg, ---- 0%, —— 10%.

Ethanol, it is agreed, is considered detrimental to yeast cells and is thought to act upon the cell membrane, permeabilising it and thus one would expect cellular ion leakage to increase on exposure to ethanol. Figures 5.28 and 5.29 show cellular magnesium and calcium leakage over a 1h stress period of ethanol shock (10%) with and without magnesium additions to the growth medium. The pattern of magnesium leakage (Fig. 5.28) over the shock period for the various concentrations of magnesium is not dissimilar to that of the control (no shock) culture *i.e.* an initial release of Mg^{2+} ions over the first 15min, followed by a steady uptake of ions by the cells throughout the shock period. Only 5mM and 10mM Mg cultures exhibited a release of ions throughout the stress period. The pattern for calcium leakage (Fig. 5.29) follows a similar story, except for cells in media with higher concentrations of magnesium. Ethanol shocked controls (2mM) show a pattern similar to that of Mg leakage (Fig. 5.28) however, cultures with 5/10 and 15mM Mg mimic the control (no shock) culture with an initial uptake of calcium (v. slight) followed by drastic calcium leakage and then a period of cellular uptake at the end of the shock period. Cultures of 20mM and 50mM Mg, however, exhibit an initial calcium release followed by a steady extracellular level throughout shock. These results go some way towards confirming the role of ethanol in membrane permeabilisation/membrane damage with the ionic leakage pattern exhibited in shock cultures at low Mg levels (2mM). Some level of protection is afforded by increasing Mg levels, however, due to chelation, etc., in the medium these results are difficult to interpret fully.

Exposure to 10% ethanol causes the formation of stress proteins (Fig. 5.30 & Fig. 5.25) as part of a stress response. These stress proteins were visible at: 104, 70, 46, 30 and 26kDa (Table 5.7). The effect of altered magnesium concentration on production of stress proteins at 10% ethanol in *Sacch. cerevisiae* (L-2226) was assessed (Fig. 5.30) and reduction in levels of these proteins was observed following media supplementation with levels of Mg^{2+} greater than 10mM, *i.e.* stress proteins were observed in the control (2mM) and following elevation of magnesium levels to 10mM (Fig. 5.30/Table 5.7). Above this concentration of concurrent addition, however, repression of the induction of stress protein synthesis was witnessed, suggesting that these concentrations of magnesium are protecting the cells from

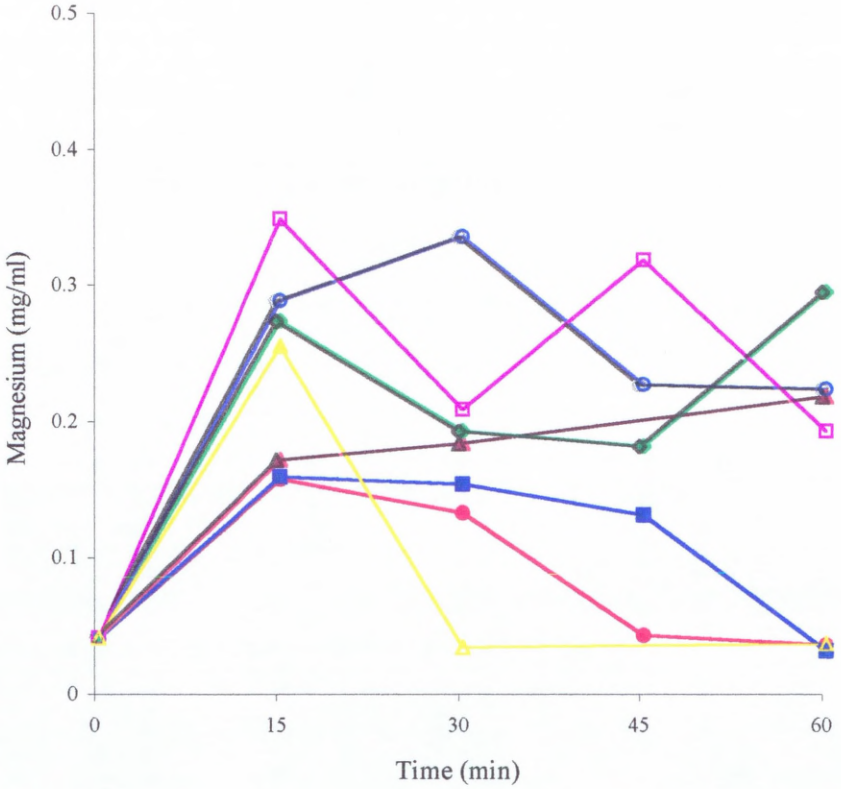


Figure 5.28 Leakage of cellular magnesium during a 1h period of ethanol shock (10%) with concurrent magnesium addition. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

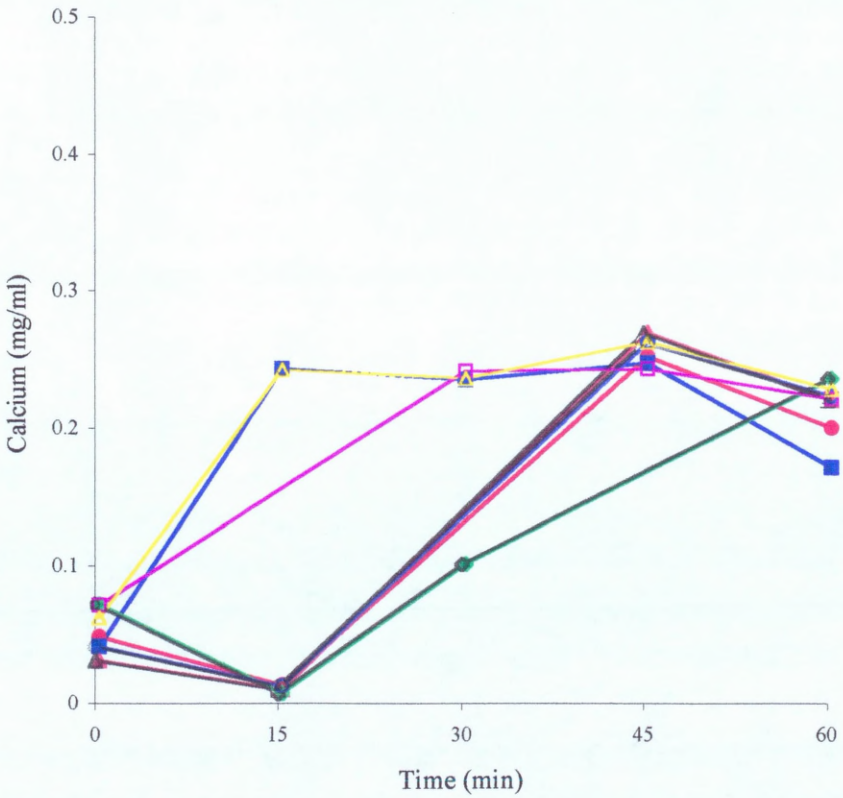


Figure 5.29 Leakage of cellular calcium during a 1h period of ethanol shock (10%) with concurrent magnesium addition. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

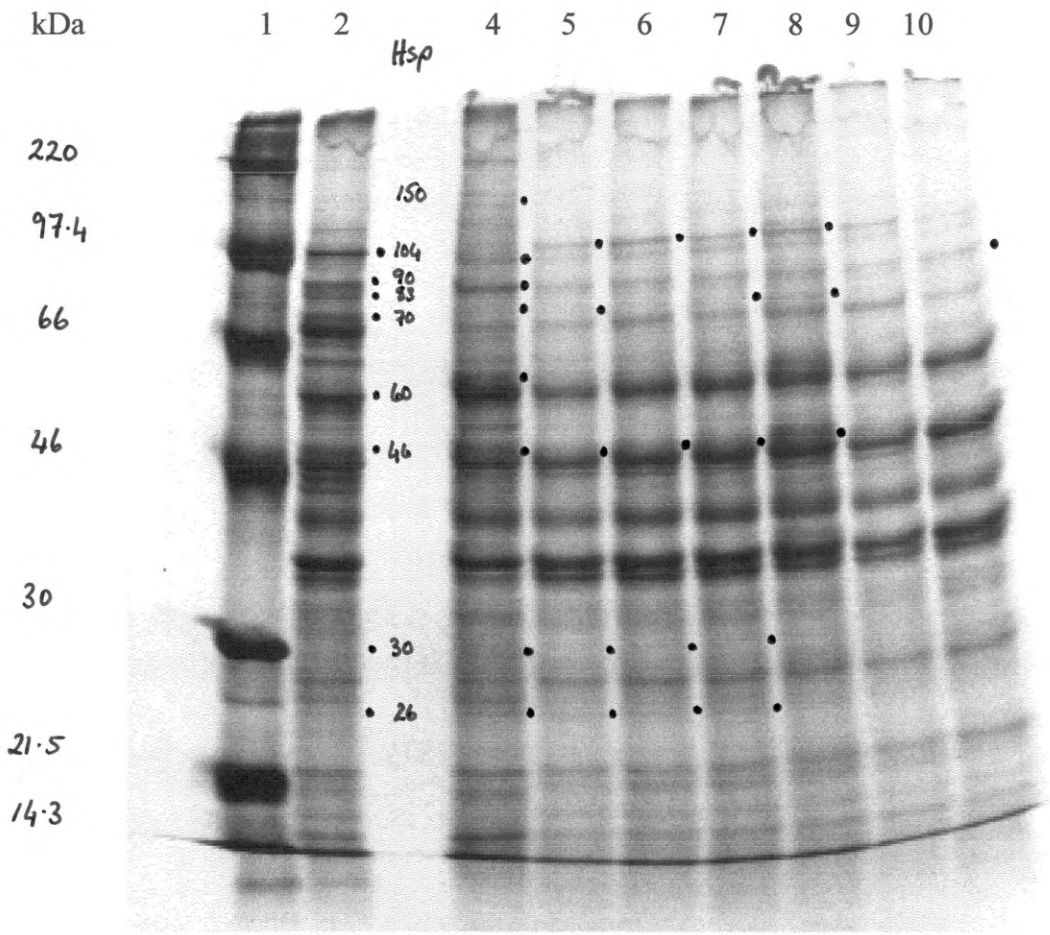


Figure 5.30 Effect of altered magnesium concentration on production of stress proteins at 10% ethanol in wine yeast (L-2226). Lanes: 1) Rainbow™ Marker, 2) & 4) Heat shock (42^{o}C), 5) 2mM Mg, 6) 5mM Mg, 7) 10mM Mg, 8) 15mM Mg, 9) 20mM Mg, 10) 50mM Mg. Lanes 5)-10) @ 10% Ethanol.

this ethanol stress thus negating the need for stress proteins. Heat shock proteins, as a comparison, were exhibited at: 150, 104, 83, 46 and 30kDa (Fig. 5.30).

Table 5.7 Effect of concurrent additions of varying concentrations of magnesium on stress protein production, under ethanol stress (10%) in wine yeast (L-2226).

Stress Protein	Concurrent Magnesium					
(kDa)	2mM	5mM	10mM	15mM	20mM	50mM
hsp150	✗	✗	✗	✗	✗	✗
hsp104	✓	✓	✓	✓	✗	✗
hsp90	✗	✗	✗	✗	✗	✗
hsp83	✗	✗	✗	✗	✗	✓
hsp70	✓	✗	✓	✓	✗	✗
hsp60	✗	✗	✗	✗	✗	✗
hsp46	✓	✓	✓	✓	✗	✗
hsp30	✓	✓	✓	✗	✗	✗
hsp26	✓	✓	✓	✗	✗	✗
hsp12	✗	✗	✗	✗	✗	✗

Note: ✓ = protein present, ✗ = protein absent

The effect of attempting to create 'ion stores' in cells by preconditioning with elevated concentrations of magnesium to establish a protection system against ethanol was investigated. This essential ion is obviously important in the stress protection scenario based on this research, however the question as to whether intra- or extra-cellular levels of Mg are more important in response to ethanol stress is unclear. In an attempt to address this problem, preconditioning of cells was carried out and the effects on various aspects of the stress response observed. Viability of cells undergoing stress was much improved over control levels following preconditioning of cells with levels of Mg at 20mM (Fig. 5.31). This reduction in mortality, with viability levels remaining >60% over a prolonged (5h) period of stress and above 40% following long term (24h) exposure, is of interest industrially in terms of the maintenance of vitality of cultures during high gravity fermentations.

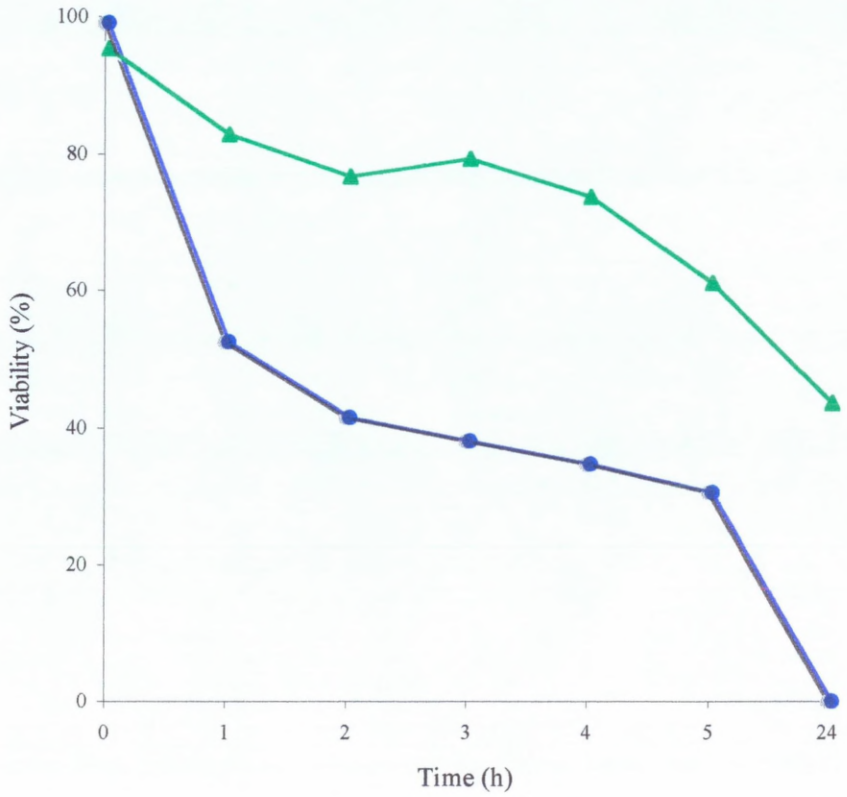


Figure 5.31 Effect of magnesium-preconditioning on the induction of ethanol (10%) protection in wine yeast (L-2226). -●- 2mM Mg, -▲- 20mM Mg.

Trehalose levels during ethanol stress of magnesium-preconditioned wine yeast (L-2226) were observed to be remarkably different to previous results. Figure 5.32 illustrates that at 0% ethanol (no shock) cells preconditioned with 20mM Mg maintain low levels of trehalose initially followed by a steady rise in levels after prolonged (3h) shock. Preconditioned cells undergoing ethanol shock, however, experience an increase in levels of trehalose over the first 2h of stress followed by a decline in levels, then a further increase before a dramatic decline over long term (24h) stress. This erratic behaviour leads one to conclude that although trehalose does have a role in yeast stress responses the exact situation is difficult to assess. Overall, however, alterations of magnesium levels has little effect on trehalose production due to ethanol stress.

Ion leakage of preconditioned cells during ethanol stress on first glance appears quite erratic (Figs. 5.33 & 5.34). Figure 5.33 shows leakage patterns of magnesium over a 1h period of ethanol shock (10%). Control cultures (no shock) exhibit a steady uptake of magnesium followed by a release into the medium towards the latter stages of the assay period. Preconditioned cells follow a similar pattern on ethanol shock to that of the control cells, however, the initial magnesium uptake was more pronounced as magnesium concentration increased. In cultures preconditioned with levels of Mg >10mM the period of magnesium leakage was shorter and the latter stages of ethanol stress witnessed cellular uptake of Mg. This corresponds to the requirement of cells for magnesium for efficient cellular functioning and further supports the positive role of Mg in stressful environments. Calcium leakage patterns (Fig. 5.34) appear quite similar to those of Mg suggesting that ethanol stress does damage membranes allowing an uncontrolled leakage of ions from stressed cells.

On a molecular level, the repression of stress proteins formed at 10% ethanol (Fig. 5.35) was observed following preconditioning of cells with levels of Mg^{2+} greater than 5mM *i.e.* stress proteins were observed in the control (2mM) and following preconditioning with 5mM Mg (Fig. 5.35). At these magnesium concentrations stress proteins were visualised at: 150, 104, 90, 70, 46, 30 and 26kDa at 2mM Mg and 150, 104, 90, 46 and 26kDa in cultures preconditioned to 5mM Mg (Table 5.8). Repression of all stress proteins did not

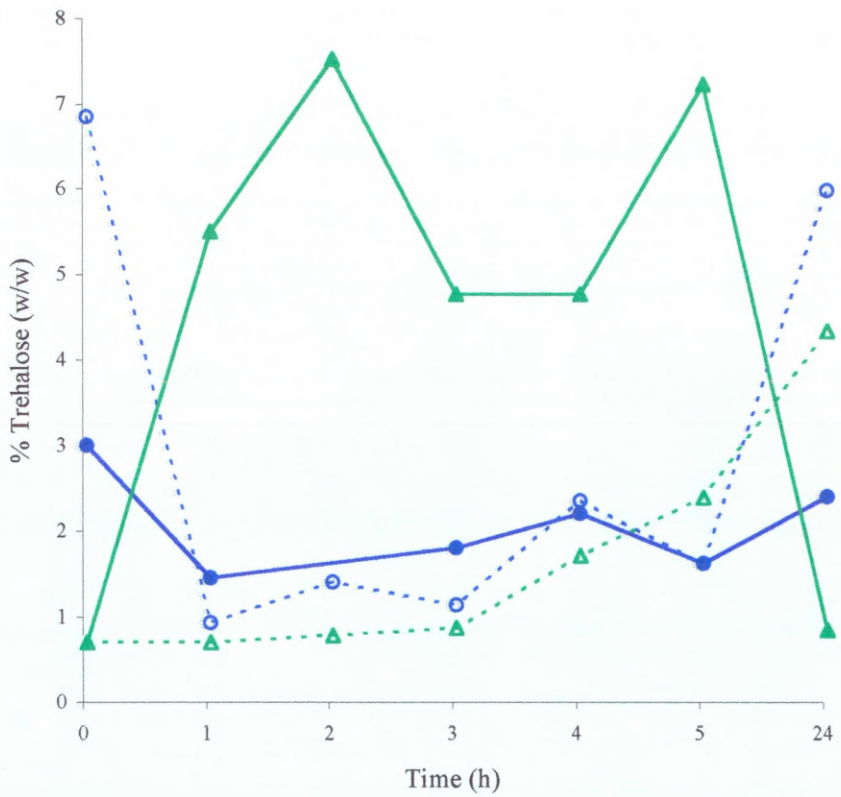


Figure 5.32 Trehalose levels during ethanol stress of magnesium-preconditioned wine yeast (L-2226). ●/○ 2mM Mg, ▲/△ 20mM Mg, ---- 0%, —— 10%.

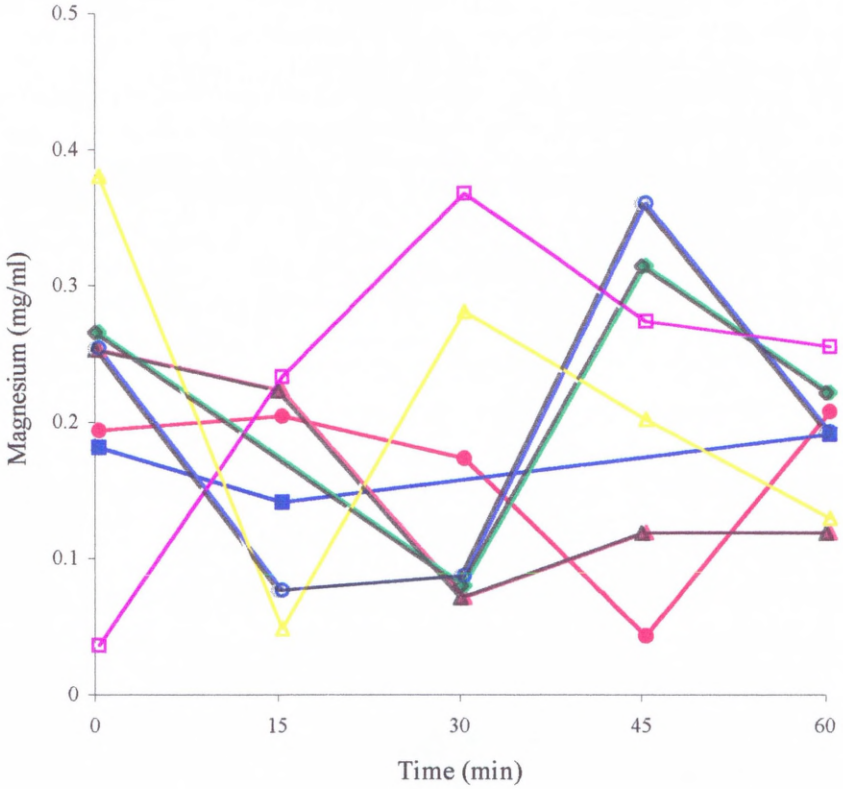


Figure 5.33 Leakage of cellular magnesium over a 1h period of ethanol shock (10%) with magnesium-preconditioning. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

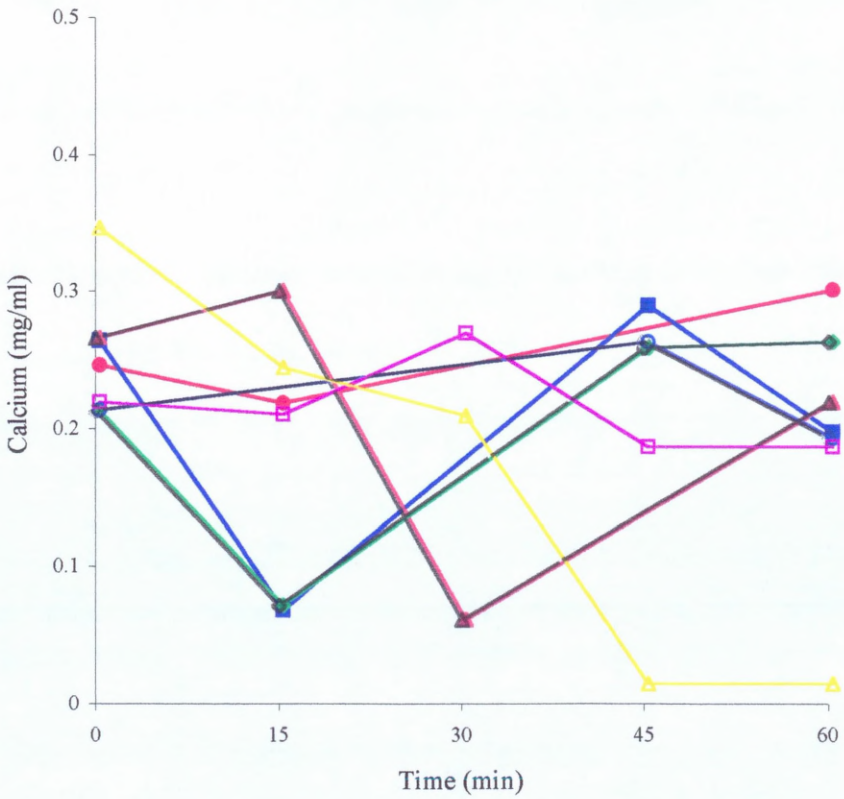


Figure 5.34 Leakage of cellular calcium over a 1h period of ethanol shock (10%) with magnesium-preconditioning. -●- Control (no shock), -■- 2mM Mg, -▲- 5mM Mg, -◆- 10mM Mg, -○- 15mM Mg, -□- 20mM Mg, -△- 50mM Mg.

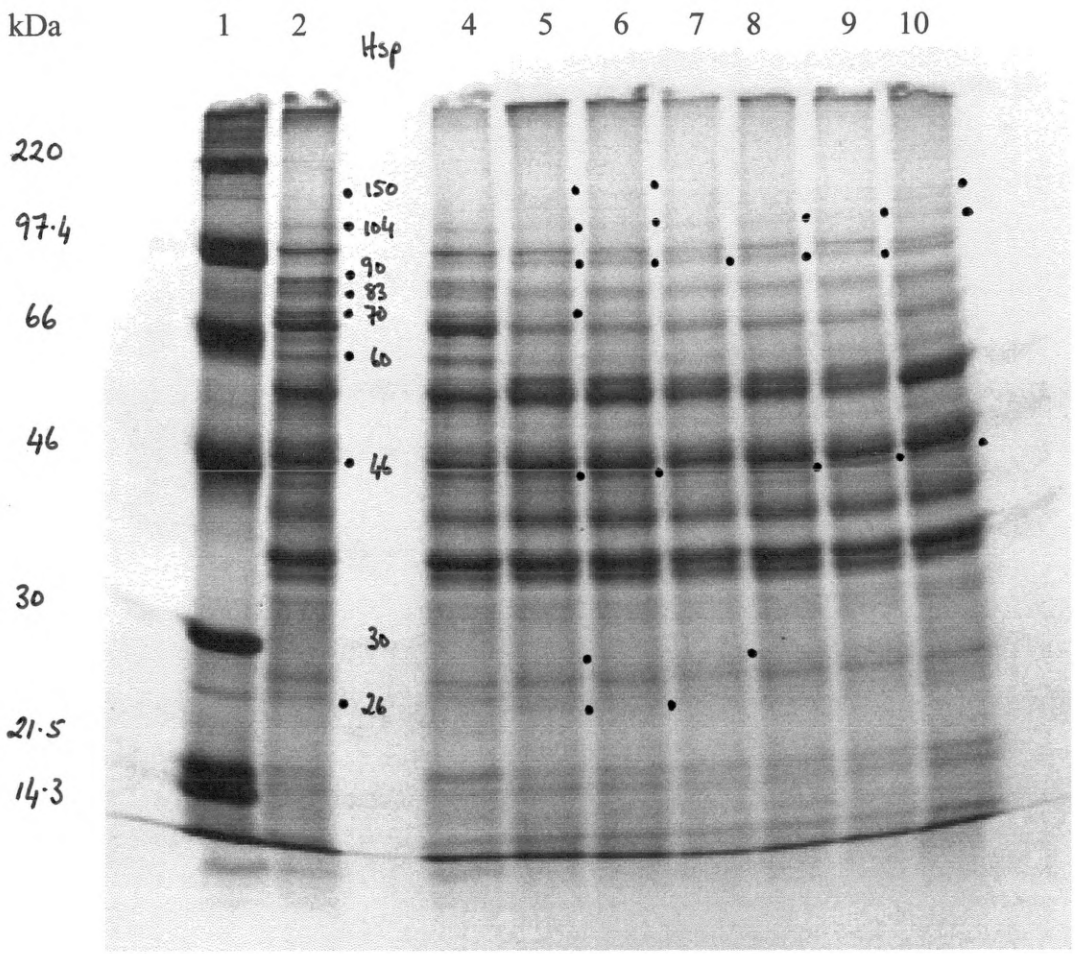


Figure 5.35 Effect on stress protein production at 10% ethanol levels, of magnesium-preconditioning of wine yeast (L-2226). Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 4) Control (30°C), 5) 2mM Mg, 6) 5mM Mg, 7) 10mM Mg, 8) 15mM Mg, 9) 20mM Mg, 10) 50mM Mg. Lanes 5)-10) @ 10% Ethanol.

occur, but the elevation of magnesium levels reduced the extent of stress protein synthesis occurring as a result of ethanol shock. Further evidence to suggest a protective role for magnesium with regard to chemical stresses experienced by industrial yeast.

Table 5.8 Effect of magnesium-preconditioning on stress protein production under ethanol stress (10%) in wine yeast (L-2226).

Stress Protein	Preconditioned Magnesium					
(kDa)	2mM	5mM	10mM	15mM	20mM	50mM
hsp150	✓	✓	×	×	×	✓
hsp104	✓	✓	×	✓	✓	✓
hsp90	✓	✓	✓	✓	✓	×
hsp83	×	×	×	×	×	×
hsp70	✓	×	×	×	×	×
hsp60	×	×	×	×	×	×
hsp46	✓	✓	×	✓	✓	✓
hsp30	✓	×	✓	×	×	×
hsp26	✓	✓	×	×	×	×
hsp12	×	×	×	×	×	×

Note: ✓ = protein present, × = protein absent

Previous results have considered concurrent additions and preconditioning of cells with elevated Mg without questioning the importance of these types of addition. The graph in Figure 5.36 shows that there is little difference in the effect of time of addition of magnesium on the induction of ethanol protection in wine yeasts. Both concurrent additions at the time of shock and preconditioning of cells with 20mM Mg maintain a reduced level of mortality (>40%) on exposure to prolonged (5h) or long term (24h) ethanol stress, compared with 2mM Mg. This concludes that both intra- and extra-cellular levels of magnesium are equally important in conferring protection to ethanol stress (10%).

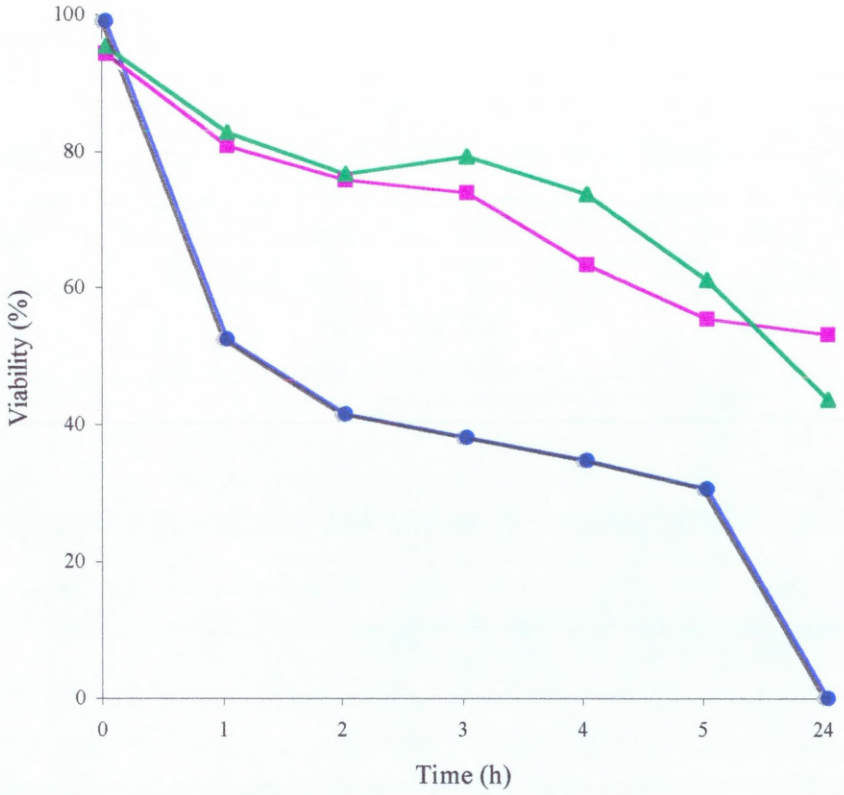


Figure 5.36 Comparison of the effect of time of addition of magnesium on the induction of ethanol (10%) protection in wine yeast (L-2226). -●- 2mM Mg, -■- 20mM Mg concurrent, -▲- 20mM Mg preconditioned.

The molecular and morphological effects of ethanol stress were evaluated in more detail and the beneficial effects of elevation of magnesium levels (intra- and extra-cellularly) assessed. Western analysis, specific for hsp70, hsp60 and hsp27, was carried out on ethanol shocked (10%) *Sacch. cerevisiae* (L-2226). Figure 5.37 shows the presence of hsp60 in the positive control (GroEL) and heat shocked samples but not in ethanol stressed samples and this result corresponds with the analysis of SDS-PAGE gels (Figs. 5.25; 5.30 & 5.35). Similarly samples tested with hsp70 and hsp27 antibodies gave negative results for ethanol shocked cells, however, the specificity of these two antibodies for yeast was questionable. Antibodies specific for yeast hsp30 or hsp26 were unavailable, so confirmation of the presence of these proteins by this analysis was unfeasible.

Cell surface damage was observed under conditions of ethanol stress. It can be seen from Plate 5.4b) that in minimal media pronounced damage can be seen after exposure to 10% v/v ethanol, compared to the control (Plate 5.4a) and again the concurrent addition of Mg^{2+} (Plate 5.4c) reduced the extent of this cellular disruption. The same phenomenon was exhibited by preconditioned cells (Plate 5.4d). In grape must (complex media) a similar response can be seen (Plate 5.5a-d). Plate 5.5b) shows dramatic pitting and cracking of yeast cells exposed to 10% v/v ethanol. On elevation of magnesium levels either by concurrent addition (Plate 5.5c) or preconditioning (Plate 5.5d) this cell surface damage is reduced and cells under these conditions resemble the appearance of control cells. Intracellular disruption of *Sacch. cerevisiae* (L-2226) in grape must under conditions of 10% ethanol shock can be seen in Plate 5.6b)-d). Although the results here are not as pronounced as with heat shock, some intracellular granules can be seen in Plate 5.6b) and magnesium-preconditioning (Plate 5.6d) restored cells to an appearance resembling control cells, illustrating the protective role of magnesium at an intracellular level.

Notwithstanding that the stress response is a transient response involving gene expression; transcription and post-translational regulation, not visible at the DNA level, CHEF analysis was carried out on *Sacch. cerevisiae* (L-2226) shocked with 10% ethanol. CHEF analysis has been suggested to be effective in detecting chromosomal variations among pure cultures

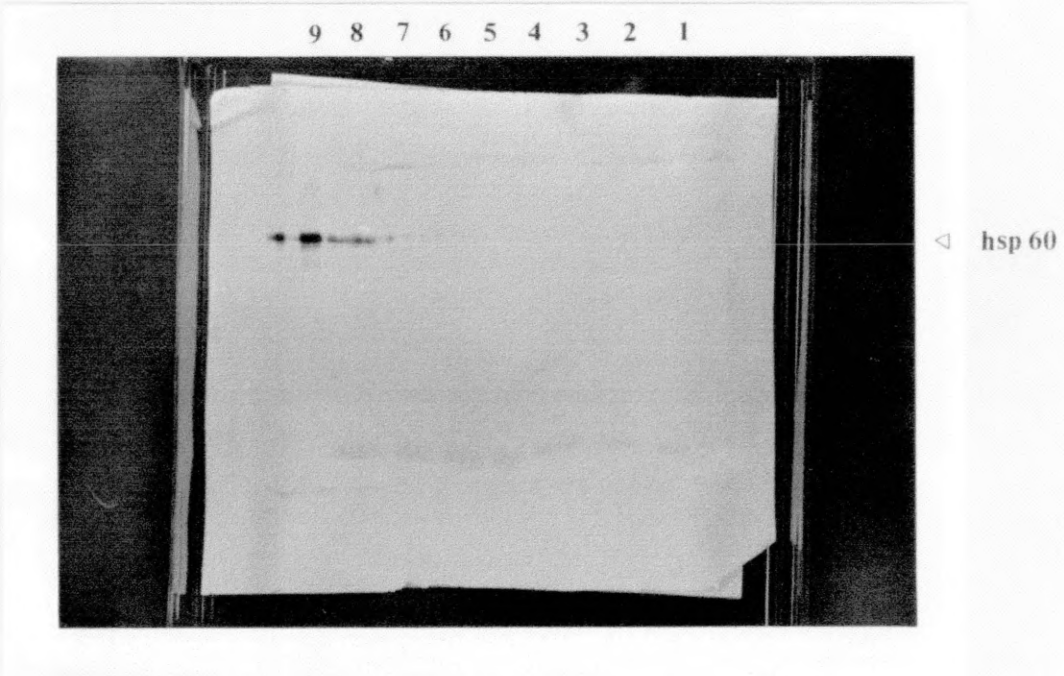
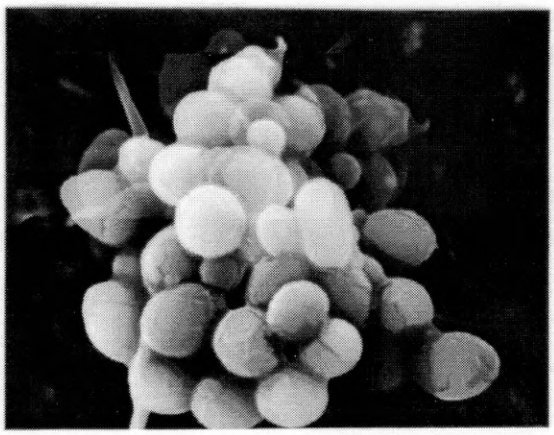
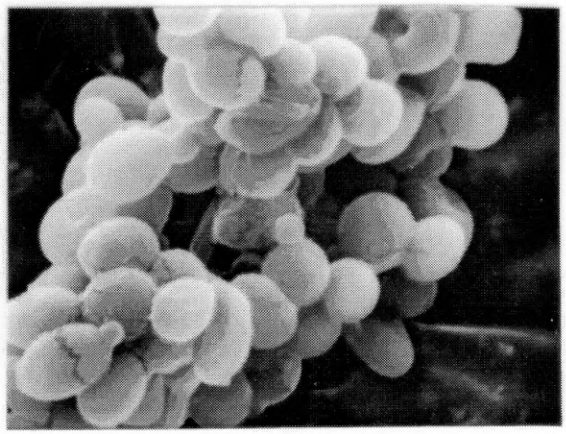


Figure 5.37 Western analysis of ethanol shocked (10%) wine yeast (L-2226) specific for hsp60. Lanes: 1) Rainbow™ Marker, 2) Control (30°C), 3) 2mM Mg, 4) 5mM Mg, 5) 20mM Mg, 6) preconditioned 5mM Mg, 7) preconditioned 20mM Mg, 8) Heat shock (42°C), 9) GroEL. Lanes 3)-7) @ 10% Ethanol.

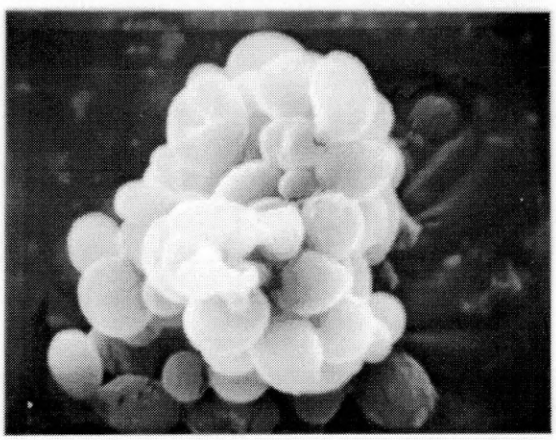
a)



b)



c)



d)

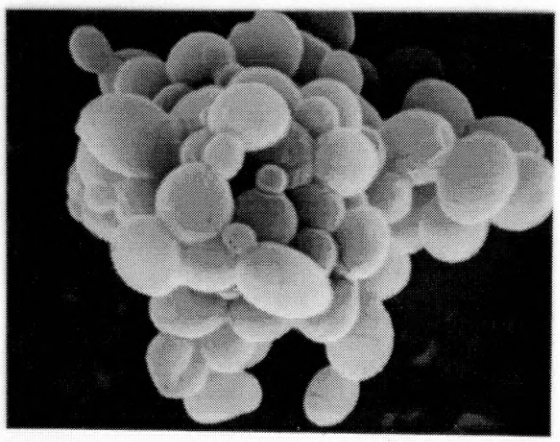
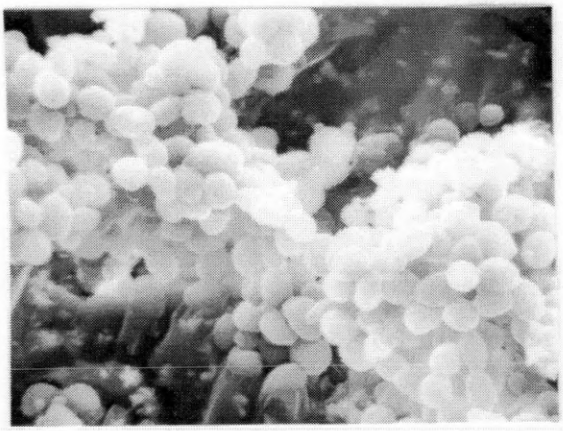


Plate 5.4 Cellular damage of *Sacch. cerevisiae* (L-2226) in minimal media, under conditions of; a) Control (30^{o}C), b) Ethanol shock (10%), c) Ethanol shock (10%) with elevated magnesium (20mM) and d) Ethanol shock (10%) preconditioned with elevated magnesium (20mM).
(Magnification: a)-d) x3165).

a)



b)

c)

d)

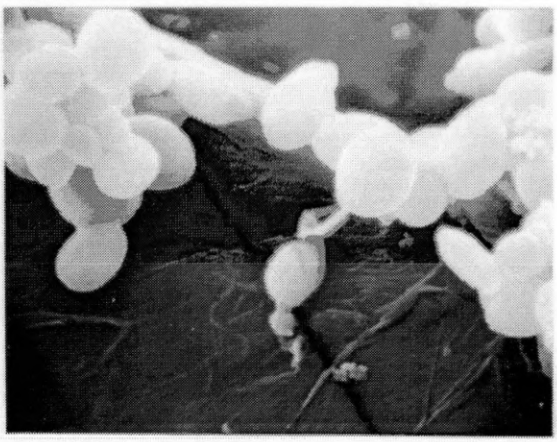


Plate 5.5 Cellular damage of *Sacch. cerevisiae* (L-2226) in grape must, under conditions of; a) Control (30^{o}C), b) Ethanol shock (10%), c) Ethanol shock (10%) with elevated magnesium (20mM) and d) Ethanol shock (10%) preconditioned with elevated magnesium (20mM). (Magnification: a) x1582.5, b)-d) x3165).

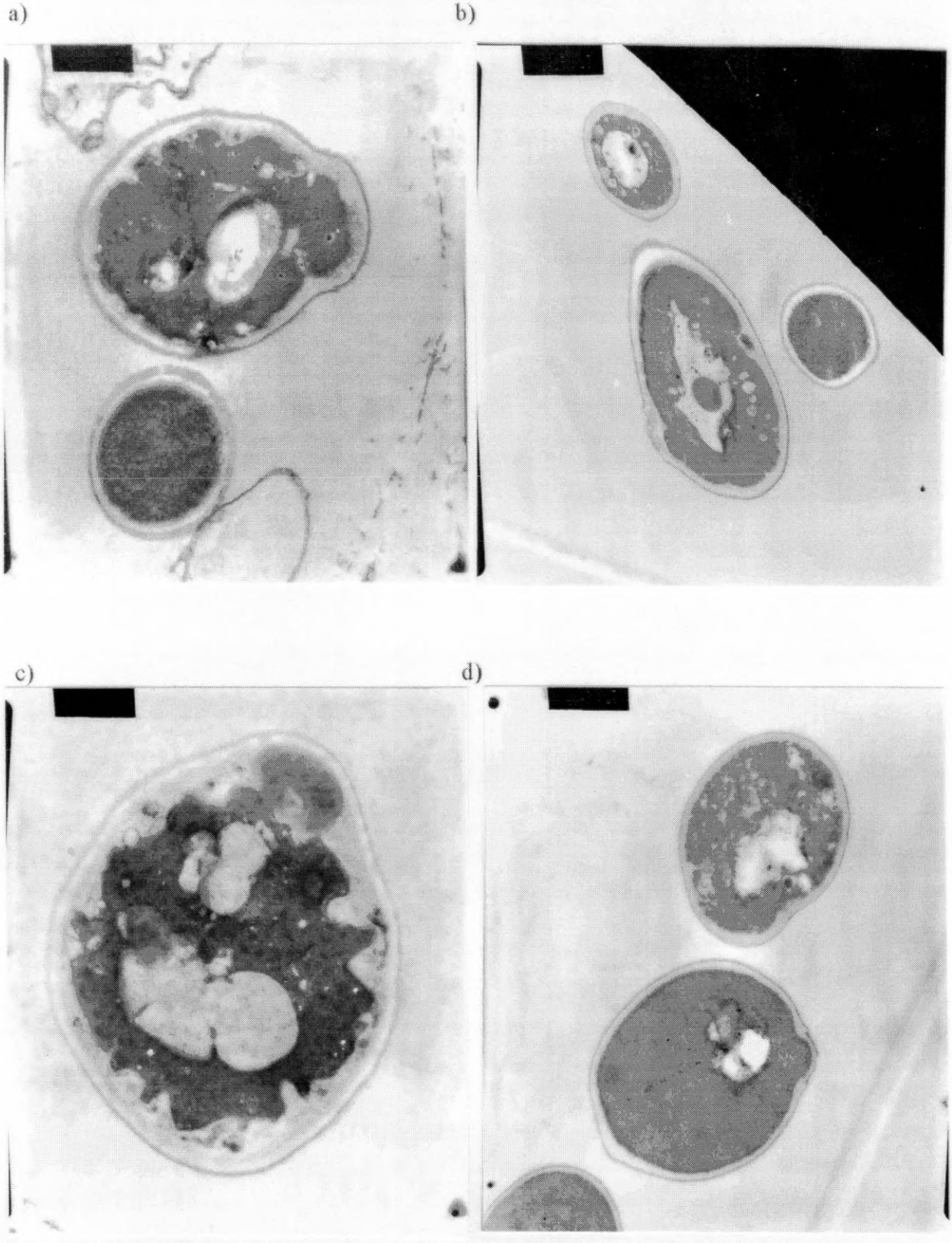


Plate 5.6 Intracellular disruption of *Sacch. cerevisiae* (L-2226) in grape must, under conditions of; a) Control (30^{o}C), b) Ethanol shock (10%), c) Ethanol shock (10%) with elevated magnesium (20mM) and d) Ethanol shock (10%) preconditioned with elevated magnesium (20mM).
(Magnification: a) x17000, b) x13000, c) x28000, d) x13000).

of industrially fermenting strains and since the ethanol produced during such fermentations could constitute stress as examined here, this analysis is a valid test. It can be seen from Figure 5.38 that cultures undergoing ethanol stress do not exhibit a chromosomal shift. The overall conclusion drawn from the comparison of pulse-chase experiments (Fig. 5.30 & 5.35) and CHEF analysis (Fig. 5.38) confirms that ethanol shock has an effect at the level of gene expression (transcription, translation) and/or on the protein stability, but not at the levels of chromosome integrity. Whether CHEF analysis could be utilised, as suggested, in detecting variations in industrially fermenting strains is not within the scope of this work, but from the results of the stress experiment this is questionable. Cells in a fermentation are all at various stages of growth and physiological states and will therefore react differently to stress or even changes in growth conditions thus this technique could only show mass effects in the population. These results would therefore disprove the theories of the applicability of PGFE for monitoring genetical variations within a fermenting population using this technique since only a mass genetic variation could be seen, conclusions confirmed by another institute (G. Cardinali; personal communication).

Ethanol, at levels experienced by wine yeast in normal fermentations constitute a stress. The results of this stress are; reduction of viability, increased ion leakage, cellular damage and stress protein production. Elevation of magnesium levels (either intra- or extra-cellularly) results in an improvement of cell status. Magnesium appears to exert a protective effect on stressed cells resulting in reductions in; cell mortality, leakage of essential ions and cell surface damage, the maintenance of intracellular integrity and a repression of repair mechanisms, e.g. stress proteins. It is thought to act in countermanding the damaging effects of ethanol on cell membranes, maintaining structural integrity of cells and protecting them from the permeabilising effect of ethanol stress.

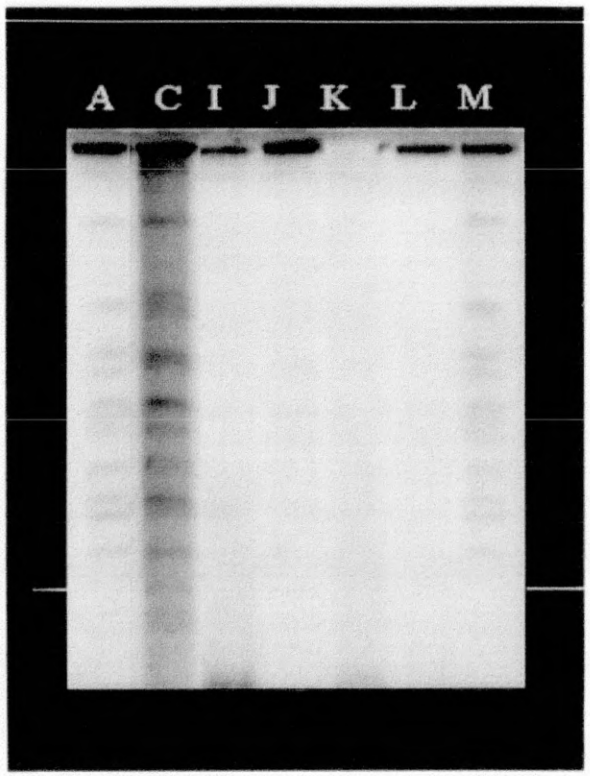


Figure 5.38 Analysis of *Sacch. cerevisiae* (L-2226) following heat (42^{o}C) and ethanol (10%) shock by CHEF electrophoresis. Lanes: A) DBV6768 (CHEF DNA Standard), C) L-2226 (YEPG), I) L-2226 (CMMM)-Control (30^{o}C), J) L-2226 (CMMM)-Heat shock (42^{o}C), K) L-2226 (CMMM)-Ethanol shock (10%), L) L-2226 (CMMM)-Heat and Ethanol shock, M) DBV6768 (YEPG).

5.3.3 NUTRIENT AND METAL ION RELATED STRESS

Stress tolerance of *Sacch. cerevisiae* varies greatly with the physiological state of cells and culture conditions, *i.e.* the nutrient supply to the cells. Cells growing rapidly are more sensitive to stresses, e.g. physical or chemical stresses, than cells that are growing slowly or resting (Craig 1985, Watson 1990).

5.3.3.1 NUTRIENT STARVATION

Nutrient limitation is the major factor responsible for the decline in fermentative activity during the early stages of fermentation in yeast (Dombek & Ingram 1986a). The limiting nutrient will more likely be an ionic species essential for enzyme activity, e.g. Mg^{2+}, or some other essential cellular process, or it will be the exhaustion of nitrogenous compounds or the carbon-source itself. Whatever the cause, nutrient stress is a serious environmental stress and as such triggers an action from the stress response.

Figure 5.39 shows stress protein production in a wine strain of *Sacch. cerevisiae* (L-2226) under nutrient stress. It can be seen from the autoradiograph of labelled cultures (Fig. 5.39) that certain stress proteins are produced under different conditions and that some stresses illicit a greater response than others. Table 5.9 illustrates the occurrence of several 'typical' hsps. Heat shock ($42^{o}C$) as a positive control, expresses the majority of these hsps, whereas the nutrient stresses express only some of the important stress proteins. Cells undergoing N_2-starvation exhibited stress proteins at: 60, 30 and 26kDa. Walker & McWilliams (1989) showed a similar response in cells of *S. pombe* and these resultant stress proteins were shown to play a similar role to hsps in protecting cells from environmental insults. Under carbon (glucose) starvation, a situation quite common to occur in fermentative or industrial situations, stress proteins of: 150, 60, 46, 30 and 26kDa are induced and the production of these five hsps corresponds to the work of Boucherie (1985) on glucose starvation in yeast. Glucose limitation (250μm), on the other hand, only results

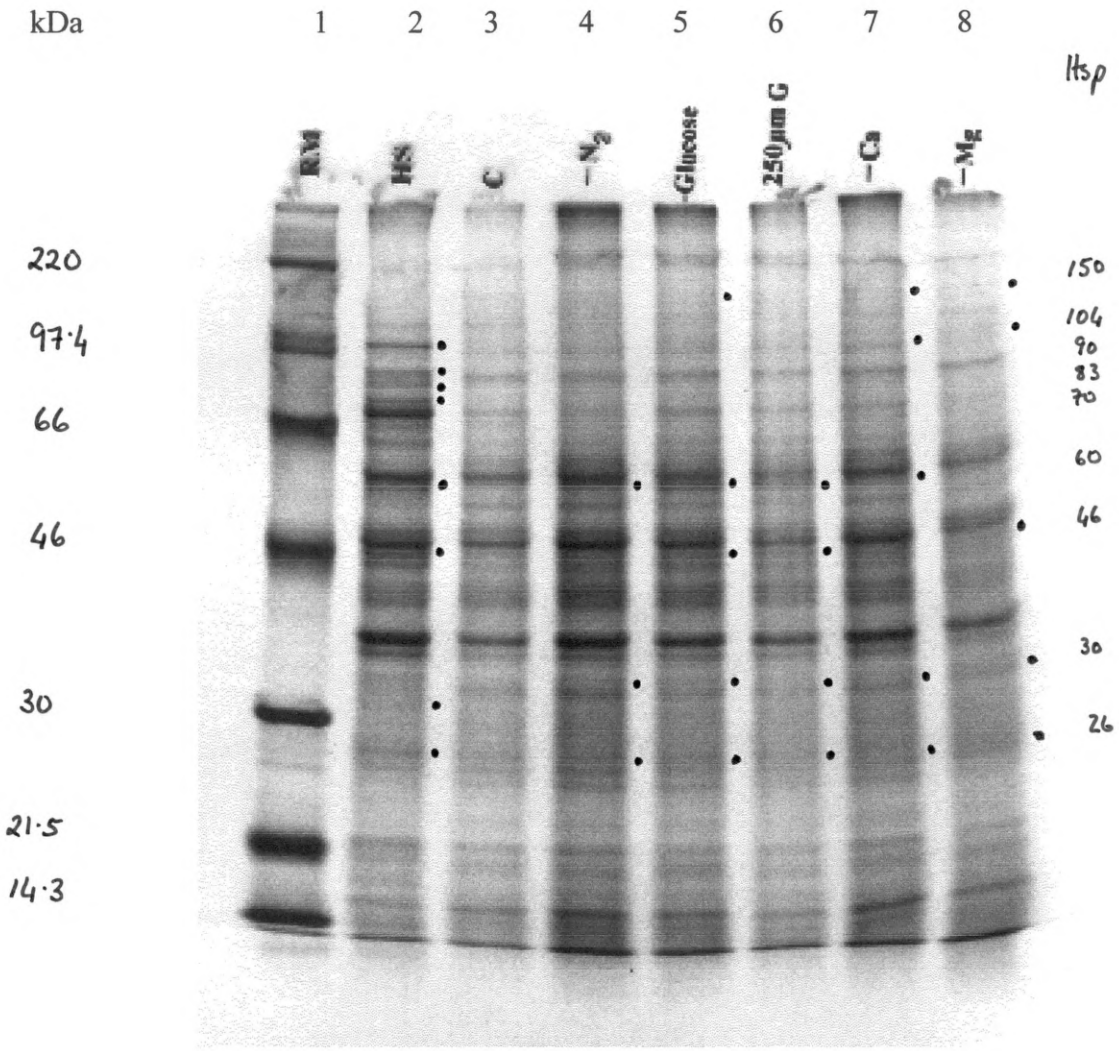


Figure 5.39 Stress protein production in wine yeast (L-2226) due to nutrient limitation/starvation. Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 3) Control (30°C), 4) N_2 starvation, 5) Glucose starvation, 6) Glucose limitation (250µm), 7) Calcium starvation, 8) Magnesium starvation.

in four hsps of: 60, 46, 30 and 26kDa in size. Another important nutrient stress is ionic limitation/starvation. Calcium and magnesium starvation of *Sacch. cerevisiae* (L-2226) both resulted in a similar response, *i.e.* the production of five or six hsps of varying size. Calcium starvation elicited the induction of stress proteins at: 150, 104, 60, 30 and 26kDa and magnesium starvation those at: 150, 104, 70, 46, 30 and 26kDa.

Table 5.9 Induction of stress protein production in wine yeast (L-2226) by various nutrient limitations/ starvations.

Stress Protein (kDa)	Heat Shock (42°C)	N_2 Starvation	C Starvation	C Limitation	Ca Starvation	Mg Starvation
hsp150	✗	✗	✓	✗	✓	✓
hsp104	✓	✗	✗	✗	✓	✓
hsp90	✓	✗	✗	✗	✗	✗
hsp83	✓	✗	✗	✗	✗	✗
hsp70	✓	✗	✗	✗	✗	✓
hsp60	✓	✓	✓	✓	✓	✗
hsp46	✓	✗	✓	✓	✗	✓
hsp30	✓	✓	✓	✓	✓	✓
hsp26	✓	✓	✓	✓	✓	✓
hsp12	✗	✗	✗	✗	✗	✗

Note: ✓ = protein present, ✗ = protein absent

The stress response is not therefore restricted to heat shock of cells. Cells undergoing nutrient stress induce stress proteins as a repair mechanism for cells. These results serve to illustrate that the physiological state of cells is of utmost importance. In industrial situations yeast will experience a range of physical and chemical stresses and these compounded by nutrient stress would result in reduced performance or even cell death. The nutritional aspects of yeast growth therefore are of fundamental importance, not least that of ionic nutrition.

5.3.3.2 MAGNESIUM : CALCIUM RATIOS

The effects of altering Mg:Ca ratios in the media have been observed previously (Chapter 3) in terms of growth and fermentation parameters of industrial strains of *Sacch. cerevisiae*. In addition to this, the stress effect of gross alterations of media ratios was assessed. Using the same ratio ranges as for the growth experiments, SDS-PAGE was carried out on a wine strain (L-2226). Figure 5.40 shows the pattern of stress protein production following the alteration of Mg:Ca ratios in minimal media. Control and heat shocked cultures were included as negative and positive controls. Table 5.10 shows the induction of certain 'typical' yeast stress proteins and as can be seen, increasing calcium ratios to extreme levels (1:100 & 1:1000) with only very low magnesium levels (0.1mM) results in the production of the most stress proteins at: 104, 90, 30 and 26kDa.

Growth at 1:1000 Mg:Ca ratios was noted to be very weak suggesting that cells cannot grow satisfactorily in such high levels of calcium, compared to magnesium, as this ion will saturate all the binding sites available thus blocking binding of even the small amounts of magnesium present in the medium, if not a range of essential ions. In this scenario, efficient cellular functioning cannot take place therefore cell growth will be reduced and as can be seen from these results the stress response is initiated. Growth of cells in very high levels of Mg also result in the induction of the stress response with the production of stress proteins at: 104, 60 and 30kDa. Stress proteins are produced, although type and levels of expression vary, across the range of ratios. This may be explained by the requirement of cells for a balance of essential ions at adequate levels. In conclusion, therefore, these results show that a balance of ions is required for maintenance of cells in a correct and unstressed physiological state.

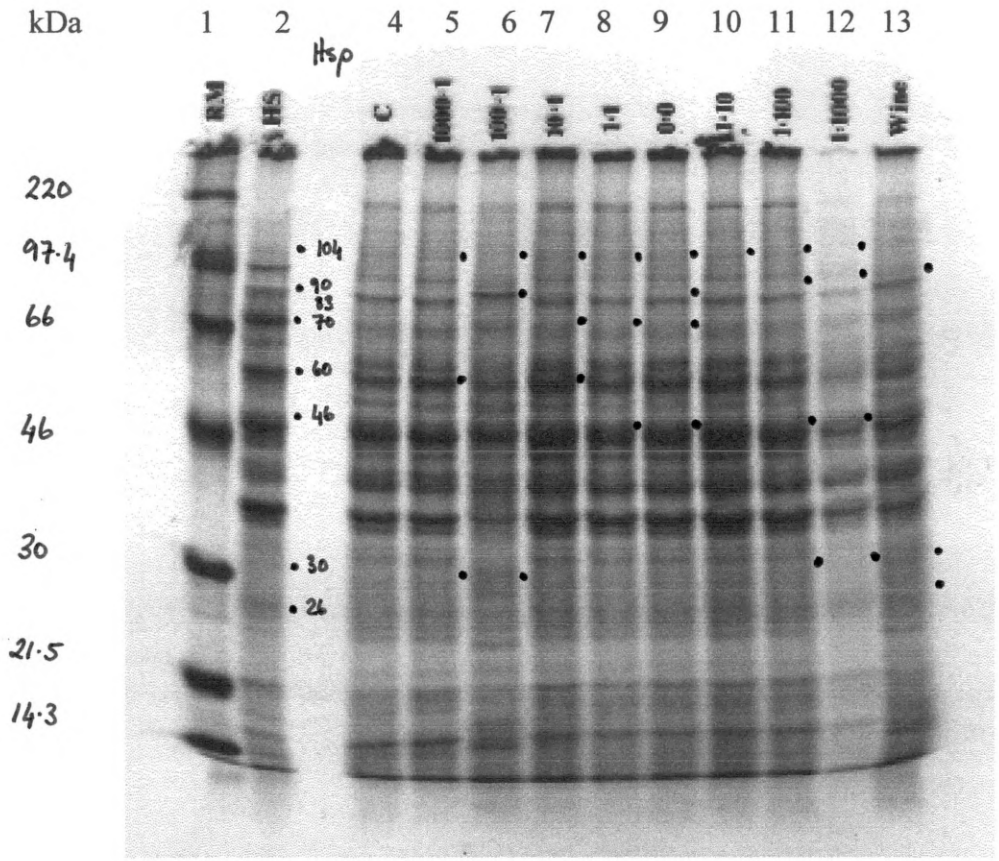


Figure 5.40 Stress protein production in wine yeast (L-2226) following alteration of Mg:Ca ratios in minimal media (CMMM). Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 4) Control (30°C), 5) 1000:1 Mg:Ca, 6) 100:1 Mg:Ca, 7) 10:1 Mg:Ca, 8) 1:1 Mg:Ca, 9) 0:0 Mg:Ca, 10) 1:10 Mg:Ca, 11) 1:100 Mg:Ca, 12) 1:1000 Mg:Ca, 13) Synthetic wine.

Table 5.10 Induction of stress protein production in wine yeast (L-2226) by altering Mg:Ca ratios in minimal media (CMMM).

Stress Protein (kDa)	Heat Shock (42°C)	Mg:Ca Concentration Ratio								
		1000:1	100:1	10:1	1:1	0:0	1:10	1:100	1:1000	Wine
hsp150	×	×	×	×	×	×	×	×	×	×
hsp104	✓	✓	✓	✓	✓	✓	✓	✓	✓	×
hsp90	✓	×	×	×	×	×	×	✓	✓	×
hsp83	✓	×	✓	×	×	✓	×	×	×	✓
hsp70	✓	×	×	✓	✓	✓	×	×	×	×
hsp60	✓	✓	×	✓	×	×	×	×	×	×
hsp46	✓	×	×	×	✓	✓	×	✓	✓	×
hsp30	✓	✓	✓	×	×	×	×	✓	✓	✓
hsp26	✓	×	×	×	×	×	×	×	×	✓
hsp12	×	×	×	×	×	×	×	×	×	×

Note: ✓ = protein present, × = protein absent

5.3.3.3 CALCIUM SHOCK

Further to the study of Mg:Ca ratios and expanding on the results observed for excess calcium levels in growth media, a study was carried out to assess the effect of calcium shock on yeast. Figure 5.41 shows stress protein production in wine yeast (L-2226) on calcium shock over a range of concentrations up to 100mM Ca. Table 5.11 records the presence of 'typical' stress proteins compared to the control culture. From this it can be seen that elevation of calcium concentration to excessive levels in the growth medium sends the cells into a shocked state. As calcium concentration is increased the number of stress proteins induced increases. For example, stress proteins are produced at: 104 and 30kDa at 2.5mM (normal levels in complex and laboratory media) and 150, 104, 46, 30 and 26kDa in concentrations of calcium of 50mM and 100mM. The results of this type of stress is similar to that of nutrient limitation, simply because it is a form of this. By elevating calcium concentration to such a high degree cells are saturated with this ion and other essential ions, such as magnesium, are thus limited because the cell cannot take up the concentrations of these ions that they require on account of the calcium ions being bound to all the available cell membrane binding sites. Ca^{2+} are in competition with Mg^{2+} and due to the higher concentration out compete other ions. Calcium is known to work in an antagonistic manner towards magnesium and this is further proof for this concept.

5.3.3.4 METAL ION REMOVAL: CHELATORS

Ion limitation is of great importance to yeast cells, as with all living cells. Ionic species are essential in various aspects of cell physiology, metabolism and growth. They are required for enzyme activation, cell division processes, maintaining structural integrity, etc., their roles are many-fold and they are essential for optimum growth and fermentation of yeast and as such metal ion removal would therefore constitute a stress. The induction of the stress response in association with ionic limitation was investigated by SDS-PAGE autoradiography.

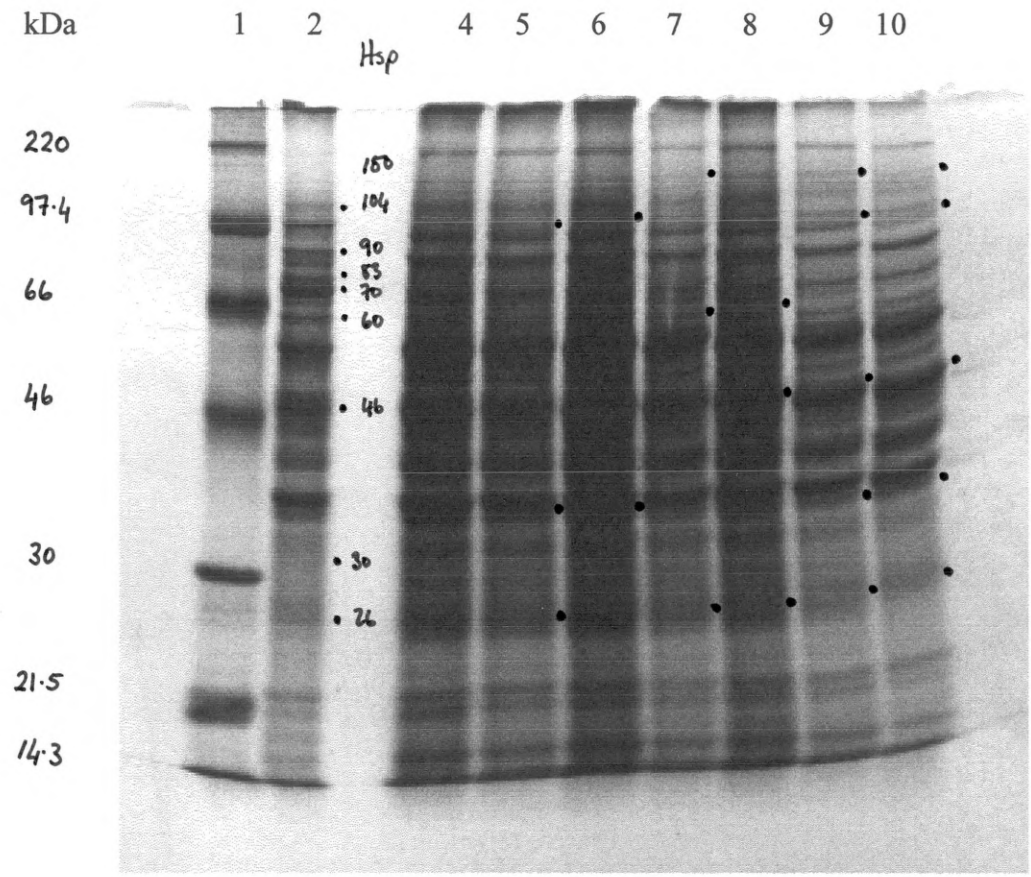


Figure 5.41 Stress protein production in wine yeast (L-2226) on calcium shock. Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 4) Control (30°C), 5) 1mM Ca, 6) 2.5mM Ca, 7) 5mM Ca, 8) 10mM Ca, 9) 50mM Ca, 10) 100mM Ca.

Table 5.11 Induction of stress protein production in wine yeast (L-2226) on calcium shock.

Stress Protein (kDa)	Heat Shock (42°C)	Calcium Concentration 1mM	2.5mM	5mM	10mM	50mM	100mM
hsp150	✗	✗	✗	✓	✗	✓	✓
hsp104	✓	✓	✓	✗	✗	✓	✓
hsp90	✓	✗	✗	✗	✗	✗	✗
hsp83	✗	✗	✗	✗	✗	✗	✗
hsp70	✓	✗	✗	✗	✗	✗	✗
hsp60	✓	✗	✗	✓	✓	✗	✗
hsp46	✓	✗	✗	✗	✓	✓	✓
hsp30	✓	✓	✓	✗	✗	✓	✓
hsp26	✓	✓	✗	✓	✓	✓	✓
hsp12	✗	✗	✗	✗	✗	✗	✗

Note: ✓ = protein present, ✗ = protein absent

Figure 5.42 illustrates the protein profiles of a wine strain of *Sacch. cerevisiae* (L-2226) following treatment with a range of chelating agents. The chemical stress resulting from the complexing of essential ionic species (not just magnesium and calcium) results in the induction of various stress proteins (Table 5.12). Chelation of ion species by EDTA resulted in the induction of stress proteins at: 104 and 30kDa, whereas EGTA also induced the production of hsp46. 8-Hydroxyquinoline and sodium pyrophosphate both induced the stress proteins: hsp104, 30 and 26 and citric acid appears to be the only chelator to induce production of hsp60 (as well as 30 and 26kDa stress proteins). The induction of different stress proteins will depend on the action of the chelator and which ionic species they are specific for. Different ionic species fulfil different jobs in the cell and likewise different stress proteins have different modes of action, repairing different aspects of cellular damage therefore the chelation of different ionic species will result in the induction of different hsps.

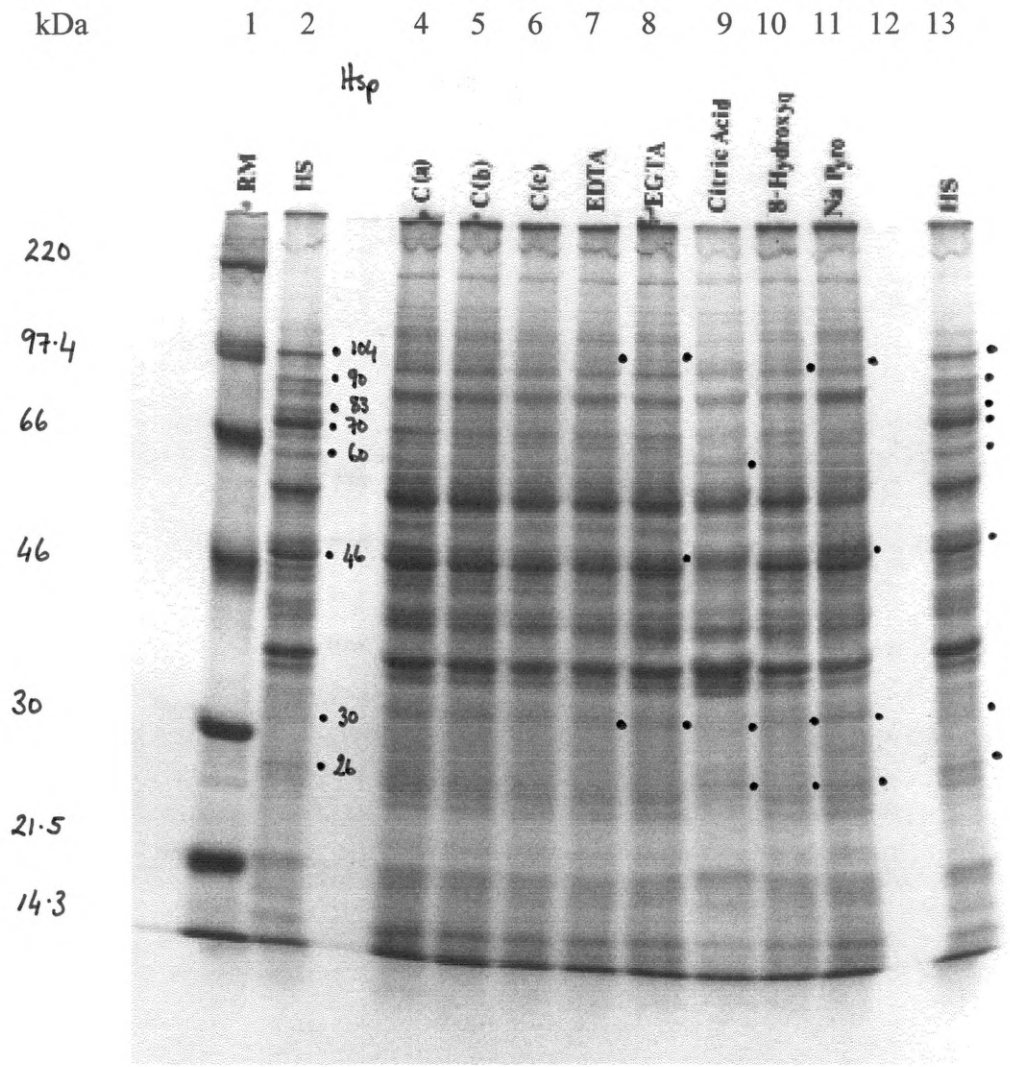


Figure 5.42 Stress protein production in wine yeast (L-2226) on metal ion removal by chelators. Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 4) Control (30°C), 5) Control + 0.1mM NaOH, 6) Control + 0.1mM HCl, 7) EDTA 30mM, 8) EGTA 15mM, 9) Citric acid 40mM, 10) 8-Hydroxyquinoline 0.05mM, 11) Sodium pyrophosphate 40mM.

Table 5.12 Induction of stress protein production in wine yeast (L-2226) on metal ion removal by chelators.

Stress Protein (kDa)	Heat Shock (42°C)	EDTA	EGTA	Citric Acid	8-Hydroxy-quinoline	Sodium pyrophosphate
hsp150	✗	✗	✗	✗	✗	✗
hsp104	✓	✓	✓	✗	✓	✓
hsp90	✓	✗	✗	✗	✗	✗
hsp83	✗	✗	✗	✗	✗	✗
hsp70	✓	✗	✗	✗	✗	✗
hsp60	✓	✗	✗	✓	✗	✗
hsp46	✓	✗	✓	✗	✗	✓
hsp30	✓	✓	✓	✓	✓	✓
hsp26	✓	✗	✗	✓	✓	✓
hsp12	✗	✗	✗	✗	✗	✗

Note: ✓ = protein present, ✗ = protein absent

METAL ION REMOVAL: IONOPHORES

Ionophores also remove essential ionic species from culture media and two such ionophores were examined in the study: Mg Ionophore (Calbiochem) and A23187. Various concentrations of these two ionophores were investigated in terms of their effect on stress protein induction over a 1h period. Figure 5.43 illustrates stress protein production in *Sacch. cerevisiae* (L-2226) on metal ion removal by the ionophore: Calbiochem Mg Ionophore, over a range of concentrations (0.05-1.0μg/ml) and Figure 5.44 illustrates that of: A23187. It can be seen from these two autoradiographs that some stress proteins are produced. From Table 5.13 it can be seen that increasing ionophore concentration increases stress protein induction, both in terms of degree of induction and number of proteins induced, when compared to control cultures. The modes of action of these two ionophores appear to be different from the protein profile patterns displayed. Levels of ionophores above 0.75μg/ml appear to be the most damaging producing stress proteins at: 150, 104,

60, 46 and 30kDa for Mg Ionophore and 150, 104, 60, 46, 30 and 26kDa for A23187. Low Ca^{2+} has a marked protective effect on toxicity of A23187, cell killing directly dependent on presence of Ca^{2+} at time of treatment (Landry *et al.* 1988).

These results give more evidence to the concept that stress on cells is dependent on the physiological status of the cells initially and that stresses are not just physical, e.g. heat, or chemical, e.g. ethanol, but that nutrient stress is an important factor for consideration. Cells require adequate ionic nutrition as well as sufficient supplies of macro-nutrients. The story of ionic limitation/shock gives support to the claims made earlier that magnesium plays an important role in the stress response in yeast cells. This ion has a protective role to play negating the need for stress protein synthesis and the point is illustrated that without other physical or chemical stresses the simple removal of this essential ion (amongst others) results in the induction of the yeast stress response cumulating in the production of stress proteins.

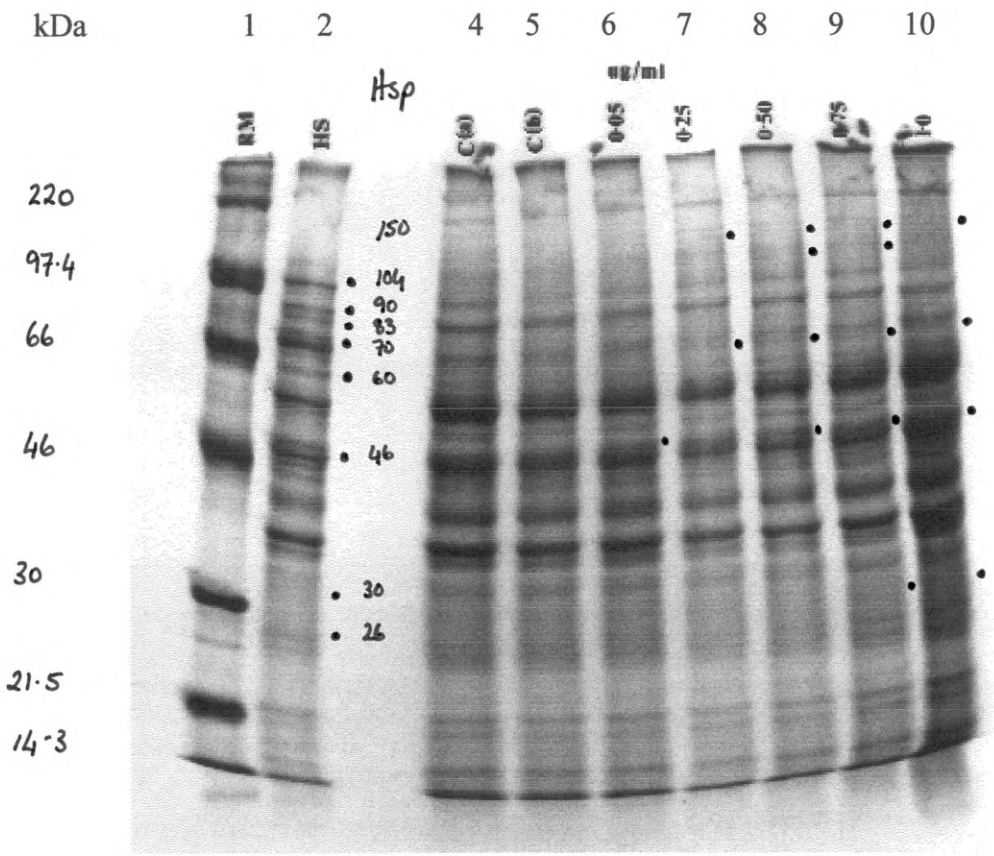


Figure 5.43 Stress protein production in wine yeast (L-2226) on metal ion removal by ionophore: Calbiochem Mg Ionophore. Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 4) Control (30°C), 5) Control + acetone/ethanol, 6) 0.05μg/ml, 7) 0.25μg/ml, 8) 0.50μg/ml, 9) 0.75μg/ml, 10) 1.0μg/ml.

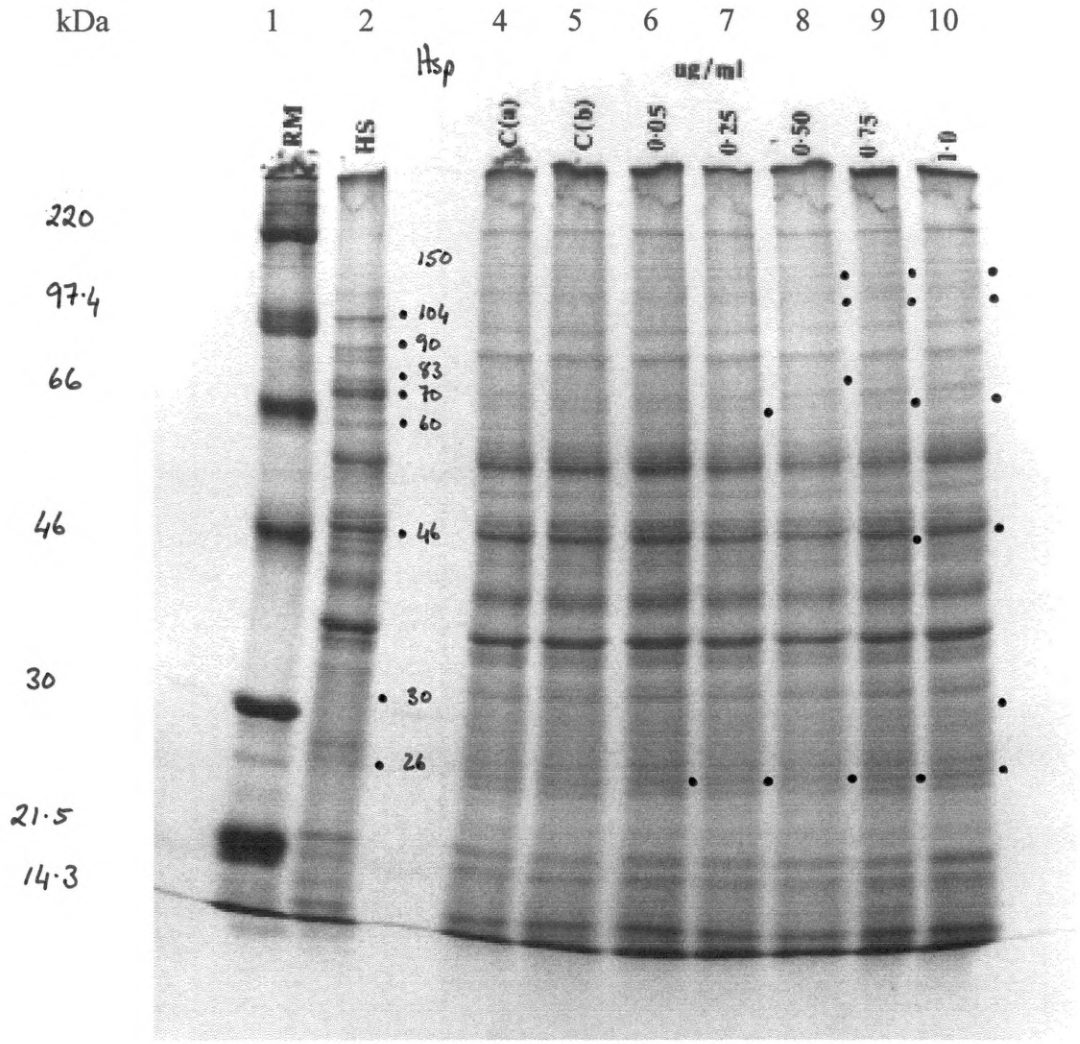


Figure 5.44 Stress protein production in wine yeast (L-2226) on metal ion removal by ionophore: A23187. Lanes: 1) Rainbow™ Marker, 2) Heat shock (42°C), 4) Control (30°C), 5) Control + acetone/ethanol, 6) 0.05µg/ml, 7) 0.25µg/ml, 8) 0.50µg/ml, 9) 0.75µg/ml, 10) 1.0µg/ml.

Table 5.13 Induction of stress protein production in wine yeast (L-2226) on metal ion removal by ionophores.

Stress Protein	Heat Shock	Mg Ionophore (μg/ml)					A23187 (μg/ml)				
(kDa)	($42^{o}C$)	0.05	0.25	0.50	0.75	1.0	0.05	0.25	0.5	0.75	1.0
hsp150	✗	✗	✓	✓	✓	✓	✗	✗	✓	✓	✓
hsp104	✓	✗	✗	✓	✓	✗	✗	✗	✓	✓	✓
hsp90	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
hsp83	✓	✗	✗	✗	✗	✗	✗	✗	✓	✗	✗
hsp70	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
hsp60	✓	✗	✓	✓	✓	✓	✗	✓	✗	✓	✓
hsp46	✓	✓	✗	✓	✓	✓	✗	✗	✗	✓	✓
hsp30	✓	✗	✗	✗	✓	✓	✗	✗	✗	✗	✓
hsp26	✓	✗	✗	✗	✗	✗	✓	✓	✓	✓	✓
hsp12	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗

Note: ✓ = protein present, ✗ = protein absent.

5.3.4 COMPARATIVE STRESS RESPONSES IN WINE YEASTS.

Stress responses have been examined under a variety of environmental insults which yeast cells may be subjected to during growth and fermentation in an industrial setting, however these studies have been carried out on only one wine strain of *Sacch. cerevisiae*. A comparison was therefore carried out between two wine strains of this yeast; *Sacch. cerevisiae* (L-2226) and *Sacch. cerevisiae* (DBVPG2168) to assess whether the responses observed here carried through the species and would therefore be applicable in all wine strains. A range of stresses was applied to the two strains and analysis of protein profiles carried out. Figure 5.45 shows the stress responses of *Sacch. cerevisiae* (L-2226) to some chemical and physical stresses such as heat shock (42^{o}C), ethanol shock (10%), calcium excess and metal ion removal by chelators (Citric acid) and ionophores (Mg Ionophore & A23187). Figure 5.46 illustrates the response of *Sacch. cerevisiae* (DBVPG2168) to some such stresses, for example heat stress (42^{o}C), ethanol stress (15%), calcium excess and deionisation of the growth medium. Table 5.14 attempts to compare the results of these stresses on the two wine strains. The response to heat shock of the two strains is remarkably similar with only slight differences occurring in the proteins induced with hsp104, hsp70, hsp60, hsp30 and hsp26 being expressed in both strains. Ethanol shock also exhibits some differences but these are not significant, with stress proteins being produced at: 104, 46 and 30kDa in both strains. Deionisation of the medium has an effect similar to the addition of chelators or ionophores (although to a greater degree), in that they all remove essential ionic species from the medium. From the table (Table 5.14) it can be seen that the stress proteins: 46 and 26kDa are produced in all cases of ion stress and over and above these various other proteins are produced dependent on which ion species is complexed and the state of growth of the cells.

Western analysis of various stresses (heat shock, ethanol shock, calcium shock and ion removal) on both strains was carried out, specific for stress proteins hsp70, hsp60 and hsp27. Figure 5.47 shows the results for hsp60 of *Sacch. cerevisiae* (L-2226), confirmation exists (lane 4) that hsp60 is produced on exposure of cells to a sub-lethal heat shock

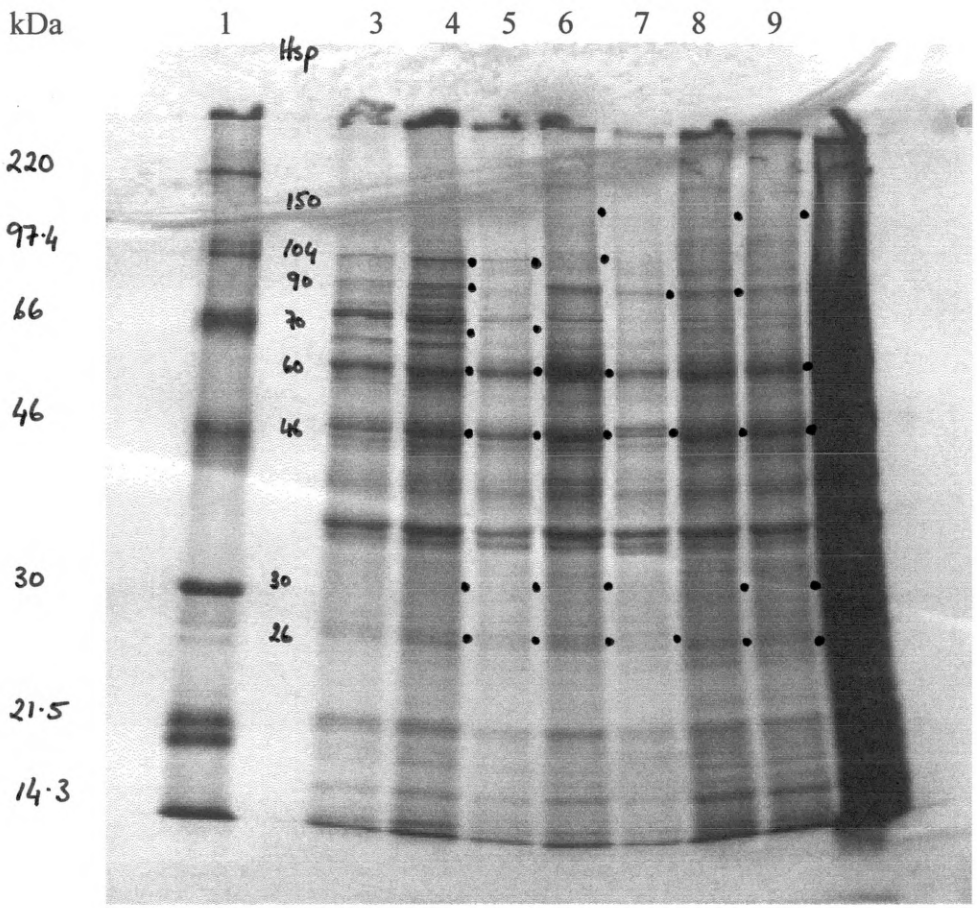


Figure 5.45 Comparison of stress response of *Sacch. cerevisiae* (L-2226) to various physical and chemical stressors. Lanes: 1) Rainbow™ Marker, 3) Control (30^{o}C), 4) Heat shock (42^{o}C), 5) Ethanol shock (10%), 6) 100mM Ca, 7) Citric acid 40mM, 8) Mg Ionophore 1μg/ml, 9) A23187 1μg/ml.

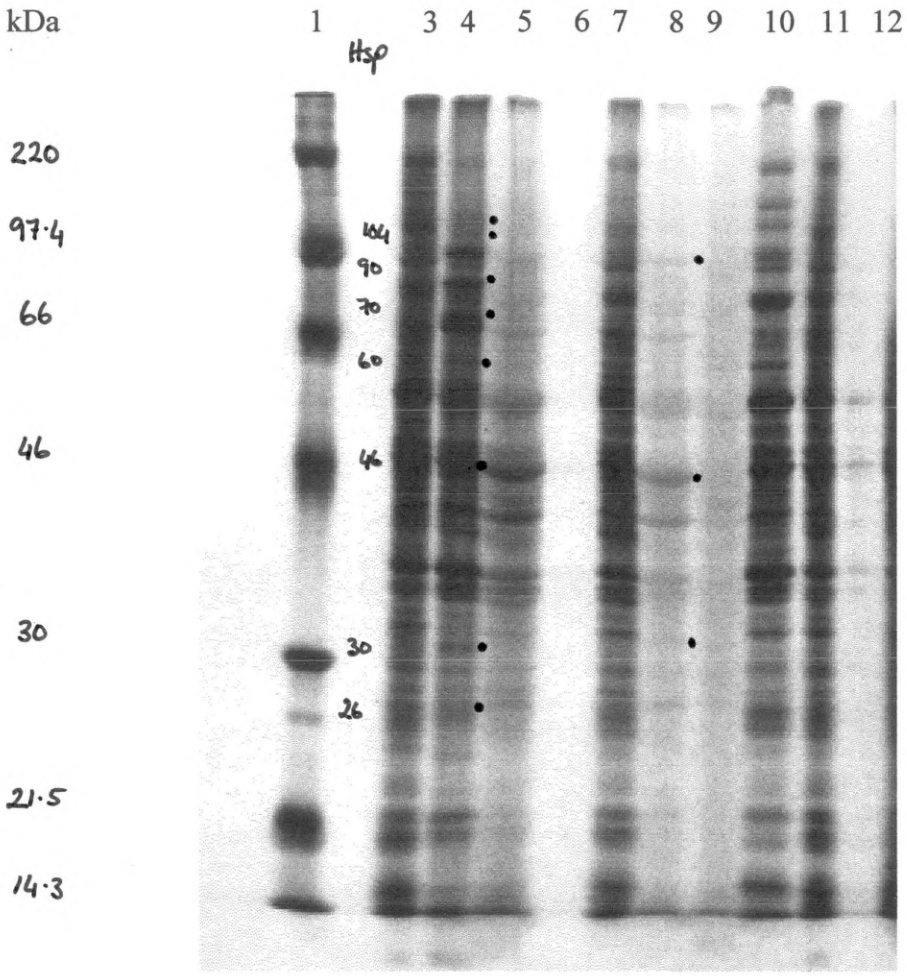


Figure 5.46 Comparison of stress response of *Sacch. cerevisiae* (DBV2168) to various physical and chemical stressors. Lanes: 1) Rainbow™ Marker, 3) Control ($30^{o}C$), 4) Heat shock ($42^{o}C$), 5) Deionised (Chelex100), 6) 1000mM Ca, 7) 1000mM Mg, 8) Ethanol shock (15%), 9) Deionised + trace elements, 10) Must [Ca]x2, 11) Must [Mg]x2.

Table 5.14 Effect of different stressors on stress protein production by wine strains *Sacch. cerevisiae* L-2226 and DBV2168.

Stress Protein	Heat Shock		Ethanol		Calcium		Chelator		Ionophore		Ionophore		Deionised	
(kDa)	(42°C)		10%	15%	100mM	1M	Citric Acid		Mg Calbiochem		A23187		Chelex 100	
Strain	L-2226	DBV2168	L-2226	DBV2168	L-2226	DBV2168	L-2226	DBV2168	L-2226	DBV2168	L-2226	DBV2168	L-2226	DBV2168
hsp150	✗	✗	✗	✗	✓	No	✗	NT	✓	NT	✓	NT	NT	✗
hsp104	✓	✓	✓	✓	✓	Growth	✗	NT	✗	NT	✓	NT	NT	✓
hsp90	✓	✗	✗	✗	✗		✗	NT	✗	NT	✗	NT	NT	✗
hsp83	✗	✗	✓	✗	✗		✓	NT	✓	NT	✗	NT	NT	✓
hsp70	✓	✓	✗	✗	✗		✗	NT	✗	NT	✗	NT	NT	✗
hsp60	✓	✓	✓	✗	✓		✗	NT	✗	NT	✓	NT	NT	✓
hsp46	✓	✗	✓	✓	✓		✓	NT	✓	NT	✓	NT	NT	✓
hsp30	✓	✓	✓	✓	✓		✗	NT	✓	NT	✓	NT	NT	✓
hsp26	✓	✓	✓	✗	✓		✓	NT	✓	NT	✓	NT	NT	✓
hsp12	✗	✗	✗	✗	✗		✗	NT	✗	NT	✗	NT	NT	✗

Note: ✓ = protein present, ✗ = protein absent, NT = not tested

(42^{o}C), but not with the other stresses and this corresponds to the results of the protein profiles of cells exposed to these stress conditions. Similar results were observed for *Sacch. cerevisiae* (DBVPG2168). Cells of either strain did not react positively with antibodies specific for hsp70 or hsp27 and again this corresponds with previous results.

Finally the response to heat and ethanol shock and a combination of these two stresses at the DNA level of the strains were compared by CHEF electrophoresis. Inherent of the heat shock response is that it is a transient short-term mechanism involving transcription and post-translational gene regulation none of which is visible at the DNA level. From LSD analysis of Figure 5.48 a) & b) it can be seen that there is not any significant difference between corresponding bands of patterns obtained from pools of heat stressed cells, nor ethanol stressed cells. The combination of heat and ethanol stress also shows no significant difference despite the fact that elevation of temperature is known to influence the ethanol tolerance of yeasts (Casey & Ingeldew 1986; D'Amore & Stewart 1987). No differences are observed in chromosomal patterns, in response to these stresses, between strains. Thus comparison between CHEF and pulse-chase experiments confirms that heat, ethanol and the combined shock have effect at the level of gene expression (transcription, translation) and/or on the protein stability, but not at the level of chromosome integrity, since as can be seen no chromosomal shift occurred in either strain (Fig. 5.48). This result was as expected due to the transient nature of the shock response and the speed at which the mechanisms of the response are put into action.

Stress responses expressed by *Sacch. cerevisiae* (L-2226) are therefore not strain specific. These results show no significant differences in the stress response between wine strains of the yeast *Sacch. cerevisiae* suggesting that the responses observed in this work are common to other yeast species, if not genus. In fact the stress response has been observed to be similar in a range of species from bacteria and yeast, to other eukaryotic organisms (Craig 1985; Lindquist 1986; Heikkila 1993).

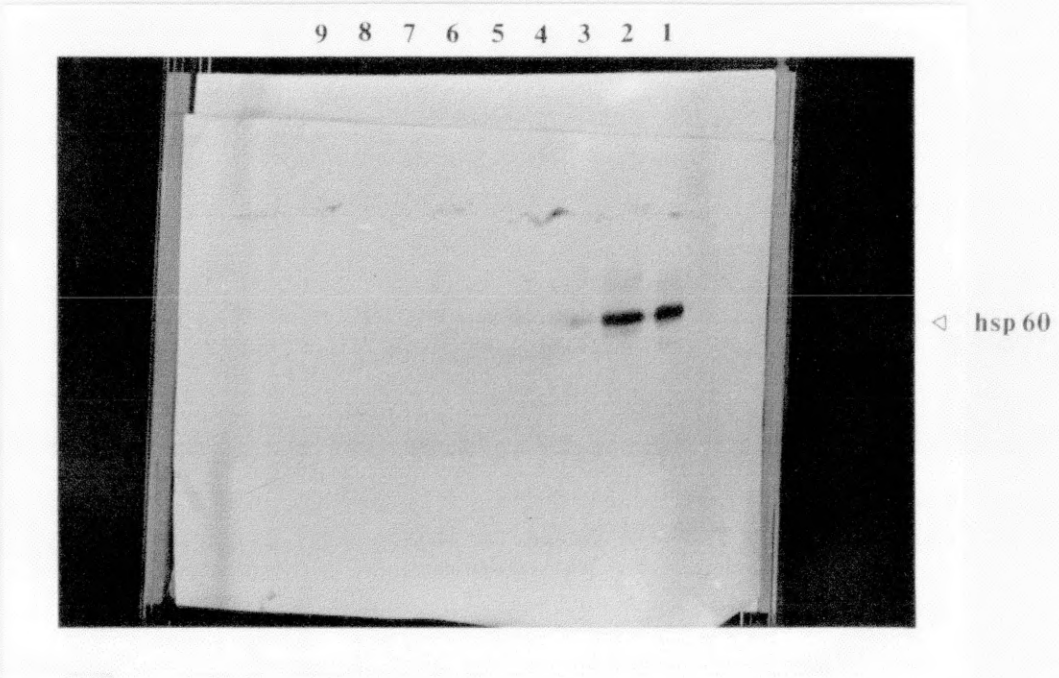


Figure 5.47 Western analysis of *Sacch. cerevisiae* (L-2226) under conditions of various stresses, specific for hsp60. Lanes: 1) GroEL, 2) Control (30°C) + GroEL, 3) Control (30°C), 4) Heat shock (42°C), 5) Ethanol shock (10%), 6) Calcium shock 100mM, 7) Citric acid 40mM, 8) Mg Ionophore 1μg/ml, 9) A23187 1μg/ml.

a) b)

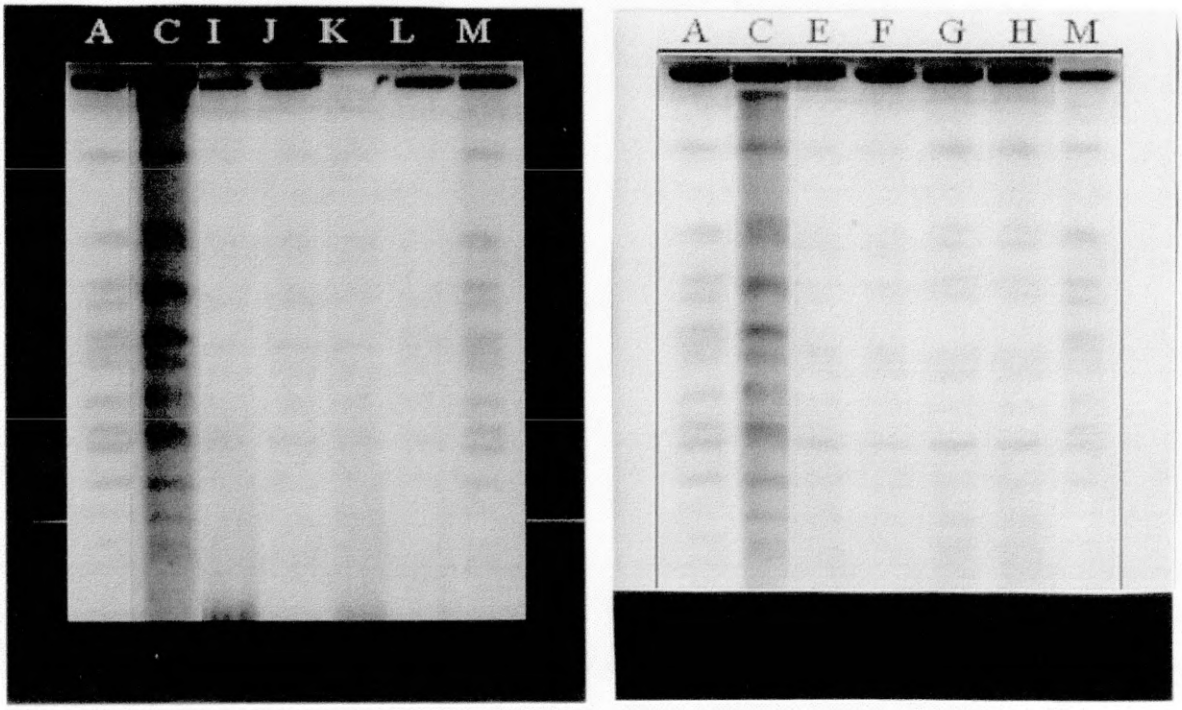


Figure 5.48 Comparison of analysis of *Sacch. cerevisiae* strains; a) L-2226 and b) DBV2168, following heat (42°C) and ethanol (10%) shock by CHEF electrophoresis. Lanes: A) DBV6768 (CHEF DNA Standard), (YEPG), C) L-2226 (YEPG), E) DBV2168 (CMMM)-Control (30°C), F) DBV2168 (CMMM)-Heat shock (42°C), G) DBV2168 (CMMM)-Ethanol shock (10%), H) DBV2168 (CMMM)-Heat and Ethanol shock, I) L-2226 (CMMM)Control (30°C), J) L-2226 (CMMM)-Heat shock (42°C), K) L-2226 (CMMM)-Ethanol shock (10%), L) L-2226 (CMMM)-Heat and Ethanol shock, M) DBV6768 (YEPG).

5.4 CONCLUSIONS

A cell's response to stress depends on the physiological status of the cell and nutrient and metal ion related stresses are equally important, if not more so than the physical or chemical stresses yeast cells may encounter. Adequate nutrition of cells is important for maximising performance and starvation or excess of essential nutrients, it has been shown, will cause stress in yeast cells.

Ionic nutrition is of great importance in this respect, in particular that of magnesium and it's possible protective role under stress conditions. Although magnesium protection of yeast cells has been suggested by several studies (Dombek & Ingram 1986a; D'Amore *et al.* 1988; Dasari *et al.* 1990) verification of this protective effect with respect to elevated temperatures and high levels of ethanol has not previously been shown for industrial strains of the yeast *Sacch. cerevisiae*.

In this study we have shown an increase in viability of cultures exposed to stress conditions, both short (1h: all stresses), intermediate (5h: heat & ethanol) and long term (24-48h: heat & ethanol) when Mg^{2+} levels are increased in the growth medium. This response can be seen with Mg^{2+}-preconditioned cells, as well as with concurrent additions on initiation of stress and it therefore suggests that both intra- and extra-cellular Mg^{2+} may offer physiological protection to yeast cells. Pre-conditioning cells with Mg^{2+} has no major advantage over concurrently adding high levels of Mg^{2+} to the growth medium suggesting that both extra- and intra-cellular levels of Mg^{2+} are equally important. From viability and SEM studies in both heat and ethanol stressed cells, elevated Mg^{2+} levels can be seen to be preventing cellular damage rather than having a role in a repair mechanism. Mg^{2+} may counteract the stress response and prevent the increase in membrane permeability caused by ethanol (Petrov & Okorokov 1990).

Protective responses induced by heat shock and ethanol stress show a high degree of similarity and these two stresses are also shown to have similar effects on the level of major integral plasma membrane proteins, e.g. hsp30. The repression of stress proteins on culturing the cells with increased levels of Mg^{2+}, suggests again that Mg^{2+} exerts a protective effect on cells. With heat and ethanol shock, cells cultured in levels of magnesium >10mM exhibited repression of various stress proteins, suggesting that Mg^{2+} counteracts the requirement for formation of damage-repairing stress proteins.

One may conclude, with regard to heat and ethanol stress that increasing Mg^{2+} availability may partially counteract stress responses by acting in a protective manner. It is hypothesised that the mode of action is stabilisation of cell membranes by Mg^{2+}.

APPENDIX 5.1 SDS-PAGE ELECTROPHORESIS

PREPARATION OF GLASS PLATES

Glass plates were immersed overnight in 0.1% DECON. The plates were then immersed for 15-30 min in 0.1% v/v sulphuric acid, rinsed thoroughly in deionised water (ultra-pure). Prior to assembly, plates were wiped with 90% ethanol to ensure the glass was grease and dust free.

10% RESOLVING GEL

25ml Protogel™ (37.5:1 Acrylamide/bis-acrylamide solution; National Diagnostics, UK)
19.5ml Resolving Gel Buffer (pH8.9)
29.7ml dH_2O
450µl 10% (w/v) Ammonium persulphate
50µl TEMED (N, N, N, N-tetraethylenediamine)
Volume sufficient to pour 2 large slab gels. Resolving gel buffer (181.5g TRIS, 4.0g SDS) was adjusted to pH8.9 with HCl at room temperature and made up to 1L with dH_2O.

3% STACKING GEL

2.5ml Protogel™ (37.5:1 Acrylamide/bis-acrylamide solution; National Diagnostics, UK)
6.5ml Stacking Gel Buffer (pH6.7)
15.7ml dH_2O
150µl 10% (w/v) Ammonium persulphate
10µl TEMED (N, N, N, N-tetraethylenediamine)
Volume sufficient to pour stacker gel for 2 large slab gels. Stacking gel buffer (59.0g TRIS, 4.0g SDS) was adjusted to pH6.7 with HCl at room temperature and made up to 1L with dH_2O.

ELECTRODE BUFFER (pH 8.3)

5L of a solution of 0.025M TRIS, 0.192M glycine and 0.1% SDS in deionised water (18Ω) was prepared per electrophoresis tank, this solution did not need the pH altered if prepared accurately.

APPENDIX 5.2 IMMUNOBLOTTING - WESTERN BLOTTING

IMMUNOBLOT SET-UP

Electrophoretic transfer to nitrocellulose sheets was carried out in a Trans Blot Transfer Cell (Biorad). Nitrocellulose sheets, 3MM Chromatography paper and Brillo™ pads were pre-soaked in Transfer Buffer before assembly. System was assembled for transfer in the following order: Brillo; 3MM; Gel; Nitrocellulose Sheet; 3MM; Brillo, from the negative electrode to the positive.

TRANSFER BUFFER (pH8.3)

3.03g TRIS
14.4g glycine
200ml methanol
Made up to 1L in a volumetric flask and cooled to $4^{o}C$. Solution will have pH of 8.3 if made accurately.

INCUBATION BUFFER (TBS)

20mM TRIS
137mM NaCl
4% 1M HCl
Adjusted to pH7.4 and made up to 1L in a volumetric flask.

TBS-TWEEN20

0.5ml/L Tween 20 was added to the TBS solution, pH adjusted to pH7.4 in 1L.
Tween 20 = Polyoxyethylenesorbitan Monolaurate).

BLOCKING SOLUTION

5% w/v Marvel™ in TBS (Skimmed Milk Powder)

DEVELOPING SOLUTION (CHROMOGEN)

1ml Chloronaphthol solution (30mg/ml in methanol)
10ml methanol
TBS was added to a volume of 50ml and then 30µl 30% hydrogen peroxide. 20ml Chromogen was required per immunoblot and the solution had to be made fresh immediately prior to use.

APPENDIX 5.3 ELECTRON MICROSCOPY

CACODYLATE BUFFER

Solution A: 0.2M $Na(CH_3)_2AsO_2$
Solution B: 0.2M HCl
To prepare buffer (0.1M) of pH7.0, 6.3ml of solution B was added to 50ml of solution A and made up to 100ml with d H_2O.

TEM RESIN

5ml Araldite (Aldrich)
6ml EPON (Aldrich)
15ml HY964 (Aldrich)
0.4ml Accelerator (Aldrich)
Mix well and place in an oven at 60^{o}C for 10min to eliminate any air bubbles.

EPON (NORMAL)

4.85g EPON
1.85g DDSA (dodecenyl-succinic-anhydride)
3.30g MNA (methyl-nadic-anhydride)
0.15g DMP30 (2,4,6 tri-di-methyl-amino-methyl-phenol)
Store at 4^{o}C.

CHAPTER 6

CONCLUDING DISCUSSION

Inorganic nutrition of the yeast *Sacch. cerevisiae* and other related strains is of fundamental importance in both laboratory and commercial cultivation and fermentation. For the yeast, it is important for cellular functioning, adequate growth and execution of cellular processes. Inorganic ions, in particular Mg^{2+}, are important in enzyme activation, cell division and a variety of other cellular processes and as such these constitute essential nutrients. On an industrial view point, it is important in maximising productivity and growth and to minimise problems of production for efficient turnover and yield, whether it be of biomass or ethanol. Commercial production of wine strains as selected starters, mainly as ADY, and the shear number of wineries around the world make consideration of the inorganic nutrition of wine yeasts of prime importance. Incorrect nutritional balances lead to various problems in yeast, e.g. poor growth, stuck or incomplete fermentations, production of acidic wines (result of excess calcium levels) and/or cellular stress.

Magnesium and calcium play a large role in yeast nutrition, in terms of effects on growth and cell physiology. From the results of these studies, it can be concluded that increasing magnesium levels in grape must (or laboratory media: semi-synthetic and minimal) is beneficial for cultures of *Sacch. cerevisiae* (and *T. delbrueckii*). Essentially this is a re-balancing of ionic ratios within the grape must since, contrary to laboratory media, Mg:Ca ratios in most complex media favour calcium (Walker 1994) and for grape must the ratios are 2:1 against magnesium. The results of this work propose that high calcium ratios generally inhibit fermentation characteristics in both semi-synthetic and complex media, slowing the fermentations and raising questions of the applicability of the nutritional make-up of industrial media. Fermentation and growth parameters were improved on increase of media magnesium levels whereas an increase in calcium levels reduced productivity and resulted in production of acidic wines.

In large scale industrial fermentations or biomass productions the balance of inorganic nutrients is critical to achieve maximal rates of growth, etc. However, complex media rarely supply the correct levels of nutrients and this can lead to various problems, such as agglomeration. Flocculant strains have some uses in wine production. Such strains were

developed and used commercially to facilitate the ridling process during Champagne and sparkling wine production (Degré 1993) and despite process developments with immobilisation of yeast these strains are still used in smaller wineries. The phenomenon of agglomeration although possessing some similarities to flocculation is different and a problem for yeast producers and wineries alike. These studies established agglomeration is a problem of the whole yeast population and not due to incompatible cell types. Ionic implications in this problem were manifested in the role played by calcium. Results of chelator studies indicated that calcium was the major ionic cofactor in agglomeration. Increasing magnesium concentration of molasses (complex media) was found to reduce the level of grit by 40-60%, with increased magnesium concentration resulting in increased reductions, suggesting an antagonistic action of magnesium vs. calcium. Magnesium saturation of cell surface binding sites is the most likely explanation for a mode of action of this antagonism, the beneficial effect being to block availability of sites to calcium. Overall, the results portrayed here substantiate the claim that calcium is an important factor in promoting agglomeration and that elevation of magnesium levels serves to reduce it. The mechanisms of agglomeration are, however, not only based on an ionic influence. An involvement at the protein level exists and results of this study identified three bands (α, β & γ) specific to gritty strains. The presence of these bands and inhibiting effect of proteinase on grit formation confirmed a protein involvement in agglomeration, therefore it would appear the best explanation for this phenomenon would be similar to those suggested for flocculation (Miki *et al.* 1982a; Kihn *et al.* 1988) *i.e.* a combination of ionic and protein involvement initiating binding of adjacent cells and that alteration of magnesium levels alleviates this problem in commercial yeast production.

Stress is a concept which underpins all aspects of yeast growth and commercial use. The stress response is one which transcends all prokaryotic and eukaryotic species. In terms of yeast, e.g. *Sacch. cerevisiae* although being of fundamental importance this effect is largely overlooked in commercial wineries and yeast production plants. Nutritional stress, heat shock and ethanol stress are all common stress situations experienced by wine yeast. Nutritional stress links back to the influence of metal ions in growth and fermentation

parameters. Ionic limitation is one of the major stresses yeast are subjected to and in response to such a stress cells initiate the stress response, viability is decreased and fermentation performance reduced.

Heat and ethanol stress are the other two main stresses experienced by yeast. Heat shock is of concern in wine production since fermentations inherently produce heat and as fermentation rate increases so does the heat. Without cooling cells become exposed to a mild heat shock which would reduce viability, productivity and cause initiation of the stress response. Ethanol stress is of major importance also since it is the major metabolite resulting from yeast fermentation and levels in wines can often reach stressful levels. Elevation of magnesium levels in the growth medium reduced cell mortality, reduced ion leakage and repressed the induction of the stress response. A similar response was observed of magnesium levels in cells exposed to ethanol shock. In addition to this, reduction of cell surface damage and intracellular disruption occurred with magnesium elevation. Intra- and extra-cellular magnesium it would seem, offer physiological protection to yeast cells and based on the results of this study a protective role in preventing cellular damage rather than having a role as a repair mechanism, as is the case with hsps, is proposed for magnesium. Magnesium is thought to counteract the stress response with regard to heat and ethanol stress, negating the requirement for stress protein induction and preventing the increase in membrane permeability. It is hypothesised that the mode of action is stabilisation of cell membranes by Mg^{2+}.

Overall, ionic influences are central to many aspects of yeast cell physiology: growth, fermentation, agglomeration and stress. Magnesium plays a role in many aspects of cellular functioning. The proposed roles for Mg^{2+} in yeast fermentation are therefore: enzyme stimulation and membrane stabilisation.

CHAPTER 7

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There is a theory which states that if ever anyone discovers exactly what the Universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable.

There is another theory which states that this has already happened.

Douglas Adams (1952-)
The Restaurant at the End of the Universe (1980)